Edited by
Wolfgang Aehle

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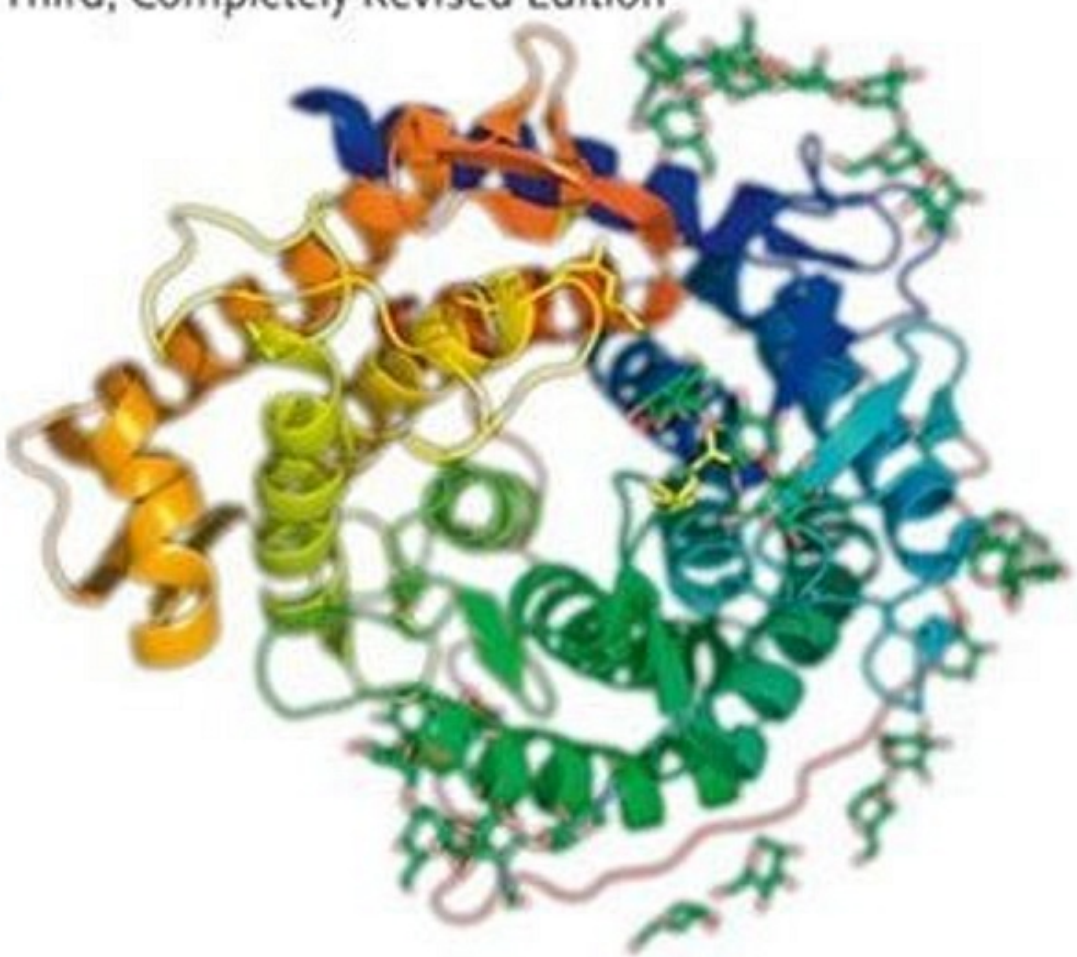
Enzymes in Industry

Production and Applications

Third, Completely Revised Edition



Enzymes in Industry
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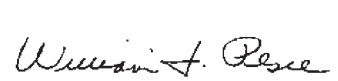

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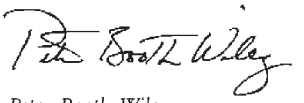

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Dr. Wolfgang Aehle
Genencor – A Danisco Division
PO Box 218
2300 AE Leiden
The Netherlands

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Preface to the Third Edition

For centuries humans have had a history of using the power of natural catalysts — enzymes. But only in the late 19th century, after the term enzymes had been coined by Kühne, did enzyme-directed research and, subsequently, understanding of enzymes start to develop. It took 50 more years until the concept of enzymes as we know it today had been fully developed. After researchers got the chance to combine observations in the three-dimensional structures with the results of the systematical modification of enzymes by using the tools of molecular biology, our knowledge base has broadened even more, and scientists now understand the function of many enzymes at the atomic level.

The industrial use of enzymes as we know it today started after the German chemist Otto Röhm discovered in 1913 the efficacy of pancreatic trypsin for the removal of proteinaceous stains from clothes. Today, microbial proteases have become the workhorses of the cleaning industry. They are contained in almost every single detergent package and catalyze the removal of stains like blood, milk, and egg from our clothes very efficiently. In other fields, enzymatic processes have completely replaced conventional chemical processes. The best example is the production of high-fructose corn syrup from cornstarch.

The table of contents of this book shows clearly in how many different applications enzymes have become a useful adjuvant. In the food industry enzymes are used to improve dairy products like cheese or to supply us with breads that have the right crumb structure and give us the right mouthfeel while eating. In nonfood applications, we not only benefit from the clean laundry that detergents deliver thanks to enzymes, but we see also the fashionable look of "stone-washed" jeans, which is achieved by treatment of jeans with cellulases. Finally the catalysis of a wide range of reactions in synthetic organic chemistry has been explored. Interestingly, enzymes find their application as parts of the molecular biology toolbox, which is necessary to enable modification of enzymes through protein engineering and in the construction of microbial production hosts for enzymes. Obviously, this book contains many more examples of enzyme usage and I leave it to the curiosity of the interested reader to discover the world of industrial enzyme use.

While writing this preface and reading the table of contents again, I realized that industrial enzyme usage is still a very rapidly emerging field. Since the previous issue of the book, new enzyme application areas have emerged. The

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production of bio-ethanol from granular cornstarch has become a fast-growing commercial application for industrial enzymes. An interesting aspect of this development is the chance to save energy during the production of high-fructose corn syrup, because the high-temperature liquefaction step is no longer necessary (see Section ((insert xref to Section 5.2.3 Enzymes in Grain Wet-Milling))). At the same time, the production of ethanol via enzyme-enabled fermentations of lignocellulosic raw materials such as corn stover was the subject of two huge research projects sponsored by the National Renewable Energy Lab of the U.S. Department of Energy. This technology has not led to major use of enzymes yet, but might become an interesting field in the near future. I expect many more industrial applications of enzymes to come, mainly because enzyme applications can help us to save energy, which, in times of rising crude oil prices, becomes a more and more interesting valuable benefit of enzyme application.

While planning the book, I strived to achieve a comprehensive overview of all aspects of enzyme usage. This includes almost all applications of enzymes in an industrial environment in its broadest sense; the discovery, modification, and production of technical enzymes; and finally a chapter about enzyme safety and regulatory considerations.

In order to have the most competent authors for each topic, I invited as many authors as possible from the enzyme-applying industry to explain usage, function, and problems of enzyme application in their field and facts about safe enzyme usage. Scientists from academia and industry describe the enabling techniques for discovery, improvement, and production of enzymes.

I would like to thank the authors for their excellent work and their dedication for keeping the information up-to-date. I have received many positive comments on the 2nd completely revised edition of this book. This is certainly a compliment to the numerous authors who contributed to it. It is another compliment to the authors that Prof. Zhanhling Lin took the initiative to find a Chinese publisher and translate the book into Chinese. I think that the authors can be proud of such an achievement.

Leiden, The Netherlands *Wolfgang Aehle*
August 2007

List of Contributors

Editor

Dr. Wolfgang Aehle
Genencor – A Danisco Division
Research & Development
P.O. Box 218
2300 AE Leiden
The Netherlands

Authors

Dr. Wolfgang Aehle
Genencor – A Danisco Division
Research & Development
P.O. Box 218
2300 AE Leiden
The Netherlands
Chapter 1, Sections 4.2, 5.4

Dr. Richard L. Antrim
Grain Processing Corporation
1600 Oregon Street
Muscatine, IA 52761-1494
USA
Section 5.2.3

Todd Becker
Genencor – A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304-1013
USA
Section 3.2.5

Dr. Rick Bott
Genencor – A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304-1013
USA
Section 4.2

Dr. Johanna Buchert
2044 VTT
Finland
Section 5.2.6

Dr. Heidi Burrows
Finfeeds International
P.O. Box 777
Wiltshire, SN8 1XN Marlborough
UK
Section 5.2.4

Alice J. Caddow
Genencor – A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304-1013
USA
Chapter 7

Dr. Gopal K. Chotani
Genencor – A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304-1013
USA
Section 3.1

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Beth Concoby
Genencor – A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304-1013
USA
Chapter 7

Dr. Hans de Nobel
Genencor – A Danisco Division
Research & Development
P.O. Box 218
2300 AE Leiden
The Netherlands
Section 4.1

Dr. André de Roos
DSM Food Specialities
P.O. Box 1
2600 MA Delft
The Netherlands
Section 5.1.4

Dr. Carlo Dinkel
SiChem GmbH
BITZ
Fahrenheitstr. 1
28359 Bremen
Germany
Section 6.1

Timothy C. Dodge
Genencor – A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304-1013
USA
Section 3.1

Prof. Dr. Karlheinz Drauz
Degussa AG
Fine Chemicals
Rodenbacher Chaussée 4
63457 Hanau-Wolfgang
Germany
Section 6.1

Prof. Dr. Saburo Fukui†
formerly Department of
Industrial Chemistry
Faculty of Engineering
Kyoto University
606-8501 Kyoto
Japan
Section 3.3

Dr. Christian Gölker
Bayer AG
Aprather Weg
42096 Wuppertal
Germany
Section 3.2

Dr. Catherine Grassin
DSM Food Specialties
15, rue des Comtesses
P.O. Box 239
59472 Seclin Cedex
France
Section 5.1.2

Dr. Harald Gröger
Degussa AG
Project House Biotechnology
Rodenbacher Chaussée 4
63457 Hanau-Wolfgang
Germany
Section 6.1

Dr. Meng H. Heng
Genencor – A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304-1013, USA
Sections 3.2.2.4, 3.2.4.1

Dr. Günther Henniger
formerly Roche Diagnostics GmbH
Nonnenwald 2
82377 Penzberg
Germany
Section 6.4

Dr. Ivan Herbots
Procter & Gamble Eurocor S.A.
Temselaan 100
1853 Strombeek-Bever
Belgium
Section 5.2.1

Dr. Andreas Herman
Terwisscha van Scheltinga
DSM-Gist Research and Development
Alexander Fleminglaan 1
2613 AX Delft
The Netherlands
Section 3.1

Dr. Brian Jones
Genencor – A Danisco Division
Research & Development
P.O. Box 218
2300 AE Leiden
The Netherlands
Section 4.1

Dr. Albert Jonke
Roche Diagnostics GmbH
Nonnenwald 2
82377 Penzberg
Germany
Chapter 2

John Kan
Genencor – A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304-1013
USA
Sections 3.2.2.4, 3.2.4.1

Dr. Christoph Kessler
Roche Diagnostics GmbH
Nonnenwald 2
82377 Penzberg
Germany
Section 6.5

Dr. Beatrix Kottwitz
R&D / Technology Laundry and
Home Care
Henkel KGaA
Henkelstr. 67
40191 Düsseldorf
Germany
Section 5.2.2

Dr. Karsten M. Kragh
Genencor – A Danisco Division
Edwin Rahrs Vej 38
8220 Brabrand
Denmark
Section 5.1.1

Dr. Georg-Burkhard Kresse
Roche Diagnostics GmbH
Pharma Research / Biology
Nonnenwald 2
82377 Penzberg
Germany
Section 6.2

Dr. Herman B. M. Lenting
TNO Science and Industry
P.O. Box 6265
5600 Eindhoven
The Netherlands
Section 5.2.5

Dr. Karl-Heinz Maurer
Henkel KGaA
Department of Enzyme Technology
Henkelstr. 67
40191 Düsseldorf
Germany
Section 5.3

Dr. Gerhard Michal
formerly Boehringer Mannheim GmbH
Research
68298 Mannheim
Germany
Chapter 2

Dr. Marja-Leena Niku-Paavola
P.O. Box 1500
2044 VTT
Finland
Section 5.2.6

Jaakko Pere
2044 VTT
Finland
Section 5.2.6

Prof. Dr. Richard N. Perham
University of Cambridge
Department of Biochemistry
80 Tennis Court Road
CB2 1GA Cambridge
England
Chapter 1

Dr. Charlotte Horsmans Poulsen
Genencor – A Danisco Division
Edwin Rahrs Vej 38
8220 Brabrand
Denmark
Section 5.1.1

Prof. Dr. Peter J. Reilly
Iowa State University
Department of Chemical Engineering
2114 Sweeney Hall
Ames, IA 50011-2230
USA
Section 5.2.3

Prof. Dr. Reinhard Renneberg
Hongkong University of Science and Technology
Department of Chemistry
Clear Water Bay
Kowlon
Hongkong
Sections 6.3, 6.4

Dr. Andrea Saettler
Henkel KGaA
Department of Enzyme Technology
Henkelstr. 67
40191 Düsseldorf
Germany
Section 5.3

Dr. Rainer Schmuck
Roche Diagnostics GmbH
Diagnostic Research
Nonnenwald 2
82377 Penzberg
Germany
Section 6.3

Dr. Carsten Schultz
EMBL
Meyerhofstr. 1
69117 Heidelberg
Germany
Section 6.1

Dr. Jorn Borch Soe
Genencor – A Danisco Division
Edwin Rahrs Vej 38
8220 Brabrand
Denmark
Section 5.1.5

Jens Frisbak Sorensen
Genencor – A Danisco Division
Edwin Rahrs Vej 38
8220 Brabrand
Denmark
Section 5.1.1

Dr. Anna Suurnäkki
2044 VTT
Finland
Section 5.2.6

Prof. Dr. Atsuo Tanaka
Department of Industrial Chemistry
Faculty of Engineering
Kyoto University
606-8501 Kyoto
Japan
Section 3.3

Dr. Liisa Viikari
2044 VTT
Finland
Section 5.2.6

Prof. Dr. Herbert Waldmann
Max-Planck-Institut für molekulare
Physiologie
Otto-Hahn-Str. 11
44227 Dortmund
Germany
Section 6.1

Dr. Jan Wilms
The Netherlands
DSM Food Specialities
Research and Developent
P.O. Box 1
2600 MA Delft
Section 5.1.3

Dr. Karl Wulff
Boeringer Mannheim GmbH
Bahnhofsstr. 9-15
82327 Tutzing
Germany
Section 6.3

Abbreviations

A:	adenosine
ACA:	acetamidocinnamic acid
ACL:	α-amino- ε-caprolactam
ADH:	alcohol dehydrogenase
ADI:	acceptable daily intake
ADP:	adenosine 5′-diphosphate
Ala:	alanine
Arg:	Arginine
AMP:	adenosine 5′-monophosphate
ATC:	D,L-2-amino- Δ^2-thiazoline-4-carboxylic acid
ATP:	adenosine 5′-triphosphate
C:	cytidine
cDNA:	copy DNA
CL:	citrate lyase
CMP:	cytidine 5′-monophosphate
CoA:	coenzyme A
CS:	citrate synthetase
CTP:	cytidine 5′-triphosphate
d:	deoxy
dam:	gene locus for E. coli DNA adenine methylase (N^6-methyladenine)
dcml:	gene locus for E. coli DNA cytosine methylase (5-methylcytosine)
dd:	dideoxy
ddNTP:	dideoxynucleoside 5′-triphosphate
DE:	dextrose equivalent
DEAE:	diethylaminoethyl
DNA:	deoxyribonucleic acid
DNase:	deoxyribonuclease
dNTP:	deoxynucleoside 5′-triphosphate
DOPA:	3-(3,4-dihydroxyphenylalanine) [3-hydroxy-L-tyrosine]

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dpm:	decays per minute
ds:	double-stranded
E.C.:	Enzyme Commission
F6P:	fructose 6-phosphate
FAN:	free alpha amino nitrogen, i.e., a measure of peptides/amino acids available for yeast to be used as nutrient
fMet:	*N*-formylmethionine
FMN:	flavin mononucleotide
$FMNH_2$:	flavin mononucleotide, reduced
G:	quanosine
GDP:	guanosine 5′-diphosphate
Glu:	glutamic acid
Gly:	glycine
GMP:	guanosine 5′-monophosphate
GOD:	glucose oxidase
GOT:	glutamate–oxaloacetate transaminase
G6P:	glucose 6-phosphate
GPT:	glutamate–pyruvate transaminase
GTP:	guanosine 5′-triphosphate
3-HBDH:	3-hydroxybutyrate dehydrogenase
HFCS:	high-fructose corn syrup
*hsd*M:	*E. coli* gene locus for methylation
*hsd*R:	*E. coli* gene locus for restriction
*hsd*S:	*E. coli* gene locus for sequence specificity
IDP:	inosine 5′-diphosphate
Ile:	isoleucine
INT:	iodonitrotetrazolium chloride
ITP:	inosine 5′-triphosphate
LDH:	lactate dehydrogenase
Lys:	lysine
^{m}(superscript):	methylated
MDH:	malate dehydrogenase
Met:	methionine
M6P:	mannose 6-phosphate
mRNA:	messenger RNA
MTT:	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

N:	any nucleotide
NAD:	nicotinamide–adenine dinucleotide
NADH:	nicotinamide–adenine dinucleotide, reduced
NADP:	nicotinamide–adenine dinucleotide phosphate
NADPH:	nicotinamide–adenine dinucleotide phosphate, reduced
NMN:	nicotinamide mononucleotide
NTP:	nucleoside 5′-triphosphate
p:	phosphate groups
^{32}P:	phosphate groups containing ^{32}P phosphorus atoms
$p_{i:}$	inorganic phosphate
°P:	degree Plato; i.e., sugar content equivalent to 1 % sucrose by weight
PEP:	phosphoenolpyruvate
6-PGDH:	6-phosphogluconate dehydrogenase
Phe:	phenylalanine
PMS:	5-methylphenazinium methyl sulfate
poly(dA):	poly(deoxyadenosine 5′-monophosphate)
$pp_{i:}$	inorganic pyrophosphate
Pro:	proline
PRPP:	phosphoribosyl pyrophosphate
Pu:	purine
Py:	pyrimidine
r:	ribo
RNA:	ribonucleic acid
RNase:	ribonuclease
SAM:	S-adenosylmethionine
SMHT:	serine hydroxymethyltransferase
ss:	single-stranded
T:	thymidine
TMP:	thymidine 5′-monophosphate
tRNA:	transfer RNA
TTP:	thymidine 5′-triphosphate
U:	uridine
UMP:	uridine 5′-monophosphate
UTP:	uridine 5′-triphosphate
Val:	valine

Plasmids
pBR322
pBR328
pSM1
pSP64
pSP65
pSPT18, pSPT19
pT7–1, pT7–2
pUC 18, pUC 19
pUR222

Bacteriophages
fd
ghl
M13
N4
PBS1
PBS2
SPO1
SP6
SP15
T3
T4
T5
T7
XP12
λ
λgt11
*Φ*SM11
*Φ*X174

Eukaryotic viruses
Ad2
SV40

1
Introduction

Enzymes are the catalysts of biological processes. Like any other catalyst, an enzyme brings the reaction catalyzed to its equilibrium position more quickly than would occur otherwise; an enzyme cannot bring about a reaction with an unfavorable change in free energy unless that reaction can be coupled to one whose free energy change is more favorable. This situation is not uncommon in biological systems, but the true role of the enzymes involved should not be mistaken.

The activities of enzymes have been recognized for thousands of years; the fermentation of sugar to alcohol by yeast is among the earliest examples of a biotechnological process. However, only recently have the properties of enzymes been understood properly. Indeed, research on enzymes has now entered a new phase with the fusion of ideas from protein chemistry, molecular biophysics, and molecular biology. Full accounts of the chemistry of enzymes, their structure, kinetics, and technological potential can be found in many books and series devoted to these topics [1–5]. This chapter reviews some aspects of the history of enzymes, their nomenclature, their structure, and their relationship to recent developments in molecular biology.

1.1
History

Detailed histories of the study of enzymes can be found in the literature [6], [7].

Early Concepts of Enzymes The term ''enzyme'' (literally ''in yeast'') was coined by Kühne in 1876. Yeast, because of the acknowledged importance of fermentation, was a popular subject of research. A major controversy at that time, associated most memorably with Liebig and Pasteur, was whether or not the process of fermentation was separable from the living cell. No belief in the necessity of vital forces, however, survived the demonstration by Buchner (1897) that alcoholic fermentation could by carried out by a cell-free yeast extract. The existence of extracellular enzymes had, for reasons of experimental accessibility, already been recognized. For example, as early as 1783, Spallanzani had demonstrated that gastric juice could digest meat in vitro, and Schwann (1836) called the active substance pepsin. Kühne himself appears to have given trypsin its present name, although its existence in the intestine had been suspected since the early 1800s.

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Enzymes as Proteins By the early 1800s, the proteinaceous nature of enzymes had been recognized. Knowledge of the chemistry of proteins drew heavily on the improving techniques and concepts of organic chemistry in the second half of the 1800s; it culminated in the peptide theory of protein structure, usually credited to Fischer und Hofmeister. However, methods that had permitted the separation and synthesis of small peptides were unequal to the task of purifying enzymes. Indeed, there was no consensus that enzymes were proteins. Then, in 1926, Sumner crystallized urease from jack bean meal and announced it to be a simple protein. However, Willstätter argued that enzymes were not proteins but ''colloidal carriers'' with ''active prosthetic groups.'' However, with the conclusive work by Northrop *et al.*, who isolated a series of crystalline proteolytic enzymes, beginning with pepsin in 1930, the proteinaceous nature of enzymes was established.

The isolation and characterization of intracellular enzymes was naturally more complicated and, once again, significant improvements were necessary in the separation techniques applicable to proteins before, in the late 1940s, any such enzyme became available in reasonable quantities. Because of the large amounts of accessible starting material and the historical importance of fermentation experiments, most of the first pure intracellular enzymes came from yeast and skeletal muscle. However, as purification methods were improved, the number of enzymes obtained in pure form increased tremendously and still continues to grow. Methods of protein purification are so sophisticated today that, with sufficient effort, any desired enzyme can probably be purified completely, even though very small amounts will be obtained if the source is poor.

Primary Structure After the protein nature of enzymes had been accepted, the way was clear for more precise analysis of their composition and structure. Most amino acids had been identified by the early 20th century. The methods of amino acid analysis then available, such as gravimetric analysis or microbiological assay, were quite accurate but very slow and required large amounts of material. The breakthrough came with the work of Moore and Stein on ion-exchange chromatography of amino acids, which culminated in 1958 in the introduction of the first automated amino acid analyzer [8].

The more complex question–the arrangement of the constituent amino acids in a given protein, generally referred to as its primary structure–was solved in the late 1940s. The determination in 1951 of the amino acid sequence of the β-chain of insulin by Sanger and Tuppy [10] demonstrated for the first time that a given protein does indeed have a unique primary structure. The genetic implications of this were enormous. The introduction by Edman of the phenyl isothiocyanate degradation of proteins stepwise from the N-terminus, in manual form in 1950 and subsequently automated in 1967 [11], provided the principal chemical method for determining the amino acid sequences of proteins. The primary structures of pancreatic ribonuclease [12] and egg-white lysozyme [13] were published in 1963. Both of these enzymes, simple extracellular proteins, contain about 120 amino acids. The first intracellular enzyme to have its primary structure determined was glyceraldehyde 3-phosphate dehydrogenase [14], which has an amino acid sequence of 330 residues and represents a size (250–400 residues) typical of many enzymes. Protein sequencing is increasingly performed

by liquid chromatography/mass spectrometry (LC/MS) techniques, and several tools and software packages are now available for protein identification and characterization. The methods of protein sequence analysis are now so well developed that no real practical deterrent exists, other than time or expense, to determination of the amino acid sequence of any polypeptide chain [9].

A more recent fundamental concept called proteome (*prote*in complement to a gen*ome*) will enable researchers to unravel biochemical and physiological mechanisms of complex multivariate diseases at the functional molecular level. A new discipline, *proteomics*, complements physical genome research. Proteomics can be defined as ''the qualitative and quantitative comparison of proteomes under different conditions to further unravel biological processes'' [15].

Active Site The fact that enzymes are highly substrate specific and are generally much larger than the substrates on which they act quickly became apparent. The earliest kinetic analyses of enzymatic reactions indicated the formation of transient enzyme–substrate complexes. These observations could be explained easily if the conversion of substrate to product was assumed to occur at a restricted site on an enzyme molecule. This site soon became known as the active center or, as is more common today, the active site.

Particular compounds were found to react with specific amino acid side chains and thus inhibit particular enzymes. This suggested that such side chains might take part in the catalytic mechanisms of these enzymes. An early example was the inhibition of glycolysis or fermentation by iodoacetic acid, which was later recognized as resulting from reaction with a unique cysteine residue of glyceraldehyde 3-phosphate dehydrogenase, which normally carries the substrate in a thioester linkage [16].

Many such group-specific reagents have now been identified as inhibitors of individual enzymes; often they are effective because of the hyper-reactivity of a functionally important side chain in the enzyme's active site. However, a more sophisticated approach to the design of enzyme inhibitors became possible when the reactive group was attached to a substrate; in this way, the specificity of the target enzyme was utilized to achieve selective inhibition of the enzyme [17]. Such active-site-directed inhibitors have acquired major importance not only academically in the study of enzyme mechanisms but also commercially in the search for a rational approach to selective toxicity or chemotherapy.

Three-Dimensional Structure Chemical studies showed that the active site of an enzyme consists of a constellation of amino acid side chains brought together spatially from different parts of the polypeptide chain. If this three-dimensional structure was disrupted by denaturation, that is, without breaking any covalent bonds, the biological activity of the enzyme was destroyed. In addition, it was found that all the information required for a protein to fold up spontaneously in solution and reproduce its native shape was contained in its primary structure. This was part of the original ''central dogma'' of molecular biology.

The X-ray crystallography of proteins [18] demonstrated unequivocally that a given protein has a unique three-dimensional structure. Among the basic design principles

Fig. 1 A molecular model of the enzyme lysozyme: the arrow points to the cleft that accepts the polysaccharide substrate (Reproduced by courtesy of J. A. Rupley)

was the tendency of hydrophobic amino acid side chains to be associated with the hydrophobic interior of the folded molecule, whereas charged side chains were almost exclusively situated on the hydrophilic exterior or surface. The first high-resolution crystallographic analysis of an enzyme, egg-white lysozyme, confirmed these principles and led to the proposal of a detailed mechanism [19]. The active site was located in a cleft in the structure (Fig. 1), which has subsequently proved to be a common feature of active sites. According to this, the enzymatic reaction takes place in a hydrophobic environment, and the successive chemical events involving substrate and protein side chains are not constrained by the ambient conditions of aqueous solution and neutral pH.

1.2 Enzyme Nomenclature

Strict specificity is a distinguishing feature of enzymes, as opposed to other known catalysts. Enzymes occur in myriad forms and catalyze an enormous range of reactions. By the late 1950 s the number of known enzymes had increased so rapidly that their nomenclature was becoming confused or, worse still, misleading because the same enzyme was often known to different workers by different names; in addition, the name frequently conveyed little or nothing about the nature of the reaction catalyzed.

To bring order to this chaotic situation, an International Commission on Enzymes was established in 1956 under the auspices of the International Union of Biochemistry (IUB). Its terms of reference were as follows: ''To consider the classification and nomenclature of enzymes and coenzymes, their units of activity and standard methods of assay, together with the symbols used in the description of enzyme kinetics.'' The Commission's recommendations have formed the basis of enzyme nomenclature since its first report in 1961 [1].

Responsibility for enzyme nomenclature passed to the Nomenclature Committee of IUB in 1977, which has subsequently published several reports, e.g., [20] and

supplements, e.g., [21]; it is expected that further supplements will be published from time to time in the *European Journal of Biochemistry*. The growth in scale can be appreciated from the fact that the 1961 *Report of the Enzyme Commission* listed 712 enzymes, whereas the 1992 version of Enzyme Nomenclature listed 3196. The most recent information about changes or additions to enzyme nomenclature is available at http://www.chem.qmw.ac.uk/iubmb/, which offers also an up-to-date version of the *Enzyme Nomenclature* list.

1.2.1
General Principles of Nomenclature

The accepted system for classification and nomenclature of enzymes embodies three general principles.

The first is that enzyme names, especially those ending in *-ase,* should be used only for single enzymes, i.e., single catalytic entities. They should not be applied to systems containing more than one enzyme.

The second general principle is that an enzyme is named and classified according to the reaction it catalyzes. This refers only to the observed chemical change produced by the enzyme, as expressed in the chemical equation. The mechanism of action is ignored, and intermediate cofactors or prosthetic groups are not normally included in the name. Thus, an enzyme cannot be named systematically until the reaction it catalyzes has been identified properly.

The third general principle is that enzymes are named and classified according to the type of reaction catalyzed, which enables Enzyme Commission (E.C.) code numbers to be assigned to enzymes to facilitate subsequent unambiguous identification. For the purpose of systematic nomenclature, all enzymes in a particular class are considered to catalyze reactions that take place in a given direction, although only the reverse direction may have been demonstrated experimentally. However, the recommended name for the enzyme may well be based on the presumed direction of the reaction in vivo.

Thus, a given enzyme often has two names, one systematic and the other recommended or trivial. The latter is generally the name in current usage, shorter and more readily applied. After its systematic name and E.C. code number have identified an enzyme, the recommended name can be used without fear of ambiguity. This practice is now generally followed in the literature.

1.2.2
Classification and Numbering of Enzymes

According to the report of the first Enzyme Commission in 1961, enzymes are divided into six main classes according to the type of reaction catalyzed. They are assigned code numbers, prefixed by E.C., which contain four elements separated by points and have the following meaning:

1. the number first indicates to which of the six classes the enzyme belongs,
2. the second indicates the subclass,

3. the third number indicates the sub-subclass, and
4. the fourth is the serial number of the enzyme in its sub-subclass.

The six classes are distinguished in the following manner:

1. *Oxidoreductases*
   This class encompasses all enzymes that catalyze redox reactions. The recommended name is *dehydrogenase* whenever possible, but *reductase* can also be used. *Oxidase* is used only when O_2 is the acceptor for reduction. The systematic name is formed according to *donor : acceptor oxidoreductase*.

2. *Transferases*
   Transferases catalyze the transfer of a specific group, such as methyl, acyl, amino, glycosyl, or phosphate, from one substance to another. The recommended name is normally *acceptor grouptransferase* or *donor grouptransferase*. The systematic name is formed according to *donor : acceptor grouptransferase*.

3. *Hydrolases*
   Hydrolases catalyze the hydrolytic cleavage of C–O, C–N, C–C, and some other bonds. The recommended name often consists simply of the substrate name with the suffix *-ase*. The systematic name always includes *hydrolase*.

4. *Lyases*
   Lyases catalyze the cleavage of C–C, C–O, C–N, and other bonds by elimination. The recommended name is, for example, *decarboxylase, aldolase, dehydratase* (e-limination of CO_2, aldehyde, and water, respectively). The systematic name is formed according to *substrate group-lyase*.

5. *Isomerases*
   Isomerases catalyze geometric or structural rearrangements within a molecule. The different types of isomerism lead to the names *racemase, epimerase, isomerase, tautomerase, mutase,* or *cycloisomerase*.

6. *Ligases*
   Ligases catalyze the joining of two molecules, coupled with the hydrolysis of a pyrophosphate bond in ATP or another nucleoside triphosphate. Until 1983, the recommended name often included *synthetase,* but the current recommendation is that names of the type *X–Y ligase* be used instead, to avoid confusion with the name *synthase* (which is not confined to enzymes of class 6). The systematic name is formed according to *X : Y ligase* (ADP-forming).

A few examples will serve to illustrate how this system works. (The full list can be found in *Enzyme Nomenclature* 1992 [20].)

The enzyme alcohol dehydrogenase (recommended name) catalyzes the reaction

$$\text{Alcohol} + \text{NAD}^+ \rightleftharpoons \text{Aldehyde or Ketone} + \text{NADH} + \text{H}^+$$

The enzyme has been assigned E.C. number 1.1.1.1. It may also be called aldehyde reductase, but its systematic name is alcohol: NAD^+ oxidoreductase.

Similarly, the enzyme hexokinase (recommended name), which catalyzes the reaction

$$\text{ATP} + \text{D-Hexose} \rightleftharpoons \text{ADP} + \text{D-Hexose 6-phosphate}$$

has been given the E.C. number 2.7.1.1. It has such other names as glucokinase and hexokinase type IV, and its systematic name is ATP: D-hexose 6-phosphotransferase.

1.3 Structure of Enzymes

Enzymes are proteins (for an exception, see Section 1.3.4) and, as such, are amenable to structural analysis by the methods of protein chemistry, molecular biology, and molecular biophysics.

1.3.1 Primary Structure

The primary structure of enzymes can be determined by direct chemical methods which, in sensitivity and automation, have reached very high levels of sophistication [9], [22]. However, for many proteins, particularly those with long polypeptide chains, direct sequence analysis would be very time-consuming; others may be available only in very small amounts. In these cases, a more profitable approach is to clone the relevant structural gene and determine its DNA sequence [9], [23], [24]. From this, the amino acid sequence can be inferred. Whenever possible, this sequence should be checked, e.g., for genetic reading frame, against whatever amino acid sequence information is available from direct methods. The recombinant DNA approach is so quick and so powerful, however, that amino acid sequence information about enzymes is growing much more rapidly from this source than from direct chemical analysis [25], [26]. Indeed, the information now available is so large in total that computer data banks are required to store it and make it available for systematic access [27].

1.3.2 Three-Dimensional Structure

The three-dimensional structure of an enzyme can be obtained at high resolution by X-ray crystallography [28] and, for molecules up to ca. 300 amino acids in length, by NMR spectroscopy. By this means, the detailed structures of many enzymes have been determined, and a broad understanding of the principles of protein structure has resulted [29], [30]. Proteins are generally well ordered; their interiors are well-packed (comparable to other crystalline organic molecules) to produce a hydrophobic core with a dielectric constant similar to that of a hydrocarbon. Proteins vary in the amount of regular secondary structure (α-helix and β-sheet) they contain and can be grouped into four classes according to the combination and packing of these structural features [31]. Although the number of possible combinations of amino acids in a given protein is virtually unlimited, it is estimated that there are not more 1000 different families of folding patterns for protein structures [32].

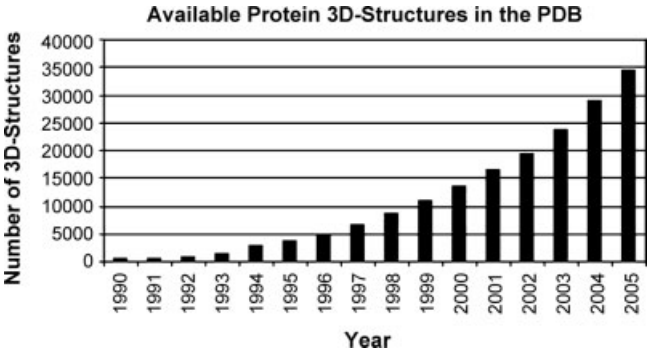

Fig. 2 Cumulative number of published 3D structures of proteins and nucleic acids in the Protein Databank from 1990 to 2005. Until May 1, 2006, 1892 3D structures were added to the 2005 data point in this graph. (Data taken from http://pdbbeta.rcsb.org/pdb/contentGrowthChart.do?content=total=100)

Despite their close-packed and generally well-ordered structure, enzymes are usually not entirely rigid molecules, and some conformational flexibility in solution is widely observed, particularly by NMR spectroscopy [33–37]. These conformational changes may be limited to a molecular ''breathing'' or flexing of the structure, they may involve various ''hinge-bending'' motions, or they may extend to more substantial conformational mobility in parts of the polypeptide chain. All such motions, contribute to the mechanisms of enzyme catalysis [2], [38].

As of April 25, 2006, 36 247 3D structures of biological macromolecules (proteins, nucleic acids, and protein nucleic acid complexes) were freely accessible from the website of the Protein Databank (http://www.rcsb.org/pdb/) [39]. The number of published 3D protein structures is growing rapidly, almost exponentially (see Fig. 2), and this will certainly help to understand the whole proteome in the near future on the atomic level. The site of the Protein Databank offers several software tools for analysis and visualization of protein (and nucleic acid) 3D structures for various computer operating systems.

1.3.3 Quaternary Structure, Folding, and Domains

Many enzymes consist of more than one polypeptide chain (or subunit), and these must form an aggregate, usually with relatively simple symmetry, before full (or even any) biological activity is conferred (Table 1). The subunits within an oligomer or multimer are often identical or at least limited to a few different types. Aggregation is generally some form of self-assembly dictated by coherent binding patterns between the subunits, which provide the necessary recognition sites in sorting out the subunits required for assembly [29], [40].

Table 1. Quaternary structures of some typical enzymes

Enzyme	E.C. number [*CAS registry number*]	Source	Number of subunits	Point symmetry	
				Crystallographic symbol	Schönflies symbol
Alcohol dehydrogenase	1.1.1.1 [9031-72-5]	horse liver	2	2	C_2
Glutathione reductase	1.6.4.2 [9001-48-3]	human red blood cells	2	2	C_2
Triose phosphate isomerase	5.3.1.1 [9023-78-3]	chicken muscle	2	2	C_2
Lactate dehydrogenase	1.1.1.27 [9001-60-9]	dogfish muscle	4	222	D_2
Glyceraldehyde 3-phosphate dehydrogenase	1.2.1.12 [9001-50-7]	*Bacillus stearothermophilus*	4	222	D_2
Pyruvate kinase	2.7.1.40 [9001-59-6]	cat muscle	4	222	D_2
Aspartate carbamoyltransferase	2.1.3.2 [9012-49-1]	*Escherichia coli*	6 + 6	32	D_3
Dihydrolipoamide acetyltransferase	2.3.1.12 [9032-29-5]	*Escherichia coli*	24	432	0
Dihydrolipoamide acetyltransferase	2.3.1.12 [9032-29-5]	*Bacillus stearothermophilus*	60	532	Y

The complexity of this sorting process in a cell becomes evident from the fact that many intracellular enzymes are dimers or tetramers. Increasingly more complicated structures are being recognized and their design principles analyzed. These range from enzymes with simple cyclic symmetry up to those with the most elaborate cubic point group symmetry, e.g., octahedral and icosahedral [29], [40].

The folding of polypeptide chains, along with their aggregation into ordered structures, is a spontaneous process in solution, and this implies that it is exergonic [39]. However, calculation of the time required for a protein to explore all possible structures during the folding process indicates that the search for the ''right'' structure cannot be entirely random. Thus, even for a small protein such as bovine pancreatic ribonuclease (124 amino acid residues), such a search might take around 10^{95} years, whereas the experimentally determined time in vivo is a few milliseconds. This dramatic discrepancy led to the concept of *kinetic pathways* during folding. Such pathways have been experimentally explored, and intermediates identified for various proteins. The stable structure of a protein in solution is therefore identified as the lowest free energy form of the kinetically accessible structures [29], [30], [40].

A typical enzyme is not an entity completely folded as a whole, as is evident from the growing catalogue of three-dimensional protein structures determined by X-ray crystallography. On the contrary, enzymes frequently consist of apparently autonomous or semiautonomous folding units, called *domains* (Fig. 3). Sometimes, these may be

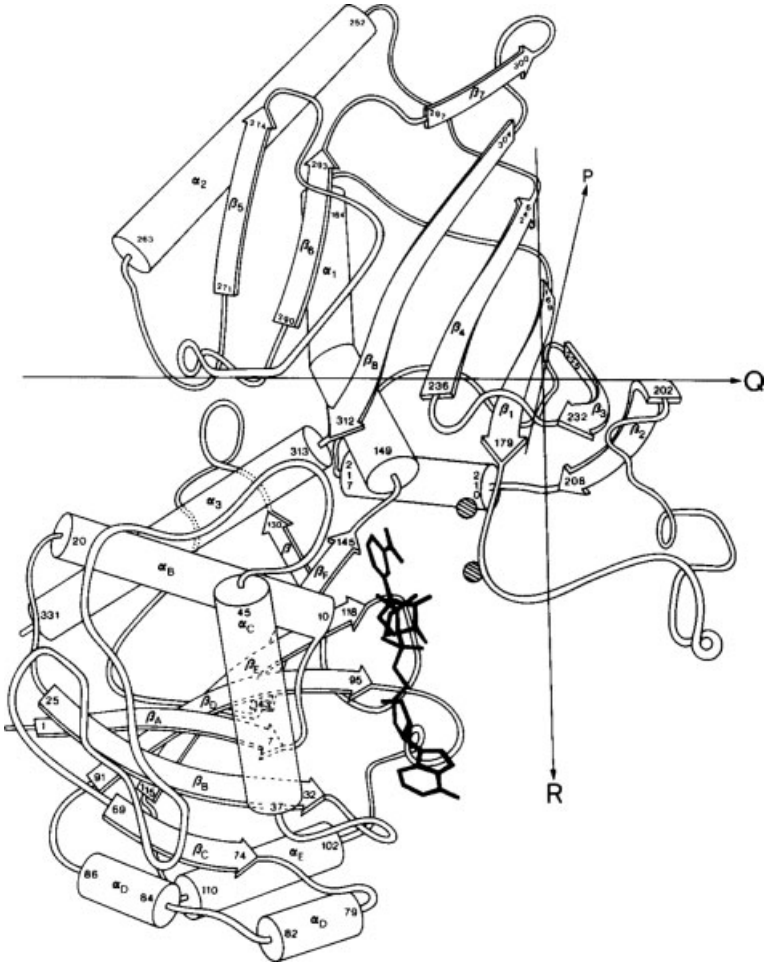

Fig. 3 The domains in glyceraldehyde 3-phosphate dehydrogenase from *Bacillus stearothermophilus* [483] Reproduced with permission

identified as products of limited proteolysis, i.e., regions of the polypeptide chain that can be excised from the chain with retention of their biological properties. Indeed, this has proved in many instances to be a valuable guide to the actual activity contributed by that part of the enzyme. Classical examples of such functional domains can be found in the study of muscle contraction and antibody-antigen recognition [29], [30].

In other cases, domains are not readily released as biologically active entities, and their existence must be inferred from the three-dimensional structure of the enzyme. Most globular proteins can in fact be subdivided into such regions, which generally have molecular masses of 20 000 or less [29]. The active site of an enzyme is often located at the interface between two such domains as, for example, in the well-known cleft of lysozyme (Fig. 1) or in glutathione reductase. Other domains appear to

represent favored folding patterns in the assembly of proteins, but biological activity associated with them can often be inferred from comparison of the structures of related proteins: a typical example is the NAD-binding domain present in dehydrogenases.

Structural domains may be regions of the polypeptide chain that fold independently of each other. Functional domains, as defined above, do indeed fold independently; and individual subunits of oligomeric enzymes appear to fold before association [29], [30], [40], [41].

1.3.4 The Ribozyme

Enzymes are proteins, but the specific involvement of RNA molecules in certain reactions concerned with RNA processing in vivo is worth noting. Until Cech *et al.* [42] and Altman *et al.* [43] published their observations, it was generally accepted knowledge that the major duties in a biological system, namely, to encode information and to catalyze chemical reactions, are neatly split, one being performed by nucleic acids, the other by proteins. With the discovery of special RNAs which store genetic information and can also catalyze reactions on themselves or on other RNAs, this dogma was destroyed [42], [43]. Over the years, it has become evident, that group I and group II introns, catalyze various transesterifications. In cellular systems these reactions facilitate their excision from pre-RNAs and the ligation of flanking exons (self-splicing). In vitro these intron RNAs perform a variety of reactions in cis (i.e., on the same strand of the RNA genome) and in trans (i.e., on another RNA), such as cleavage and ligation of RNAs, transfer of nucleotides between RNAs, polymerization, and editing-like reactions. These RNAs thus can act as enzymes and are therefore called ''ribozymes'' [44].

In *Escherichia coli*, tRNA precursors are cleaved by ribonuclease P to generate the correct 5'-ends of the mature tRNA molecules, and the enzyme contains an essential RNA moiety that can function in the absence of the protein. In fact, this RNA moiety fulfills all the criteria of an enzyme [45]. Similarly, the ribosomal RNA of *Tetrahymena thermophila* undergoes self-splicing to perform a highly specific intramolecular catalysis in the removal of an intervening sequence. A truncated version of the intervening sequence, lacking the first 19 nucleotides of the original excised RNA, can then behave as an enzyme in vitro, capable of acting as an RNA polymerase and a sequence-specific ribonuclease under appropriate conditions [46].

The structure of the ribosome's large subunit has since been solved. This largest unique structure established that the ribosome is a ribozyme in which the ribosomal RNA, and not the protein, performs catalytic functions, including the peptidyl transferase reaction that forms the peptide bond [47], [48]. One of the most remarkable findings to emerge from this is that although enzymes composed entirely of protein promote virtually all chemical reactions that occur in living organisms, the protein synthesis reaction that occurs on the ribosome is due to the two-thirds of its mass that is RNA, not the one-third that is protein. In addition to enhancing the understanding of protein synthesis, this work will have significant medical implications, because the ribosome is a major target for antibiotics [49].

Ribozymes also offer an excellent opportunity to compare and contrast the behavior of RNA enzymes with that of protein enzymes. The differences between the RNA and protein enzymes highlight features that are distinct and thus enable a better understanding of each of these classes of biological macromolecules. On the other hand, the features of protein and RNA enzymes that are similar may represent aspects that are fundamental to biological catalysis. Indeed, these studies have suggested that RNA enzymes, like their protein counterparts, can use binding interactions remote from the site of bond transformation to facilitate that transformation [50]. Beyond this, recent results suggest that RNA enzymes are ideally suited for exploration of the energetic origins of this interconnection between binding and catalysis [51]. This use of binding energy provides a natural connection between rate enhancement and specificity, the two hallmarks of biological catalysis. Finally, ribozymes will not only offer new clues about evolution [52], but also offer the potential for specific inactivation of disease-associated mRNAs or viral RNA genomes that, unlike conventional therapeutics, require no knowledge of the structure or function of proteins that target RNAs encode [53].

1.4 Enzymes and Molecular Biology

1.4.1 Biosynthesis of Enzymes

Enzymes are synthesized in cells by the normal machinery of protein synthesis. The structure of any given enzyme is encoded by a structural gene, whose DNA base sequence is transcribed into a messenger RNA, and the mRNA is translated from its triplet code into the amino acid sequence of the desired protein by the ribosomes and associated factors [54], [55]. The enzyme then folds spontaneously into its active conformation. Posttranslational modifications may be required to target an enzyme to its ultimate intracellular or extracellular location.

1.4.2 Enzymes and DNA

For many years, the chemical manipulation of DNA lagged behind that of proteins. The chemical complexity and variety of proteins, with up to 20 different naturally occurring amino acids, served to make them more amenable to increasingly sophisticated methods of analysis. On the other hand, DNA, composed of only four different nucleotides, appeared dauntingly large, with few structural features to make it yield to available methodology. Paradoxically, this very lack of variety in the nature of the constituent nucleotides of DNA has permitted the revolution in genetic engineering, in which the enzymology of DNA [56] has played a prominent part. For example, the discovery and purification of restriction enzymes enabled DNA to be cleaved selectively into defined fragments; phosphatases and ligases permit the fragments to be rejoined selectively; and DNA polymerases allow DNA to be synthesized and sequenced at astonishing speed, all in vitro [23], [54–56].

2
Catalytic Activity of Enzymes [57, 58, 60]

The theory of enzyme-catalyzed reactions proposed by MICHAELIS and MENTEN in 1913 [61] is based on the assumption that the enzyme (the catalyst, E) and the substrate (the reactant, S) form a complex (ES) by a reversible reaction. The complex is then converted into the product (P) with the reaction rate k_2, when practically no product is present.

$$S + E \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \overset{k_2}{\rightarrow} P + E \tag{2.1}$$

Under commonly used conditions of enzyme activity measurement, c_{ES} can be considered to be sufficiently constant during the observed reaction period (steady-state assumption, BRIGGS and HALDANE [104]):

$$\frac{d(c_{ES})}{dt} = 0 = (k_1 \cdot c_E \cdot c_S) - (k_{-1} \cdot c_{ES}) - (k_2 \cdot c_{ES}) \tag{2.2}$$

By using a term for the total concentration of enzyme

$$c_{Et} = c_E + c_{ES} \tag{2.3}$$

one obtains

$$0 = k_1 \cdot (c_{Et} - c_{ES}) \cdot c_S - (k_{-1} \cdot c_{ES}) - (k_2 \cdot c_{ES}) \tag{2.4}$$

or

$$c_{ES} = \frac{k_1 \cdot c_{Et} \cdot c_S}{k_{-1} + k_2 + k_1 \cdot c_S} \tag{2.5}$$

By introducing the Michaelis constant K_M

$$K_M = \frac{k_{-1} + k_2}{k_1} \tag{2.6}$$

the reaction rate as a function of c_{ES}

$$v = k_2 \cdot c_{ES} \tag{2.7}$$

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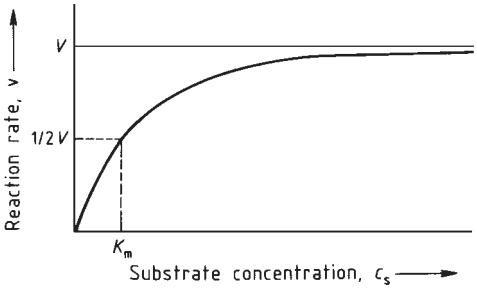

Fig. 4 Reaction rate as a function of substrate concentration (enzyme at constant concentration) For explanation of symbols, see text

and the maximum reaction rate V, which is reached, when all of the enzyme is saturated with substrate ($c_{ES} = c_{Et}$)

$$V = k_2 \cdot c_{Et} \tag{2.8}$$

one obtains the so-called Michaelis–Menten equation

$$v = \frac{V \cdot c_S}{K_M + c_S} \tag{2.9}$$

which shows the dependency of the reaction rate on the substrate concentration (first-order reaction). The plot of this relationship is given in Figure 4.

In the case of frequently occurring two-substrate reaction, a similar derivation leads to the formula:

$$v = \frac{V}{1 + \frac{(K_M)_{S1}}{c_{S1}} + \frac{(K_M)_{S2}}{c_{S2}} + \frac{(K_M)_{S1S2}}{c_{S1} \cdot c_{S2}}} \tag{2.10}$$

This is called a second-order reaction. If, however, the concentration of the second substrate is kept at a level many times of the respective Michaelis constant, then the third and fourth term of the denominator are practically zero and the equation is identical with Equation (2.9), allowing the same evaluation as with a one-substrate reaction.

Michaelis Constant As can be derived from Equation (2.9), the Michaelis constant equals the substrate concentration at half the maximal reaction rate. The value K_M can be obtained by plotting the experimentally measured reaction rate against the various substrate concentrations (Fig. 4). A more convenient way is the plot according to Lineweaver and Burk [105], using a reciprocal of the Michaelis–Menten equation

$$\frac{1}{v} = \frac{k_M}{V} \cdot \frac{1}{c_S} + \frac{1}{V} \tag{2.11}$$

A schematic plot is shown in Figure 4. The intersections with abscissa and ordinate allow the determination of the values for K_M and V. For further discussion, see [62]. The Michaelis constant approaches the dissociation constant K_s of the enzyme–substrate complex and is therefore valuable for estimating individual reaction kinetics.

Michaelis constants for enzymes usually range from 10^{-2} to 10^{-5} mol/L; a low K_M indicates a high affinity between enzyme and substrate.

Molar or Molecular Activity (Turnover Number). The efficiency of an enzyme-catalyzed reaction is indicated by the molar activity, formerly called turnover number, which is defined as the number of substrate molecules converted in 1 min by one enzyme molecule under standardized conditions. This can be calculated from the specific activity of a particular enzyme if its molecular mass is known (cf. Section 2.2.3). The average molar activity ranges from 10^3 to 10^4; peak values have been measured for acetylcholinesterase (E.C. 3.1.1.7) [9000-81-1] at 1×10^6 and for catalase (E.C. 1.11.1.6) [9001-05-2] at 5×10^6.

2.1
Factors Governing Catalytic Activity [63]

2.1.1
Temperature

The temperature dependence of enzyme-catalyzed reactions exhibits an optimum because the thermodynamic increase of reaction rate (1 in Fig. 5) is followed by a steep drop caused by thermal denaturation of the enzyme (2 in Fig. 5). The optimum is generally between 40 and 60 °C. Some temperature-insensitive enzymes may exhibit an optimum at almost 100 °C. Data on various frequently used enzymes are given in [64]. Figure 5 illustrates temperature dependence [60].

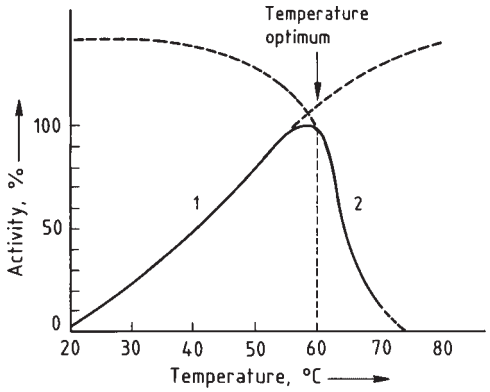

Fig. 5 Temperature optimum of enzyme activity

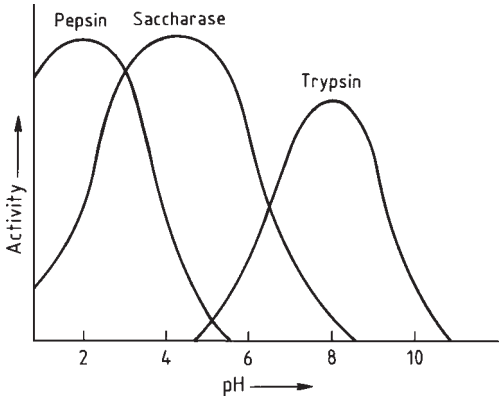

Fig. 6 Activity of various enzymes as a function of pH

2.1.2 Value of pH

All enzymes have an optimum pH range for activity. The optimum depends not only on pH but also on ionic strength and type of buffer. It may also be influenced by temperature, substrate, and coenzyme concentrations. For most enzymes, the pH optimum lies in the range from 5 to 7. Extreme values of 1.5 and 10.5 have been found for pepsin (E.C. 3.4.23.1) [9004-07-3] and for alkaline phosphatase (E.C. 3.1.3.1) [9001-78-9], respectively. Figure 6 shows some examples [60].

2.1.3 Activation

Many chemical effectors activate or inhibit the catalytic activity of enzymes. In addition to substrates and coenzymes many enzymes require nonprotein or, in some cases, protein compounds to be fully active. Enzyme activation by many inorganic ions has been adequately described. The activating ion may be involved directly in the reaction by complexing the coenzyme or cosubstrate (e.g., Fe ions bound to flavin or the ATP–Mg complex). In other cases, the ion is part of the enzyme and either acts as a stabilizer for the active conformation (e.g., Zn ions in alkaline phosphatase) or participates directly at the active site (e.g., Mn ions in isocitrate dehydrogenase (E.C. 1.1.1.42) [9028-48-2] and Zn or Co ions in carboxypeptidases).

2.1.4 Inhibition [65]

In vivo and in vitro inhibition studies of enzymatic reactions contributed important knowledge to various fields of biochemistry. For example, the mechanism of action of

many toxic substances and antidotes has been found to affect enzymes directly. In many cases, the importance of these enzymes for metabolism has been revealed. On the other hand, discovery of end-product inhibition elucidated many metabolic pathways. Enzyme inhibition can be either reversible or irreversible. Depending on the type of inhibitory effect, the following mechanisms of enzyme inhibition may be distinguished.

Irreversible Inhibition An irreversible inhibitor frequently forms a stable compound with the enzyme by covalent bonding with an amino acid residue at the active site. For example, diisopropyl fluorophosphate (DIFP) reacts with a serine residue at the active site of acetylcholinesterase to form an inactive diisopropylphosphoryl enzyme. Alkylating reagents, such as iodoacetamide, inactivate enzymes with mercapto groups at their active sites by modifying cysteine.

Reversible Inhibition Reversible inhibition, in contrast, is characterized by an equilibrium between enzyme and inhibitor. Several main groups of reversible inhibitory mechanisms can be differentiated.

Competitive Inhibition The inhibitor competes with the substrate or coenzyme for the binding site on the active center by forming an enzyme-inhibitor complex EI. In most cases, the chemical structure of the inhibitor resembles that of the substrate. Inhibition can be made ineffective by excess substrate, as is the case for inhibition of succinate dehydrogenase (E.C. 1.3.99.1) [9002-02-2] by malonate.

Noncompetitive Inhibition The inhibitor decreases the catalytic activity of an enzyme without influencing the binding relationship between substrate and enzyme. This means that inhibitor and substrate can bind simultaneously to an enzyme molecule to form ES, EI, or ESI complexes. Noncompetitive inhibition is dependent solely on the inhibitor concentration and is not overcome by high substrate concentration. An example is the blocking of an essential cysteine residue by such heavy metals as copper or mercury.

Uncompetitive Inhibition The inhibitor reacts only with the intermediary enzyme–substrate complex. An example is the reaction of azide with the oxidized form of cytochrome oxidase (E.C. 1.9.3.1) [9001-16-5]. Lineweaver–Burk plots of the reciprocal initial reaction rate $1/v_0$ versus the reciprocal substrate concentration for these three modes of reversible inhibition are shown in Figure 7 A–C [57]. The inhibitor constant K_I characterizes the inhibiting activity. The respective formulae (reciprocal from according to Lineweaver–Burk [105] are:

Competitive inhibition:

$$\frac{1}{v} = \frac{K_M \cdot \left(1 + \frac{c_I}{K_I}\right)}{V \cdot c_S} + \frac{1}{V} \tag{2.12}$$

Noncompetitive inhibition:

$$\frac{1}{v} = \frac{K_M}{V \cdot c_S} \cdot \left(1 + \frac{c_I}{K_I}\right) + \frac{1}{V} \cdot \left(1 + \frac{c_I}{K_I}\right) \tag{2.13}$$

Uncompetitive inhibition:

$$\frac{1}{v} = \frac{K_m}{V \cdot c_S} + \frac{1 + \frac{c_I}{K_I}}{V} \tag{2.14}$$

For details, see [62].

Substrate Inhibition High concentration of substrate (or coenzyme) may decrease the catalytic activity of an enzyme. Examples are the action of ATP on phosphofructokinase (E.C. 2.7.1.11) [9001-80-3] or of urea on urease (E.C. 3.5.1.5) [9002-13-5]. The Lineweaver–Burk plot [105] is given in Figure 7.

End-Product Inhibition In many multienzyme systems, the end product of the reaction sequence may act as a specific inhibitor of an enzyme at or near the beginning of the sequence. The result is that the rate of the entire sequence of reactions is determined by the steady-state concentration of the end product (Fig. 8). This type of inhibition is also called *feedback inhibition* or *retroinhibition*.

2.1.5
Allostery [66]

Cosubstrates with a central role in metabolism, such as acetyl-CoA, ATP, or AMP, may also influence the rate of reaction sequences by allosteric regulation. For example, phosphofructokinase, the first enzyme in the energy-supplying Embden–Meyerhof–Parnass pathway, is inhibited by a high concentration of ATP (i.e., positive energy balance). A high concentration of AMP, on the other hand, (i.e., energy deficiency) terminates this inhibition. Allosterically regulated enzymes have a quaternary structure and are composed of two or more structurally similar or identical subunits (protomers), each with a binding site for the substrate and another independent binding site for the allosteric effector. The binding of the effector modifies the conformation of the subunit and its active center, which then affects the conformation and hence the catalytic activity of the entire molecule. The cooperation of substrate and effector regulates the overall catalytic activity of the enzyme depending on the concentration of metabolite.

Allosteric enzymes usually do not show the classical Michaelis–Menten kinetic relationship of c_s, V, and K_M. Many allosteric enzymes give a sigmoid plot of initial rate vs. substrate concentration rather than the hyperbolic plots predicted by the Michaelis–Menten equation. The shape of the curve is characteristically changed by an allosteric activator (positive cooperativity) or an allosteric inhibitor (negative cooperativity), as shown in Figure 9.

This sigmoidal curve implies that within a certain range of substrate concentration, the enzyme is able to respond to small concentration changes by great activity changes.

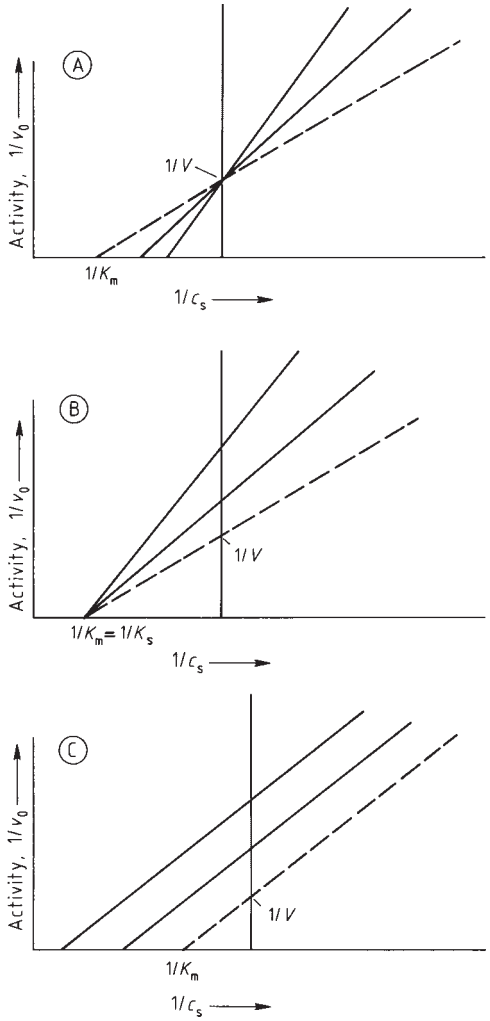

Fig. 7 Lineweaver–Burk graphs of reversibly inhibited enzyme reactions
A) Competitive inhibition;
B) Noncompetitive inhibition;
C) Uncompetitive inhibition
– – – Uninhibited reaction

2.1.6
Biogenic Regulation of Activity

In principle, enzyme activity may also be controlled by regulating the amount of enzyme in the cell. This can be accomplished by regulating the biosynthesis of individual enzymes or of several functionally related enzymes by induction or repression, or by specific attack of proteolytic enzymes (Fig. 8).

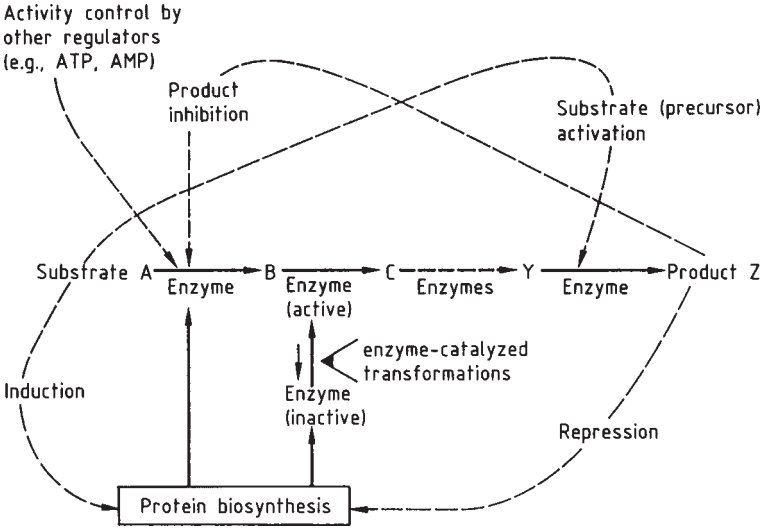

Fig. 8 Natural mechanisms for regulating enzyme activity

Chymotrypsin (E.C. 3.4.21.1) [9004-07-3], for example, is synthesized as inactive zymogen and converted to the active enzyme by trypsin (E.C. 3.4.21.4) [9002-07-7], a typical protease. A large number of natural protease inhibitors have been isolated and characterized. They act as protease antagonists and are capable of selectively affecting many proteolytic reactions. These proteinase inhibitors are proteins with molecular masses of 5000–25 000; they inhibit proteases by specific complex formation [67].

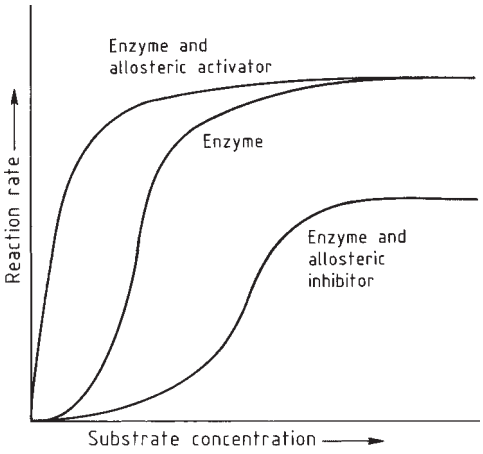

Fig. 9 Activity of an allosteric enzyme as a function of substrate concentration in absence and presence of an allosteric activator or inhibitor

Another important regulation mechanism is the modification of activity by phosphorylation and dephosphorylation, which allows quick adaptation to changing environmental conditions. For example, this takes place with hydroxymethylglutaryl-CoA reductase (NADPH) (E.C. 1.1.1.34), which is phosphorylated by [hydroxymethylglutaryl-CoA reductase (NADPH)] kinase (E.C. 2.7.1.109) [106] and dephosphorylated by [hydroxymethylglutaryl-CoA reductase (NADPH)] phosphatase (E.C. 3.1.3.47) [107]. In addition, this enzyme is regulated at the protein synthesis level and by controlled degradation.

2.2 Enzyme Assays

2.2.1 Reaction Rate as a Measure of Catalytic Activity

As biological catalysts, enzymes increase the rate of a reaction or permit it to proceed. Therefore, the conversion rate of substrates, v, is measured to determine the catalytic activity. The respective formulae for enzyme kinetics are given in Equations (2.9) and (2.10).

If in Equation (2.9) the substrate concentration is kept at a level considerably above the Michaelis constant ($c_S \gg K_M$), then all of the enzyme is saturated with substrate and the reaction proceeds with the constant and the maximum rate $v = V$ (zero order reaction). Consequently, catalytic activity is linearly dependent on the amount of enzyme used. This situation is attempted to be reached in enzyme activity measurements.

2.2.2 Definition of Units

Originally, units were defined by the investigator who first discovered and described an enzyme. Therefore, in the older literature, enzyme activity was expressed in arbitrary units, e.g., changes in absorbance, increase of reducing groups, amount of converted substrate expressed in milligrams or micromoles. These parameters were related to various time units, such as 1 min, 30 min, or 1 h.

To obtain standardized values for each enzyme, in 1961 the Enzyme Commission of the International Union of Biochemistry defined the *International Unit* U as the activity of an enzyme which, under optimized standard conditions, catalyzes the conversion of 1 μmol of substrate per minute. With respect to basic SI units, the Expert Panel on Quantities and Units (EPQU) of the International Federation of Clinical Chemists (IFCC) and the Commission on Quantities and Units in Clinical Chemistry (CQUCC) of IUPAC defined the base unit *katal* as the catalytic amount of any enzyme that catalyzes a reaction (conversion) rate of 1 mol of substrate per second in an assay system [68]. This unit, however, is not in common use.

The temperature must be stated for each assay. As a general rule, the rate of enzymatically catalyzed reactions is about doubled by a temperature increase of

10 °C in the range of 0–40 °C. The temperature must be controlled precisely and kept constant to achieve reproducible results [63].

However, for practical reasons, many enzyme reactions cannot be monitored by measuring the stoichiometric amount of substrate consumed or product formed. Therefore, the catalytic activity of a particular enzyme may be impossible to express in International Units.

The definition of units for most enzymes used in molecular biology is rather arbitrary. For example, the unit of a restriction endonuclease (E.C. 3.1.21.3–5), is defined as the catalytic activity of the enzyme that yields a typical cleavage pattern, detectable after electrophoresis, with a precise amount (usually 1 μg) of a particular DNA under defined incubation conditions [67], [70]. Other parameters that define the activity of such enzymes are the degradation of a nucleic acid or the incorporation of nucleotides into a nucleic acid, expressed in micrograms, nanomoles, absorbance units, or number of base pairs. For practical reasons, different time periods such as 1, 10, 30, or 60 min, may be chosen as references.

2.2.3 Absorption Photometry [71]

Basic Considerations Because of its simple technique and reliable, reasonably priced instruments, photometry is today one of the preferred methods of enzyme assay. It can be carried out most quickly and conveniently when the substrate or the product is colored or absorbs light in the ultraviolet region because the rate of appearance or disappearance of a light-absorbing product or substrate can be followed with a spectrophotometer.

According to the Bouguer–Lambert–Beer law, which is valid for very dilute solutions, the following relationship exists between absorbance A and concentration:

$$A = \log \frac{I_0}{I} = \varepsilon \cdot c \cdot d \tag{2.15}$$

and

$$c = \frac{A}{\varepsilon \cdot d} \tag{2.16}$$

where c is the concentration in millimoles per liter, ϵ the millimolar absorption coefficient in liters per mole and per millimeter, and d the path length in millimeters.

The catalytic activity z then corresponds to the absorbance change per minute.

$$z = \frac{\Delta A \cdot V \cdot 1000}{\varepsilon \cdot d \cdot \Delta t} \tag{2.17}$$

where V is the assay volume in liters and t the time in minutes. The unit of z is then micromoles per minute and corresponds to the definition of the International Unit U given in the previous section.

Example 1: Assay of Lactate Dehydrogenase (*LDH*). In the reaction catalyzed by LDH (E.C. 1.1.1.27) [9001-60-9], hydrogen is transferred from NADH to pyruvate, to yield L-lactate and NAD [72]:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{L-Lactate} + \text{NAD}^+ \tag{2.18}$$

The reduced coenzyme NADH absorbs at 340 nm, whereas the oxidized form NAD, lactate, and pyruvate do not. Thus the progress of Reaction (2.18) can be followed by measuring the decrease in light absorption at 340 nm with a mercury line photometer emitting at 334 or 365 nm. The enzyme can also be measured by monitoring the reverse reaction under slightly alkaline conditions. However, the reverse reaction is much slower than the reaction starting with pyruvate [73].

The principle of the optical assay may also be used to follow an enzymatic reaction in which neither the substrate nor the product has any characteristic light absorption maxima. In that case, the reaction is coupled to some other enzymatic reaction which can be followed easily by photometry. The activity of the nonabsorbing enzyme system can then be measured if the enzyme considered is made the rate-determining component by appropriate choice of assay conditions.

Example 2: Assay of Pyruvate Kinase (*PK*). The reaction between phosphoenolpyruvate and ADP yields pyruvate and ATP by transfer of a phosphate group; it is catalyzed by pyruvate kinase (E.C. 2.7.1.40) [9001-59-6]:

$$\text{Phosphoenolpyruvate} + \text{ADP} \rightarrow \text{Pyruvate} + \text{ATP} \tag{2.19}$$

This reaction is easily measured when a large excess of LDH and NADH is added to the system, which couples Reactions (2.19) and (2.18) [74]. The formation of pyruvate in Reaction (2.19) is followed by the very rapid reduction of pyruvate to lactate in Reaction (2.18). For each molecule of pyruvate formed and reduced, one molecule of NADH is oxidized to NAD, causing a decrease in light absorption at 340 nm.

The catalytic activity of enzymes such as phosphatases [75], whose natural substrates do not have suitable spectral properties, can be determined by using a colorless synthetic substrate, which is split enzymatically to yield a colored product.

Example 3: Assay of Phosphatase. For the assay of phosphatases, glycosidases, and several other hydrolases, colorless 4-nitrophenyl compounds are incubated, and 4-nitrophenolate is formed under alkaline conditions with a characteristic maximum between 400 and 420 nm. In this way, the catalytic activity of such enzymes can be measured conveniently at this wavelength:

$$\text{4-Nitrophenyl phosphate} + \text{H}_2\text{O} \rightarrow \text{4-Nitrophenol} + \text{Phosphate} \tag{2.20}$$

$$\text{4-Nitrophenol} + \text{OH}^- \rightarrow \text{4-Nitrophenolate} \tag{2.21}$$

Another principle involves the absorption of a colored metabolite formed directly or indirectly, by the action of the enzyme being analyzed. Again, the activity of the enzyme can be determined only if it is made the rate-determining component by proper choice of conditions.

Example 4: Assay of Glycerol Phosphate Oxidase. Among the class of oxidoreductases utilizing O_2 as the sole final electron acceptor, many enzymes exist that generate H_2O_2 during oxidation of their individual substrates [76]. Highly sensitive and accurate colorimetric assays of H_2O_2 have been developed during the last two decades [77]; in one system, phenol gives a purple color with 4-aminoantipyrine in the presence of oxidizing agents. Therefore, this color reaction can be used for colorimetric determination of H_2O_2-generating enzyme reactions with peroxidase as oxidation catalyst and indicator enzyme. Although this principle is used predominantly to determine metabolites in body fluids, it is often employed in the assay of such enzymes as glycerol phosphate oxidase (E.C. 1.1.3.21) or glucose oxidase (E.C. 1.1.3.4) [9001-37-0].

The catalytic activity of the first is determined according to the following reaction sequence:

$$2\,\text{L-}\alpha\text{-Glycerol phosphate} + 2\,O_2 \rightarrow 2\,\text{Dihydroxyacetone phosphate} + 2\,H_2O_2 \quad (2.22)$$

$$2\,H_2O_2 + \text{4-Aminoantipyrine} + \text{Phenol} \rightarrow \text{Quinonimine dye} + 4\,H_2O \quad (2.23)$$

The broad absorption maximum of the quinonimine dye is centered around 500 nm, with an absorption coefficient of about 13×10^2 L mol^{-1} mm^{-1}. The increase in absorbance per minute at 500 nm is measured to determine the activity of glycerol phosphate oxidase.

2.2.4
Fluorometry [78]

The fluorometric method is rarely used for determining the catalytic activity of raw or purified enzyme preparations. Because of its high sensitivity, it permits the assay of small amounts of enzymes in organs or tissue sections [79]. For example, systems that depend on NAD and NADP can be measured by fluorometry; the reduced pyridine coenzymes exhibit a fluorescence of low intensity. To enhance sensitivity, the oxidized form is treated with alkali, to yield strongly fluorescing compounds; in addition, selective filters must be used. The overall sensitivity of this method is a thousand times that of absorption photometry.

2.2.5
Luminometry [80]

Luminometry uses fluorescence, phosphorescence, and chemiluminescence as detector systems. Chemiluminescence observed in living organisms is termed bioluminescence. Bioluminescence is catalyzed by enzymes called luciferases, whose substrates, known as luciferins, are converted to light-emitting products.

In luminometry, the number of photons emitted by the reaction system per unit time is measured with specially designed instruments called luminometers; they are based on single photon counting detectors, usually photomultiplier tubes.

An example is the reaction catalyzed by the luciferase from *Photinus pyralis*, *Photinus*-luciferin 4-monooxygenase (ATP-hydrolyzing) (E.C. 1.13.12.7) [61970-00-1]:

$$\text{ATP} + \text{D-Luciferin} + O_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + \text{pp} + CO_2 + 0.9\,h\nu \quad (2.24)$$

In this reaction, ATP is consumed as a substrate, and photons at a wavelength of 562 nm are emitted. The quantum yield is 0.9 einstein per mole of luciferin, i.e., for one ATP molecule consumed, approximately one photon is emitted. This reaction is therefore suitable for the assay of ATP and, hence, of enzymes that catalyze ATP-consuming or ATP-producing reactions.

To monitor enzyme activity with the aid of firefly bioluminescence, the light intensity must increase linearly for several minutes and must be strictly proportional to the catalytic activity of the enzyme. Such measuring conditions have been realized for determination of the catalytic activity of creatine kinase (E.C. 2.7.3.2) [9001-15-4]:

$$\text{Creatine phosphate} + \text{ADP} \rightarrow \text{Creatine} + \text{ATP} \tag{2.25}$$

Other reactions that depend on NAD(P) can be followed by using the bioluminescence from lucibacteria.

2.2.6 Radiometry

When radioactively labeled substrates are used, the activities of some enzymes can be determined with high sensitivity. This technique is widely employed in the field of molecular biology to monitor (1) the incorporation of radioactively labeled nucleotides into acid-insoluble nucleic acids or polynucleotides (DNA and RNA polymerases), (2) the decomposition of radioactively labeled DNA (exonuclease III), (3) the transfer of a radioactively labeled phosphate group from γ-^{32}P-ATP [2964-07-0] to the 5′-hydroxyl end of a polynucleotide (polynucleotide kinase), or (4) the exchange of radioactively labeled pyrophosphate on a carrier matrix (T_4 DNA ligase). The most common isotopes for labeling are ^{32}P, ^{14}C, ^{3}H, and to a minor degree, ^{35}S.

In experiments with radioactively labeled compounds, special safety and legal regulations must be observed. In some determinations, however, the amounts of radioactive material needed are below the limits regulated by law.

2.2.7 Potentiometry [81]

A pH-sensitive glass electrode can be used to measure reactions in which protons are produced or consumed. For this purpose, the pH is kept constant by countertitration, and the consumption of acid or base required to do this is measured. The electrode controls an automatic titrator, and this concept is the *pH-stat* technique described by Bücher [82]. A typical example is the determination of the catalytic activity of lipase (E.C. 3.1.1.3) [9001-62-1] [83]. A fat (triglyceride) is hydrolyzed by this enzyme, and the fatty acid formed is neutralized by countertitration with NaOH in a pH-stat mode:

$$\text{Triglyceride} + H_2O \rightarrow \text{Diglyceride} + \text{Fatty acid} \tag{2.26}$$

The substrate, olive oil, is incubated with the diluted sample containing lipase, and the mixture is titrated at constant pH. A recorder plots the consumption of NaOH vs. time, and the resulting slope correlates with catalytic activity. Other examples are the assay of papain (E.C. 3.4.22.2) [9001-73-4] and glucose oxidase (E.C. 1.1.3.4.) [9001-37-0].

2.2.8 Conductometry

In principle, all enzymatic reactions that lead to a change in overall ionic mobility can be measured by conductometry. In this way, the elastolytic activity of elastase (E.C. 3.4.21.36) by using unmodified elastin as substrate has been determined [84]. Other applications have also been described [85]. In this reaction, protons are liberated by cleavage of peptide bonds.

2.2.9 Calorimetry

Many enzymatic reactions evolve heat; therefore, some interest in calorimetric (enthalpimetric) methods has developed. In a suitable experimental arrangement, a temperature sensor serves as a device for measuring the catalytic activity of the enzyme. A new area of analytical chemistry has developed from this approach. Previously called microcalorimetry, it is now commonly known as enthalpimetry [86]. The method is used mainly in research.

2.2.10 Polarimetry

Polarimetry is rarely used, partly because of the inconvenience involved. However, it is required in determining the catalytic activity of mutarotase (E.C. 5.1.3.3.) [9031-76-9], which catalyzes the equilibrium between α- and β-glucose [87].

2.2.11 Manometry

Manometry is one of the classical methods in biochemistry. It is no longer used for routine assay of enzymes. Formerly, the catalytic activity of glucose oxidase [88], arginase (E.C. 3.5.3.1) [9000-96-8] [89], and other enzymes was determined by this method.

2.2.12 Viscosimetry

Viscosimetry has been practically abandoned for enzyme assays. Formerly, e.g., cellulase (E.C. 3.2.1.4) [9012-54-8] activity has been determined by the change of

viscosity per unit time [90]. Nowadays the cellulase assay is performed with a colorimetric reaction [108]:

$$\text{Cellulose} \rightarrow \text{Oligosaccharide} + n\,\text{Glucose} \rightarrow \text{Red dye} \tag{2.27}$$

2.2.13 Turbidimetry

The turbidimetric method can be adapted for different enzyme assays [109]. As an example, lysozyme (muramidase, E.C. 3.2.1.17) cleaves bacterial cell walls. A standard substrate suspension (dried germs of *Micrococcus luteus)* is used. The decrease of absorbancy is measured at 450 nm and 25 °C.

2.2.14 Immobilized Enzymes [91]

Immobilized enzymes are used in analytical chemistry and as catalysts for the production of chemicals, pharmaceuticals, and food (Section 2.2.14). They also serve as simple and well-defined models for studying membrane-bound enzymes.

Because of their particular structure, immobilized enzymes require specific assays. In addition to requiring optimal conditions different from those of soluble enzymes, particle size, particle-size distribution, mechanical and chemical structure, stability and structure of the matrix, and the catalytic activity used for immobilization must be considered.

At least two different assay procedures are used for insoluble enzymes: one employs a stirred suspension in a vessel; the other, a packed bed or a column reactor. The conditions of such assay are very close to those used in industrial applications. Enzyme activity can be assayed continuously or batchwise.

Conductometry, potentiometry, and polarimetry are better suited to detection than photometry, because they allow the reaction to be followed directly, without the need for additional indicator enzymes, coenzymes, or second substrates. An example is the assay of immobilized penicillin amidase (E.C. 3.5.1.11) [9014-06-6] [92]:

$$\text{Penicillin G} + H_2O \rightarrow \text{6-Aminopenicillanate} + \text{Phenyl acetate} + H^+ \tag{2.28}$$

2.2.15 Electrophoresis

Electrophoresis is an indispensable tool for determining the catalytic activity of nucleases, especially restriction endonucleases. It is also used for other important enzymes in genetic engineering, e.g., DNA methylases. Restriction enzymes catalyze the specific cleavage of DNA, e.g., that of *Escherichia coli* phage λ DNA, which is a typical substrate in genetic engineering (see Chap. Nonidusrial Enzyme Usage). The DNA is split into smaller fragments of defined lengths. Because the negative charge per base

pair is the same for all fragments, these can be separated according to length. The products of cleavage can be separated from intact or incompletely split molecules by electrophoresis under conditions that are carefully optimized for each specific enzyme–substrate reaction. Electrophoresis is usually performed with high-quality agarose gels as carrier material. Each resolution problem requires a specific agarose concentration, e.g., a relatively low concentration (0.5 g per 100 mL) for large fragments and a relatively high concentration (1.6–2 g per 100 mL) for small fragments. When very small fragments must be separated, polyacrylamide gels may be used as an alternative carrier material. Separated DNA fragments are visualized by staining with the fluorescent dye ethidium bromide [1239-45-8].

The fragments can be labeled by staining the gel in a separate tank after completion of electrophoresis or, more easily, by electrophoresis in the presence of the dye in the gel and buffer (e.g., at 1 μg/mL). When the gel is illuminated with long-wavelength UV light (e.g., at 366 nm), the separated fragments become visible and can be photographed.

To calculate enzyme activity, the minimum amount of enzyme must be estimated which converts a given substrate completely to the fragment pattern typical for that enzyme. For practical reasons, this amount is divided by the volume of the enzyme solution.

As an example, electrophoretic assay of the restriction enzyme *Hin*dIII is described briefly (Fig. 10) [69].

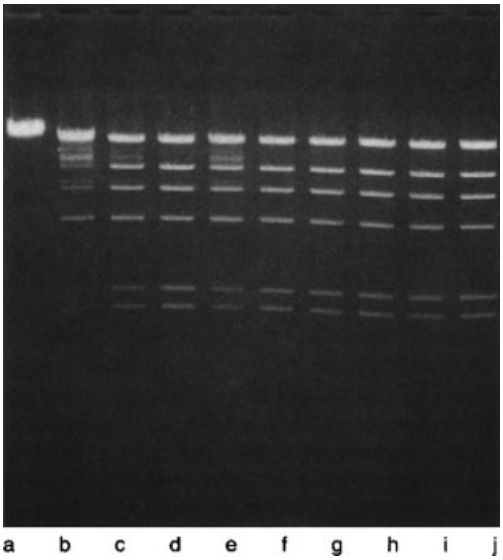

Fig. 10 Determination of the catalytic activity of *Hin*dIII on λ DNA
a) λ DNA without enzyme; b–d) 1, 2, and 3 μL, respectively, 1 : 30 dilution; e–g) 1, 2, and 3 μL, respectively, 1 : 20 dilution; h–j) 1, 2, and 3 μL, respectively, 1 : 10 dilution

Definition of unit: one unit is the catalytic activity of *Hin*dIII, which completely splits 1 μg of λ DNA in a total volume of 0.025 mL. The reaction is terminated after 60 min incubation at 37 °C in 0.025 mL of a defined buffer mixture.

Assay: different volumes (1–3 μL) of several enzyme dilutions (1 : 10, 1 : 20, 1 : 30) are incubated with 1 μg of λ DNA in a total volume of 0.025 mL. The reaction is terminated after 60 min by cooling with ice and adding 0.015 mL of a stop solution; the mixture (0.02 mL) is then placed in the slots of an agarose gel.

Agarose gel: the gel consists of 1 g of agarose per 100 mL and 1 mg of ethidium bromide per liter. The gel dimensions are 200×200 mm; total volume is 250 mL; and slots of 1×7 mm are prepared with a comb.

Electrophoresis: the apparatus is designed for submarine electrophoresis (2 h at 100 V). The buffer system is tris(hydroxymethyl)aminomethane–acetate, 40 mmol/L, and disodium ethylenediamine-tetraacetate, 2 mmol/L; pH 8.2. The buffer contains 1 mg of ethidium bromide per liter.

Detection: the gel is illuminated directly after electrophoresis with long-wavelength UV light (366 nm) and photographed (Polaroid CU 5/film type 107); the amount of enzyme is estimated at which the complete typical fragment pattern is obtained [e.g., slot (c) in Fig. 10]. The activity is calculated according to

$$\text{Activity} = \frac{\text{Dilution}}{\text{Sample volume, mL}} \times \frac{\text{Units}}{\text{Volume, mL}} \tag{2.29}$$

(of original enzyme solution) In the example, complete digestion was obtained with a minimum of 0.003 mL at 1 : 30 dilution of the original enzyme solution, resulting in an activity of 10 000 U/mL.

2.3 Quality Evaluation of Enzyme Preparations

2.3.1 Quality Criteria

The quality of enzyme preparations is characterized by activity, purity, stability, formulation, and packaging. These parameters depend on each other, but the formulation and packaging are easy to control and keep constant. The other parameters influence each other in such a way that quality is considered to be a function of activity, purity, and stability [93].

2.3.2 Specific Activity

One of the most important quality criteria of an enzyme preparation is its *specific activity,* i.e., the catalytic activity related to its protein content. Specific activity is usually expressed as units per milligram or, for less purified products, units per gram (see Section 2.2.2).

Specific activity data can be evaluated correctly only if the specific activity can be compared with that of a highly purified enzyme of the same origin. For this purpose, catalytic activities must be measured under identical conditions including the determination of protein.

2.3.3 Protein Determination

Since protein content is the most important reference point for determination of the specific activity of an enzyme preparation, several methods of protein determination are briefly mentioned in the following paragraphs [94]. All of these procedures are based on different principles and depend on the amino acid composition of the enzyme proteins. They will, therefore, yield different values.

Ultraviolet Absorption Because of their content of aromatic amino acids, proteins exhibit an absorption maximum at 270–280 nm. For many pure proteins, reference values have been established for the 280-nm absorbance of a solution containing 10 mg/mL ($A_{280}^{1\%}$). WARBURG and CHRISTIAN found a formula which accounts for the nucleic acid content [95]. For greater precision, absorbance is also measured at shorter wavelengths, e.g., 235 nm [96].

Biuret Method [97] The reaction of peptide bonds with copper ions in an alkaline solution yields a purple complex which can be determined photometrically. The intensity is a linear function of protein concentration.

BCA Method [110] The BCA method of the company Pierce is used for many protein samples. It combines the biuret method with the features of BCA:

$$\text{Protein} + \text{Cu}^{++} + \text{Bicinchoninic acid} \rightarrow \text{Red dye}\,(\text{Cu}^{+}\ \text{complex}) \qquad (2.30)$$

The complex allows the spectrophotometric quantitation of the protein in aqueous solutions.

Lowry Method [98] The *Lowry* method combines the biuret reaction of proteins with reduction of the *Folin–Ciocalteu* phenol reagent (phosphomolybdic–phosphotungstic acid) by tyrosine and tryptophan residues. The reduction is promoted by the copper–protein complex. This method is very sensitive, but it is affected by many other compounds. The method has been modified to overcome these problems and to obtain a linear relationship between absorbance and protein content.

Protein-Dye Binding Attempts have been made to determine protein concentration by using dyes. The method published by BRADFORD now predominates [99]. It is based on the shift of the absorption maximum of Coomassie Brilliant Blue G 250 [6104-58-1] from 465 to 595 nm, which occurs when the dye binds to the protein.

Kjeldahl Analysis Before colorimetric procedures were established, protein concentration was calculated from nitrogen content by using an empirical factor [97].

2.3.4
Contaminating Activities

The content of contaminating activities, that is, the presence of other enzymes in the original material, is an important quality criterion for enzymes. This is usually related to the activity of the main enzyme and expressed in percent. Since the absolute amount is often very small, it cannot be expressed as protein mass (in milligrams) and, thus, does not influence the overall specific activity of the enzyme preparation. For example, lactate dehydrogenase from rabbit muscle has a specific activity of 500 U/mg and contains 0.001 % pyruvate kinase. Even if the content of pyruvate kinase increased tenfold to 0.01 %, the corresponding change in specific activity could not be measured. However, this contaminating activity is so high that such an enzyme preparation is useless for the determination of pyruvate kinase in blood.

Depending on the special applications of an enzyme, different impurities must be determined. For example, enzymes used in genetic engineering all act on a common substrate, nucleic acid; therefore, they must be free from impurities that also act on that substrate, such as specific or unspecific nucleases or phosphatases. Traces of unspecific endodeoxyribonucleases, for example, are detected routinely by incubating 10–100 U of an enzyme for a prolonged period (e.g., 16 h) with a susceptible substrate such as the supercoiled form of a plasmid DNA, e.g., pBR322. A minimum of 50 units should not influence the structure of that substrate, whereas another application may require that only 10 units do not change substrate structure. Such impurities are usually detected by electrophoresis (see Section 2.2.15) or by radiometry (see Section 2.2.6).

2.3.5
Electrophoretic Purity

Electrophoresis is important in the evaluation of purity because of its sensitivity (detection of less than 50 μg/mL contaminating protein is possible). It is far inferior to the determination of contaminating activities. Furthermore, enzymatically inactive proteins usually do not interfere with enzymatic analyses. Electrophoresis gained importance when isoenzymes had to be analyzed which could not be distinguished by their catalytic function but only by physical properties such as electric charge. It is an indispensable tool for the identification of lactate dehydrogenase isoenzymes.

Electrophoresis is also used in the isolation of various enzymes of RNA, DNA, and protein biosynthesis. For this purpose, the introduction of disc electrophoresis on polyacrylamide gels and the use of dodecyl sulfate for the separation of enzyme complexes became valuable tools [100]. The system developed by Laemmli [101] is widely used and exhibits very high sensitivity, especially if gradient gels are employed [102]. The sensitivity of the method has been enhanced considerably by silver staining [103].

Electrophoresis with 2-dimensional gels (2D PAGE) and isoelectric focussing (IEF), which separates the proteins according to their isoelectric points are commonly used methods [111], [112]. The Phast-system (Pharmacia) [113] and the electroblotting of

biomolecules is applied for automatic gel electrophoresis. It consists in the transfer of electrophoretically separated proteins from the polyacrylamide matrix onto a ''protein absorbing'' membrane in an electrical field.

2.3.6 High-Performance Liquid Chromatography [111]

Since the 1980s the method has gained popularity for proteins. HPLC-assays are used to test enzymes for purity or to obtain information about isoenzymes. The pattern of the peaks is evaluated. Examples are peroxidase (E.C. 1.11.1.7), alkaline phosphatase (E.C. 3.1.3.1) and catalase (E.C. 1.1.1.6).

2.3.7 Performance Test

For many applications, partially purified enzyme preparations can be used, provided they do not contain any interfering contaminating activities. They are less costly and, therefore, preferred to highly purified products. However, they may contain unknown byproducts that can interfere with enzymatic analyses, for example. To avoid such problems, a performance test should be carried out. Examples are the determination of glucose with glucose oxidase and peroxidase (E.C. 1.11.1.7) [9003-99-0] or the determination of glycerol with glycerokinase (E.C. 2.7.1.30) [9030-66-4].

For some enzymes used in molecular biology, the determination of activity is not directly correlated to their application. In such cases, even highly purified enzymes must be analyzed for proper function in a typical performance experiment. An example is T_4 DNA ligase (E.C. 6.5.1.1) [9015-85-4] which functions properly by joining together fragments created by the action of a restriction endonuclease; in addition, the joined fragments can be recleaved by the same restriction endonuclease.

2.3.8 Amino Acid Analysis and Protein Sequence Analysis

Both methods are routinely used to estimate the amount and to determine the composition of enzymes. For amino acid analysis, the protein is first cleaved by complete hydrolysis (enzymatically or chemically). Then the released amino acids are assayed by quantitative chromatography. The amino acids require derivatization to improve their chromatographic behavior or their detectability. Although this is a laborious method, it is used frequently [115].

Sometimes it is necessary to characterize the enzyme sequence in detail, e.g., to check for purity this way. A useful procedure is the Edman degradation [116], [117]. With this method, the *N*-terminal amino acid is cleaved from a peptide or a protein, derivatized with phenylisothiocyanate and identified. Then the reactions are repeated with the next amino acid. Today, many instruments perform the Edman degradation automatically.

2.3.9
Stability [93]

A very important factor in the application of enzymes is their stability in concentrated or dilute form and after mixing with other substances. This applies to the manufacture of products for pharmaceutical purposes, food chemistry, or enzymatic analysis. Some enzymes can be stabilized by adding glycerol (50 vol %), ammonium sulfate (ca. 3.2 mol/L), or sodium chloride (3 mol/L) to their aqueous solution. Furthermore, many enzymes can be kept in lyophilized form for a long period of time in the presence of stabilizers such as salts, preservatives, inert proteins (predominantly bovine serum albumin), or carbohydrates.

Most enzymes used in analysis are stored at ca. 4 °C; solutions of restriction endonucleases must be kept at −20 °C or lower to maintain catalytic activity. To avoid degradation by moisture, the chilled enzyme preparation must be warmed before opening. Freezing and thawing may, in some cases, impair the activity of enzymes. Contamination by heavy metals or oxidants often inactivates enzymes, for example, by blocking mercapto groups. The activity of metalloenzymes or metal-dependent enzymes may be reduced by complexing agents such as ethylenediaminetetraacetate.

Especially for applications in commercial test kits some enzymes are modified by immobilization [118], for example, by covalent crosslinking with biopolymers like cellulose or dextrane. This improves the stability in solutions and/or the heat resistance.

2.3.10
Formulation of Enzyme Preparations

An enzyme preparation should be formulated according to its application. For analytical purposes, it should be easy to pipette and — if possible — free of stabilizers and preservatives that might impair its function. For example, glutamate dehydrogenase (E.C. 1.4.1.3) [9029-12-3] must not contain any traces of ammonia if it is to be used in the enzymatic determination of urea or ammonium.

In reagent kits employed for enzymatic analysis in clinical laboratories or for food analysis, the enzyme may be used preferably in lyophilized form. Compared to enzyme solutions, the solid material is in some cases easier to mix with other solid components and stable for a longer period of time, even at slightly elevated temperature. When immobilized enzymes are to be used in columns, their particle size must ensure fast flow.

Packaging Careful selection of packaging materials is very important for handling enzymes. Bottles and stoppers used for lyophilized enzymes must be absolutely tight to prevent access of moisture. Glass or plastic bottles as well as stoppers (rubber or plastic) should not release any traces of heavy metals or other enzyme-inactivating substances into the enzyme solution or suspension. In some cases, enzymes must be protected from light and packaged in brown glass bottles.

3
General Production Methods

3.1
Microbial Production

Use of an aerobic submerged culture in a stirred-tank reactor is the typical industrial process for enzyme production involving a microorganism that produces mostly an extracellular enzyme. This section concentrates on the typical process, with only short references to variations. Figure 11 shows the unit operations of the production process, and basic information can be found under → Biotechnology and in handbooks on microbiological principles and methods [119–121]. This section is concerned with fermentation itself, and three elements — the organism, the equipment, and the protocol for fermentation — are discussed. These elements must be arranged in the most effective way possible to realize economically optimal results in industrial processes. Optimization of biotechnological processes is a multidisciplinary effort; for further information, see [122–124].

In the past, biotechnological process development may have looked somewhat like an art because adequate process control was lacking. This chapter should show that, based on the present state of the art, a production process can be highly controlled. To achieve this level of control in fermentation, the organism must be genetically adapted, and the process protocol must match the physiological possibilities of the organism and the limitations of the apparatus.

3.1.1
Organism and Enzyme Synthesis

A variety of different microorganisms are used for the industrial production of enzymes. They cover the taxonomic gamut from eukaryotic systems such as yeasts and fungi to prokaryotic systems from both the gram-negative and gram-positive families. When biopharmaceutical enzymes are considered as well, mammalian and insect cell lines also come into play. For most of the history of enzyme applications, production occurred in the strain known to make the enzyme of interest. This explains why so many different types of microorganisms have been employed to make enzymes. Alkaline protease is naturally secreted by *Bacillus licheniformis* to break down proteinaceous substrates and resulted in one of the first commercially produced

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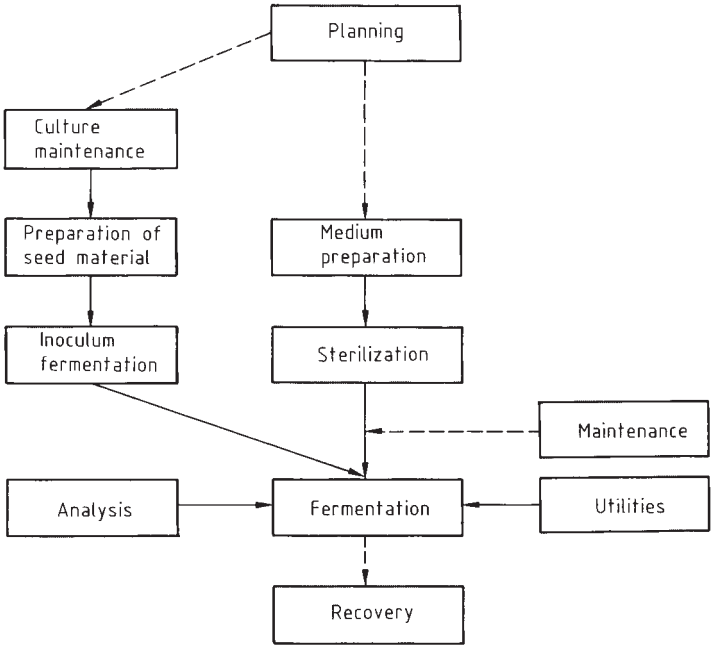


Fig. 11 Unit operations of a fermentation process

enzymes, subtilisin Carlsberg, for use in detergents. Strains were selected that produced higher levels of protease, and an industry was born. A similar story was followed for α-amylase production. Again, *Bacillus licheniformis* naturally secreted a highly thermostable α-amylase capable of breaking down starch to more easily digestible oligosaccharides. Strains of *Bacillus* have been one of the workhorses of enzyme production for decades, based mainly upon their ability to overproduce subtilisin and α-amylase [125].

Amylase from *Bacillus* has historically been used to liquefy starch. However, to break it down completely to single units of glucose, a second enzyme, namely, glucoamylase, is required. The most widely used enzyme for glucose production from starch is the glucoamylase from strains of the fungal genus *Aspergillus* [126]. Overproducing strains have been isolated over the years that have led to high production of glucoamylase.

An acid cellulase enzyme complex is found in the fungus *Trichoderma* [127]. This enzyme mixture was thought to be capable of breaking down cellulosic substrates to glucose, similar to the starch-degrading enzymes. This particular application was not initially commercialized. Instead, it has found applications in the treatment of textiles. New programs to improve the enzyme complex and its expression are resurrecting its potential use as an additive for the breakdown of cellulose. As with the other systems above, more productive strains have been isolated.

A final example is the production of glucose/xylose isomerase. Glucose isomerase catalyzes the rearrangement of glucose to fructose, yielding a product with a sweeter taste. The enzyme discovered to be capable of industrial conversion originated from a

species of *Steptomyces*. As with the others, the native host strain has been used for production of glucose isomerase.

All of the strain types described in the examples above are capable of differentiation. For example, *Bacillus* thrives in nature due to its ability to survive various insults by forming a non growing, yet viable, spore. The spore remains dormant until it reaches an environment where it can germinate and divide. Regulation of protease production is associated with this differentiation. This highly complex behavior has been difficult to model for aiding process development. The same can be said of the fungal species *Aspergillus* and *Trichoderma*. Their differentiation makes process development and modeling difficult. Although *Streptomyces* does not truly sporulate, it differentiates by forming filaments as opposed to isolated individual cells. This property also has an effect on product formation and physical properties of the fermentation broth.

An organism can be viewed as a metabolic system converting substrates into cell mass and byproducts. Enzymes function in this system as catalysts for the different reactions. Each cell is equipped with mechanisms that regulate the synthesis and activity of the enzymes to enable the cell to respond adequately to environmental changes. Therefore, in its elementary form, an organism can be described as a set of metabolic components with a mechanism for enzyme synthesis and a regulatory apparatus. The kinetics of the process are determined by structural components of the organism and by various physical and chemical factors, as shown in Figure 12. The key role that enzymes play in biological processes has led to an extensive study of both the mechanism and the regulation of their synthesis [128], [129], and some of the results are related to the problem of process development.

The basic mechanisms of enzyme synthesis, including transcription, translation, and posttranslational processing, seem to be highly conserved [124]. However, several differences exist between various classes of organisms, as well as some fundamental differences between prokaryotic and eukaryotic organisms. The enzymes themselves

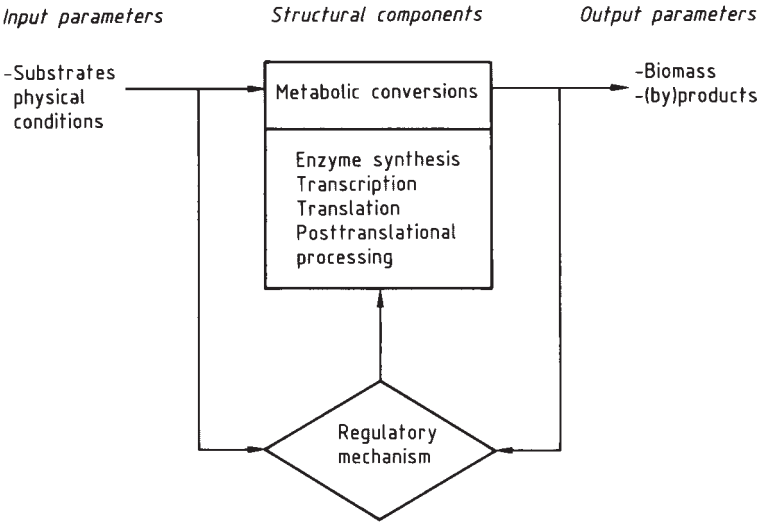


Fig. 12 Scheme of the metabolic process

differ enormously in molecular mass, number of polypeptide chains, isoelectric point, and degree of glycosylation. In addition, a variety of enzyme-producing species exist [131]. Although all the differences may influence the characteristics of synthetic patterns, the basic mechanisms underlying enzyme synthesis are similar enough to allow a general treatment of the microbiological production process. However, the differences in production kinetics among various species are large enough to make individual optimization programs necessary.

Different organisms may also differ in their suitability for fermentation; such process characteristics as viscosity or recoverability, legal clearance of the organism, and knowledge available on the selected organism, must be considered.

Because of the action of the regulatory mechanism, enzyme synthesis rates range from no synthesis to maximum synthesis allowed by the synthetic apparatus, as in a normal control loop. The complexity of mechanisms ranges from relatively simple and well-understood induction and repression systems, to very complex global regulatory networks [132].

Process development must deal with the complexity of the enzyme synthesis system either by changing the structural characteristics, including structural elements of the regulatory systems (see Section 3.1.2), or by selecting optimal environmental conditions (see Section 3.1.3).

3.1.2
Strain Improvement

Most of the strains used for enzyme production have been improved through classical selection. Mutagenesis by chemical agents or UV radiation has been used to more quickly find useful variants. Many cells must be subjected to a mutation or recombination procedure and then tested for the desired combination of characteristics by selection. The success of strain improvement programs often depends on development of an effective selection method for finding one mutant among 10 000–100 000 cells. Methods range from plate selection to the continuous culture technique [137], [138].

Mutation changes the protein structure and most probably results in a deterioration of function. Changes in structural components by mutation are therefore rarely improvements unless the specific loss of function is required for production purposes, e.g., when a loss of regulatory function results in enhanced enzyme production.

Mutation and selection are directed primarily toward higher overall productivity rather than mutation of a specific function, but a loss of regulatory function is highly probable. However, some studies describe screening for a mutation in a specific function [135].

Based on the complex interdependency of pathways and the competition for substrates in the organism, random mutation may also affect the rate of enzyme synthesis. However, only minor improvements should be expected, although a series of mutations can still result in an interesting degree of improvement. In vivo recombination techniques have been used to complement mutation techniques by bringing

together mutations in different cell lines and cleaning strains of undesired deleterious mutations [122]. Strain improvement has been revolutionized by the advent of genetic engineering. The ability to specifically improve strains by the manipulation of the host genome or the addition of extra-chromosomal DNA elements has greatly increased the speed of strain improvement. In the early stages, most work was performed in strains for which methods to manipulate DNA were established. Attempts were made to express many different enzymes in *Escherichia coli*. However, *E. coli* typically does not excrete such proteins and instead deposits them in an insoluble form (called inclusion bodies) in the cytoplasm of the cell. It proved more effective to develop DNA manipulation techniques in the native host strain of the different enzymes. Today, nearly all production strains used commercially have been genetically engineered. Strains have been improved through extra copies of the gene of interest, removal of inhibitory regulation, and enhancement of positive regulation.

If the absence of regulation is assumed, considerable improvement can be realized by increasing the turnover rate of the limiting step in synthesis. Techniques of genetic engineering can be used to increase the rate of mRNA synthesis by constructing plasmids with the desired gene (gene multiplication). The number of plasmids per cell can be very high. The rate of mRNA synthesis should be related linearly to the number of gene copies, but the rate of enzyme synthesis may be limited again by the next step in the process. These techniques also allow replacement of the promoter by a more effective one [139]. The same methods allow replacement of the leader sequence of a gene, resulting in the excretion of a formerly intracellular enzyme [140].

Genetic engineering also uses microorganisms to produce enzymes of higher organisms by placement of the corresponding gene into the microorganism. The presence of introns may then prevent proper expression of the gene, but techniques have been developed to overcome this difficulty [136]. Chymosin (E.C. 3.4.23.4) [9001-98-3], calf rennet, has been cloned by several groups either in prokaryotes or in yeast, one of the first cloned mammalian enzyme to be produced industrially by microorganisms [141].

Novel methods to improve strain performance are under development (ca. 2002). These include gene shuffling and directed evolution. Most of the literature regarding these techniques has focused on the improvement of specific enzyme properties (see Section 4.2). However, directed evolution is now being applied to the overproduction of given enzymes as well. By mutagenizing a plasmid harboring a gene for subtilisin, enzyme production was increased five fold [142]. It was shown that the structural gene was unchanged and that the effect was most likely due to an improved promoter or higher plasmid copy number leading to higher rates of transcription.

3.1.3 Physiological Optimization

Figure 12 shows that not only the structural characteristics of the system, but also the input parameters, determine its metabolic activity. Once a suitable organism has been

found, either genetically improved or not, the next task of process development is to define optimal input parameters.

The rate of enzyme synthesis r_e is defined by:

$$r_e = q_e \cdot c_x$$

where r_e is the enzyme synthesis rate in units per liter per hour (for definition of unit, see Section 2.2.2), q_e the specific enzyme synthesis rate in units per gram of biomass per hour, and c_x the biomass concentration in grams per liter.

In kinetic terms, optimization of the enzyme synthesis rate means finding the highest specific synthesis rate for a given amount of biomass. Because enzyme synthesis depends so much on primary metabolism, the complete cellular machinery must function at all times. Therefore, conditions favoring enzyme synthesis also favor growth, as suggested in Figure 12. The search for optimal conditions for enzyme synthesis can then be reduced to a search for (1) the conditions influencing growth rate and (2) the relation between specific enzyme synthesis rate and growth rate.

Growth can be expressed by a specific growth rate μ as the amount of biomass synthesized per unit biomass and unit time:

$$\mu = \frac{1}{c_x} \cdot \frac{dc_x}{dt}$$

where c_x is the biomass concentration in grams per liter and t the time in hours.

Specific enzyme synthesis rate and specific growth rate express the metabolic activities of the cell. These are abbreviated as synthesis rate q_e and growth rate μ. Monod [143] formulated the relationship between growth rate and substrate concentration as:

$$\mu = \mu_{max} \cdot \frac{c_s}{K_s + c_s}$$

where μ_{max} is the maximum obtainable growth rate per hour; K_s is the saturation, and c_s the concentration of substrate, both expressed as moles per liter.

According to this relation actual growth rates at low substrate concentration are lower than the maximum possible value determined by the structural properties of the organism. When $c_s \gg K_s$, the growth rate reaches its maximum value. If all substrates are present in excess ($c_{s_i} \gg K_{s_i}$ for any i), the growth rate is limited by internal structure. The parameters μ_{max} and K_s are dependent on pH, temperature, osmotic pressure, and such factors as medium type (minimal vs. nutrient medium). When all substrates are present in excess except one ($\mu < \mu_{max}$), that substrate is called the limiting factor; in this case, an external growth limitation exists. Nutrient-limited growth is considered the natural state for microorganisms [144].

The kinetics of enzyme synthesis cannot be expressed in one equation for all enzymes. Instead, a number of patterns of synthesis rate q_e versus growth rate μ are shown in Figure 13. The curves are somewhat idealized, but many examples can be found in the literature [145–148].

The importance of these physiological considerations is that a limiting factor can be used to control growth rate and thereby synthesis rate. If the q_e versus μ relationship is

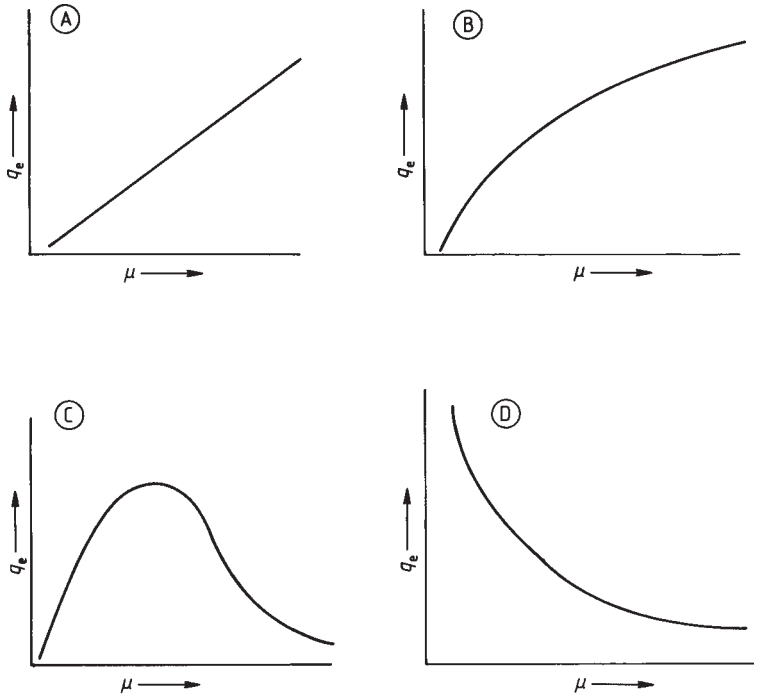


Fig. 13 Types of relationship between specific synthesis rate q_e and specific growth rate μ
A): Growth-coupled synthesis [147];
B): Saturated synthesis [148];
C): Saturated synthesis with catabolic repression [151];
D): Repressed synthesis [154]

known, the value of μ that results in a maximum q_e corresponds to the optimal physiological condition for synthesis.

Complete physiological characterization includes knowledge of the relationship of q_e to μ, the yield on different substrates, the effect of the limiting factor, the physicochemical conditions, and the possible role of regulatory agents. Because these factors are interdependent, establishment of the complete physiological characteristics of an organism is a very extensive task. In industrial practice, experience, general knowledge, and use of well-known organisms or techniques are generally employed to find shortcuts.

The previous discussion emphasizes the importance of the limiting factor for control of growth and product synthesis. Control of growth is possible if the substrate concentration c_s is in the range of the saturation concentration K_s. The K_s values for most substrates ($\pm$ 0.1 mmol/L) are rather low compared to the consumption rate ($\pm$ 10 mmol L^{-1} h^{-1} for glucose), i.e., the response time constant (less than a minute) is low. Continuous feed is therefore necessary to control growth rate by limiting substrate

concentration, as applied in fed-batch and continuous cultures. In batch cultures, all substrates are present at the start of fermentation, and the growth rate is maximal until the substrate is nearly consumed. Therefore, direct control of growth is not possible in a batch culture.

One of the applications of metabolic-pathway engineering to fermentation production technologies is the ever-expanding ''toolbox'' to enable mining of biodiversity, maximize productivity, enhance carbon efficiency, improve product purity, expand product lines, and broaden markets. Functional genomics, proteomics, metabolomics, fluxomics, physiomics, and cellomics applications holistically delineate metabolic pathways and environment within the host [149]. Functionomics-based optimization includes four key elements: (1) completion and optimization of the primary metabolic pathway to the targeted product, including removal of rate-limiting, transcriptional, and allosteric regulation; (2) genetic blockage of competing pathways; (3) enhanced carbon commitment to the primary metabolic pathway from central metabolism; and (4) modification of secondary metabolic pathways as necessary to enhance energy metabolism and availability of required enzymatic cofactors. Recently, strain-specific stoichiometry (metabolic genotype) has been woven into flux balance models to determine optimal flux distributions (metabolic phenotype) by using linear programming. A combination of such approaches may prove to be valuable for design and optimization of bioprocesses [150].

3.1.4 The Fermentor and its Limitations

Fermentation process design is interdisciplinary and uses concepts and methodologies of both chemical engineering and microbial physiology to accomplish scale-up. In practice, scale-up effects are more pronounced for aerobic, i.e., aerated and agitated environment, than for anaerobic fermentations. Therefore, as a rule of thumb, in an aerobic fermentor, the constant oxygen transfer rate and concentration of dissolved oxygen are generally maintained in the scale-up. While thermodynamic and kinetic phenomena are independent of scale, momentum, mass and heat transfer are functions of scale. Mixing, aeration, and cooling are well controlled and uniform at 10 L scale, but not all transport parameters can be maintained in this way at large scale. Further scale-up complications arise from cell response to distributed values of dissolved oxygen, temperature, pH, and nutrients.

The development of entirely new processes or the improvement of existing processes requires the evaluation of a wide range of strains and cultivation conditions in a short period of time. Shake-flask fermentation studies have a cost advantage but lack process control options (pH, nutrient addition, aeration). This often leads to use of laboratory-scale (ca. 10 L) agitated, aerated fermentors with adequate instrumentation and control. Most fermentation processes can be translated to production scale by use of laboratory-scale fermentors. However, pilot-scale fermentors are often necessary for downstream process scale-up.

For enzyme production, economy of scale leads to the use of fermentors with a volume of 20–200 m^3. The higher energy yield from aerobic metabolism results in the

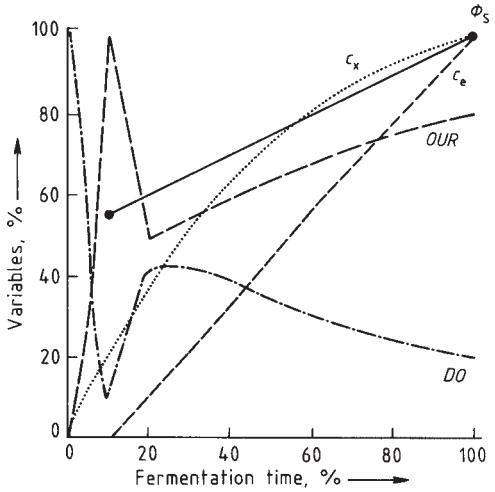


Fig. 14 Schematic representation of a fermentation This illustrates the production of amylase by *B. licheniformis*. After an inital exponential growth period, the process is controlled by an increasing feed rate which results in the oxygen uptake rate and biomass profile shown. The final result is an almost constant rate of enzyme synthesis during fermentation Variables (expressed as percent of maximum value during fermentation): c_x, biomass concentration; c_e, enzyme activity; *DO*, dissolved oxygen; *OUR*, oxygen uptake rate; Φ_s, carbon feed rate

use of aerobic processes which require continuous transfer of poorly soluble oxygen into the culture broth. The concomitant problems of mass and heat transfer are usually neglected in small fermentors and at low cell densities. However, in industrial microbiology, with the above-mentioned fermentor volumes and the economic necessity of using the highest possible cell densities, transport processes must be considered. Such processes can limit metabolic rates; for example, the oxygen supply may become limiting and the microorganism may respond by changes in its physiological pattern. Under these conditions, the desired control of microbial metabolism may be lost. In controlled operation of an industrial process, metabolic rates must be limited to a level just below the transport capacity of the fermentor. Therefore, the highest possible productivity in a fermentor is obtained at maximal transport capacity. This is reflected in low concentrations of dissolved oxygen during fermentation, as shown in Figure 14.

Maximizing the transport processes is chiefly a problem of fermentor design and is generally treated in handbooks on bioengineering [121], [123], [151], [152], [153]. In the common stirred-tank reactor, agitation and injection of compressed air are used to mix the contents of the reactor and to transfer gases. The oxygen transport rate n, for example, can be expressed as:

$$n = k_1 a[c_0(\mathrm{g}) - c_0(l)]$$

where k_1a is the transfer coefficient per hour; c_0 (g) is the oxygen concentration in the liquid phase in equilibrium with the gas phase, and c_0 (l) the actual oxygen concentration in the medium, both expressed in millimoles per liter.

The transfer coefficient k_1a shows that the main resistance to oxygen transport is the gas–liquid interface, where k_1 is the resistance coefficient in meters per hour, and a the total gas–liquid interface area in square meters per cubic meter. The transfer capacity of a fermentor can be expressed as the k_1a value. The k_1a value is determined primarily by the power input of the agitator and the air jet system [154]. Bioengineering therefore aims at the design of a fermentor with the highest k_1a value at the lowest power input.

The k_1a value is also influenced by factors such as viscosity, ionic strength, and presence of surfactants. The dependence of k_1a on viscosity explains the preference for using pellet growth to cultivate mycelium-forming microorganisms [155].

The complex relationships of factors determining transport processes make the scale-up of biotechnological processes a very difficult task [122].

3.1.5
Process Design

Process design entails the development of an optimum outcome for process performance. This typically begins at the fermentation stage, but must also take into account downstream operations such as cell harvesting, product isolation, enzyme purification, and final product formulation. The way in which these unit operations interact can have a profound effect upon optimum process performance.

The fermentation stage begins with culture storage. Cell lines can be stored for future use in a number of ways. These include cultures frozen in liquid media with an appropriate cryoprotectant, on or within solid media such as agar, as freeze-dried suspensions, and as spore suspensions. The process of reviving the stored culture is an oft-neglected phase of the fermentation process. Development time typically focuses on operation of the production fermentor, and the seed stages are run simply to grow the cells as quickly as possible. A typical seed train is shown in Figure 15. However, the physiological state in which the cells are cultivated during seed propagation can have a profound effect upon fermentor performance. This is especially true for microorganisms that can differentiate, either through filamentation or sporulation. For genetically engineered strains, maintaining strain stability is of utmost concern.

The desired outcome of the fermentation process is often to maximize the enzyme synthesis rate, or volumetric productivity. This rate is the product of the specific enzyme production rate and the cell concentration (see Section 3.1.3). Both of these factors can be influenced by process design. If the specific production rate is a strong function of growth rate, i.e., growth-associated production, maintenance of high growth rate will be of high priority. In such cases, continuous-culture techniques are quite suitable. The constant removal of cell mass allows for sustained high growth rate and specific productivity. The high metabolic rate associated with high growth rate can limit the achievable cell mass concentration. Oxygen- and

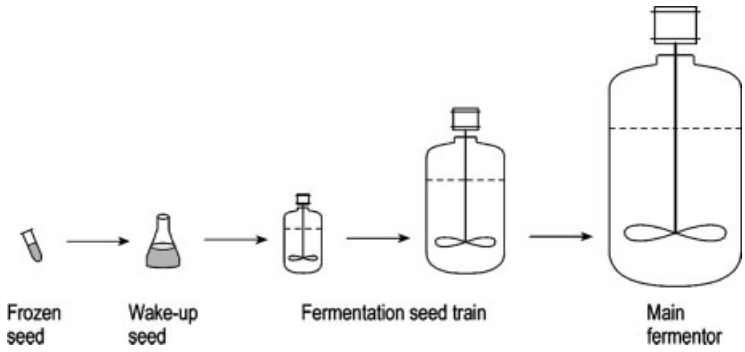


Fig. 15 Typical fermentation train

heat-transfer limitations of the fermentation equipment are usually the limiting factors.

Some enzymes are produced more as a secondary metabolite, and specific productivity may then be an inverse function of growth rate, i.e., non-growth-associated production. Here a recycling reactor may be most suitable. A recycling reactor is similar to the continuous culture, but a device is added to return a significant fraction of the cells to the reactor. Low growth rates with high cell concentration can often be achieved in such systems.

In many cases, however, the relationship between growth rate and specific productivity can be quite complex, so that neither the continuous-culture nor the recycling fermentor is a good choice. This is where fed-batch processes are most prevalent. Starting with a relatively low cell mass concentration, growth rate can be controlled throughout the course of the fermentation through feeding of a growth-limiting substrate.

The discussion above is focused on optimization of the fermentation process. Yet the goal of most processes is to produce a final formulated enzyme product and will likely include many post-fermentation unit operations. For the entire process, maximum production rate could still be the most important factor. However, lowest unit production cost could also be an important driving force. Optimization of each individual unit operation will not always lead to the optimal overall process performance, especially when there are strong interactions between unit operations [156]. Understanding of these interactions is crucial to overall process optimization. For instance, product concentration or purity in the fermentation broth can significantly impact downstream purification unit operation. If the fermentation is optimized for productivity, without taking into account its effect on the purification step, the overall process productivity can be negatively affected. The use of antifoaming agents in the fermentation process is another example of such a trade-off. By reducing foaming in the fermentation, a higher working volume can be used to optimize the fermentation unit operations. However, many antifoaming agents negatively impact ultrafiltration membranes and reduce the capacity of this recovery unit operation [157]. Knowledge of how

the fermentation process will affect other downstream unit operations is also of great importance.

3.1.6 Modeling and Optimization

Many enzyme production processes are carried out on a large scale (> 100 000 L). Small increases in performance can have a significant impact on economic viability. Therefore, process optimization is of great importance for enzyme production by fermentation. Process modeling can give a structured approach to the optimization problem so that optimal conditions can quickly be reached.

Relationships between controllable parameters and desired outputs are a prerequisite for successful process modeling. Balance equations combined with kinetic expressions have been the most widely used tools for such modeling. Monod-type kinetic expressions (see Section 3.1.3) are a typical starting point. This type of modeling has been successfully applied to the improvement of enzyme production processes. Kinetic expressions of cell growth, substrate utilization, and product formation are formulated. Whenever possible, independent experimental procedures are utilized to determine model parameters. Fermentation experiments are then performed to fit any remaining parameters. Mathematical simulations are then run to predict the performance of the system based upon known adjustable parameters, such as substrate feed rate. Optimization can be carried out ''in silico'' for any given objective function (e.g., maximum product concentration or maximum production rate). In fact, combinations of different parameters can simultaneously be optimized to meet conflicting constraints [158]. Experimental fermentations can then be used to calibrate and validate the model. Adjustments to the model architecture can be made to improve the predictive capability of the model. Continual iteration between model modification and experimental results is a trademark of such kinetic modeling.

The drawback of such models is the prerequisite for relationships between the controllable parameters and the desired outputs. One way around this impediment is the use of artifical neural networks (ANNs) [159], [160], [162]. These adaptive computer programs have the ability to learn and can be used for predictions if properly trained. Advantageously, many enzyme production processes have a large repository of past data that can be used to build and train such models. The ANN consists of a set of input and output variables determined by the process being investigated. A set of nodes is then established between the inputs and outputs, each with a different weighting factor. The optimal number of nodes is typically found through trial and error. The weighting factors are continually updated by ''training'' on actual process data. New inputs and outputs can be added to help improve the predictive power of the model. Such procedures have been used for enzymes and other microbially derived products.

ANNs can be extended by combining them with a more structured model to create hybrid models [161]. Input variables can be placed into an ANN and the output variables can then be used as the input to a set of balance equations. Such models have shorter training times as they combine the black-box approach of the ANN with known relationships between controllable parameters and desired outputs.

3.1.7
Instrumentation and Control

Like any chemical reactor, fermentors are controlled by monitoring and estimating several critical parameters such as pressure, temperature, pH, dissolved oxygen, redox potential, foam level, heat flux via coolant supply, respiration (carbon dioxide, oxygen) rate, mass- and heat-transfer coefficients (Fig. 16). Mass spectrometry is commonly used for analyzing the off-gas composition for calculating oxygen uptake rate, carbon dioxide evolution rate, oxygen transfer coefficient k_1a, respiration coefficient, and carbon balance.

Typically, fermentation is optimized by careful design of batch and feeding media, in order to regulate cell growth and maximize production. When an optimal trajectory of the feed rate has been found and desired values for physicochemical variables have been established, the process is ready for production scale. Elementary physical variables, pH, and feed rate are maintained by proper control. If process development is of reasonable quality, the resulting output will be somewhere in the expected range. In practice, however, deviations between actual and expected results may arise for several reasons: (1) the model and the experimental knowledge are never complete; (2) errors of

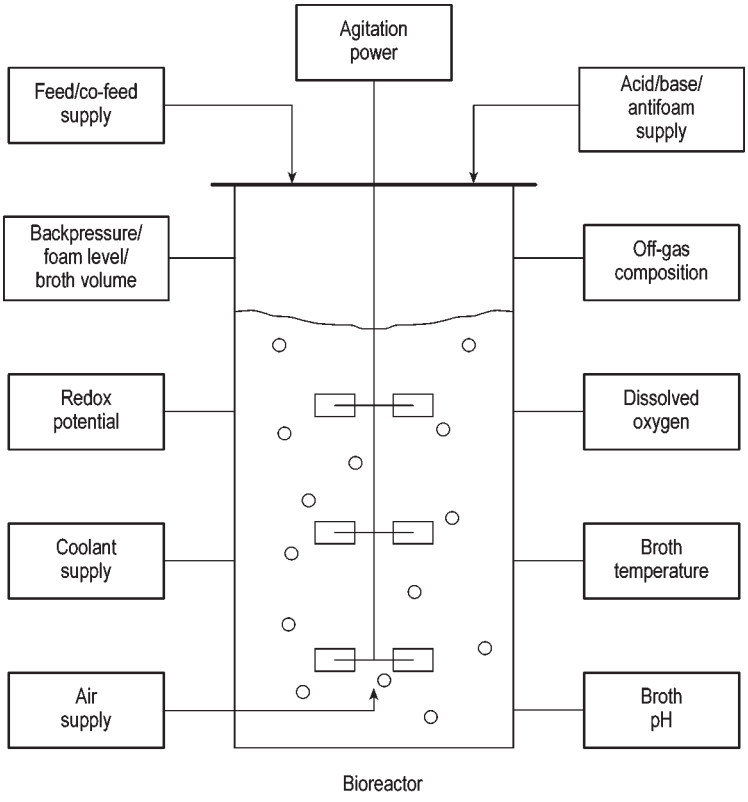


Fig. 16 Bioreactor monitoring and control

measurement and set point always exist, along with deviations in the variables regulated; (3) raw materials may vary in composition; and (4) transport conditions can cause deviations in response.

If deviations from the expected results are minimal, the process can be operated with a minimum level of instrumentation and control. If the deviations cannot be ignored, output variables may be measured to correct input variables; for example, the carbon dioxide production rate can be used for feedback regulation of the feed rate. More advanced control can be realized when a model of the process is used to relate different input and output variables. The measured input and output variables of the process can be compared with the model, and some type of regulatory action can be derived from this comparison [168].

Fermentation processes are complicated, and in principle, control of the process can be improved by measuring as many variables as possible and using them for computer-based process control and optimization. A general limitation of such control systems is lack of adequate (cheap and sterilizable) sensors for measuring the chemical and biological variables [169]. Another problem is the lack of persons to develop the software and operate such systems [170]. Optimistically, reliable measurements and models will lead to on-line estimation and control of variables not measured, using balances for mass and elements, and phenomenological relations for mass transfer and acid-base equilibria in combination with the electroneutrality condition [171].

3.2
Isolation and Purification [172–187]

The degree of purity of commercial enzymes ranges from raw enzymes to highly purified forms and depends on the application. Raw materials for the isolation of enzymes are animal organs, plant material, and microorganisms.

Enzymes are universally present in living organisms; each cell synthesizes a large number of different enzymes to maintain its metabolic reactions. The choice of procedures for enzyme purification depends on their location. Isolation of intracellular enzymes often involves the separation of complex biological mixtures. On the other hand, extracellular enzymes are generally released into the medium with only a few other components. Enzymes are very complex proteins, and their high degree of specificity as catalysts is manifest only in their native state. The native conformation is attained under specific conditions of pH, temperature, and ionic strength. Hence, only mild and specific methods can be used for enzyme isolation. Figure 17 shows the sequence of steps involved in the recovery of enzymes.

3.2.1
Preparation of Biological Starting Materials

Animal Organs Animal organs must be transported and stored at low temperature to retain enzymatic activity. The organs should be freed of fat and connective tissue before freezing. Frozen organs can be minced with machines generally used in the meat

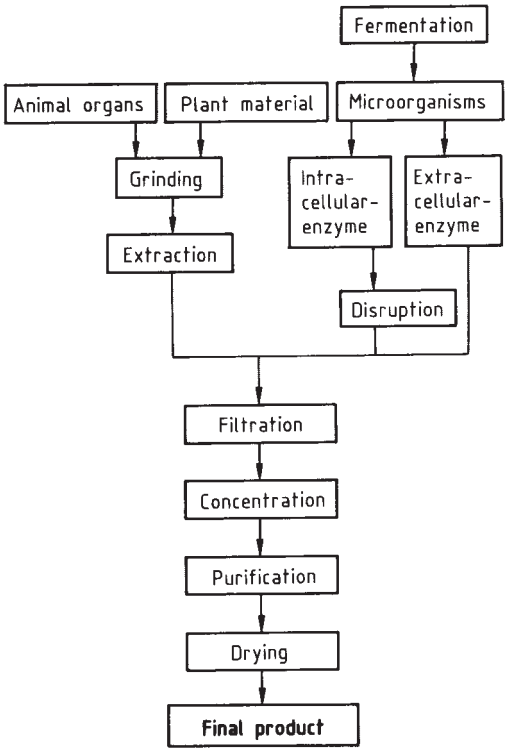


Fig. 17 Sequence of steps in the isolation of enzymes

industry, and the enzymes can be extracted with a buffer solution. Besides mechanical grinding, enzymatic digestion can also be employed [188]. Fat attached to the organs interferes with subsequent purification steps and can be removed with organic solvents. However, enzymatic activity might be influenced negatively by this procedure.

Plant Material Plant material can be ground with various crushers or grinders, and the desired enzymes can be extracted with buffer solutions. The cells can also be disrupted by previous treatment with lytic enzymes.

Microorganisms are a significant source of enzymes. New techniques, summarized under genetic and protein engineering, have much to offer the enzyme industry. A gene can be transferred into a microorganism to make that organism produce a protein it did not make naturally. Alternatively, modification of the genome of a microorganism can change the properties of proteins so that they may be isolated and purified more easily. Such modifications might, for example, cause the release of intracellular enzymes into the medium; change the net charge and, therefore, the chromatographic properties of proteins; or lead to the formation of fused proteins [189].

Most enzymes used commercially are *extracellular enzymes,* and the first step in their isolation is separation of the cells from the solution. For *intracellular enzymes*, which are

Table 2. Methods for disruption of cells

Mechanical methods	Nonmechanical methods
High pressure (Manton–Gaulin, French-press)	Drying (freeze-drying, organic solvents)
Grinding (ball mill)	Lysis
Ultrasound	physical: freezing, osmotic shock
	chemical: detergents, antibiotics
	enzymatic: enzymes (e.g., lysozyme), antibiotics

being isolated today in increasing amounts, the first step involves grinding to rupture the cells. A number of methods for the disruption of cells (Table 2) are known, corresponding to the different types of cells and the problems involved in isolating intracellular enzymes. However, only a few of these methods are used on an industrial scale.

3.2.1.1 Cell Disruption by Mechanical Methods

High-pressure homogenization is the most common method of cell disruption. The cell suspension is pressed through a valve and hits an impact ring (e.g., Manton–Gaulin homogenizer). The cells are ruptured by shearing forces and simultaneous decompression. Depending on the type of machine, its capacity ranges from 50 to 5000 L/h. The rigid cell walls of small bacteria are only partially ruptured at the pressures up to 55 MPa (550 bar) achieved by this method. Higher pressures, however, would result in further heat exposure (2.2 °C per 10 MPa). Hence, the increased enzyme yield resulting from improved cell disruption could be counteracted by partial inactivation caused by heating and higher shearing forces. Therefore, efficient cooling must be provided.

The *wet grinding* of cells in a high-speed bead mill is another effective method of cell disruption [190–193]. Glass balls with a diameter of 0.2–1 mm are used to break the cells. The efficiency of this method depends on the geometry of the stirrer system. A symmetrical arrangement of circular disks gives better results than the normal asymmetrical arrangement [194]. Given optimal parameters such as stirring rate, number and size of glass beads, flow rate, cell concentration, and temperature, a protein release of up to 90 % can be achieved in a single passage [190].

3.2.1.2 Cell Disruption by Nonmechanical Methods

Cells may frequently be disrupted by *chemical*, *thermal*, or *enzymatic lysis*. The drying of microorganisms and the preparation of acetone powders are standard procedures in which the structure of the cell wall is altered to permit subsequent extraction of the cell contents. Methods based on enzymes or autolysis have been described in the literature [195–198]. Ultrasound is generally used in the laboratory. In this procedure, cells are disrupted by shearing forces and cavitation. An optimal temperature must be maintained by cooling the cell suspension because heat is generated in the process. Additional problems may arise from generation of free radicals.

3.2.2 Separation of Solid Matter

After cell disruption, the next step is separation of extracellular or intracellular enzymes from cells or cellular fragments, respectively. This operation is rather difficult because of the small size of bacterial cells and the slight difference between the density of the cells and that of the fermentation medium. *Continuous filtration* is used in industry. Large cells, e.g., yeast cells, can be removed by *decantation*. Today, efficient *centrifuges* have been developed to separate cells and cellular fragments in a continuous process. Residual plant and organ matter can be separated with simpler centrifuges or filters.

3.2.2.1 Filtration

The filtration rate is a function of filter area, pressure, viscosity, and resistance offered by the filter cake and medium. For a clean liquid, all these terms are constant which results in a constant flow rate for a constant pressure drop. The cumulative filtrate volume increases linearly with time. During the filtration of suspensions, the increasing thickness of the formed filter cake and the concomitant resistance gradually decrease the flow rate. Additional difficulties may arise because of the compressibility of biological material. In this case, the resistance offered by the filter cake and, hence, the rate of filtration depend on the pressure applied. If the pressure applied exceeds a certain limit, the cake may collapse and total blockage of the filter can result.

Pressure Filters A *filter press* (plate filter, chamber filter) is used to filtrate small volumes or to remove precipitates formed during purification. The capacity to retain solid matter is limited, and the method is rather work-intensive. However, these filters are highly suitable for the fine filtration of enzyme solutions.

Vacuum Filters Vacuum filtration is generally the method of choice because biological materials are easily compressible. A *rotary vacuum filter* (Figure 18) is used in the continuous filtration of large volumes. The suspension is usually mixed with a filter aid, e.g., kieselguhr, before being applied to the filter. The filter drum is coated with a thin layer of filter aid (precoat). The drum is divided into different sections so that the filter cake can also be washed and dried on the filter. The filter cake is subsequently removed by using a series of endless strings or by scraper discharge (knife). The removal of a thin layer of precoat each time exposes a fresh filtering area. This system is useful for preventing an increase in resistance with the accumulation of filter cake during the course of filtration.

Cross-Flow Filtration [198], [200] In conventional methods, the suspension flows perpendicular to the filtering material (Fig. 19 A). In cross-flow filtration, the input stream flows parallel to the filter area (Fig. 19 B), thus preventing the accumulation of filter cake and an increased resistance to filtration. To maintain a sufficiently high filtration rate, this method must consume a relatively large amount of energy, in the form of high flux rates over the membranes. With the membranes now available, permeate

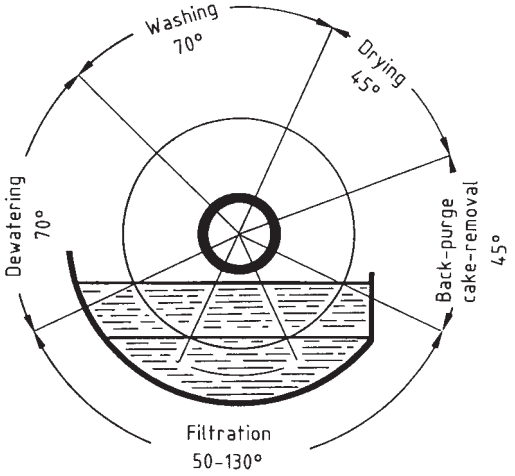


Fig. 18 Rotary vacuum filter

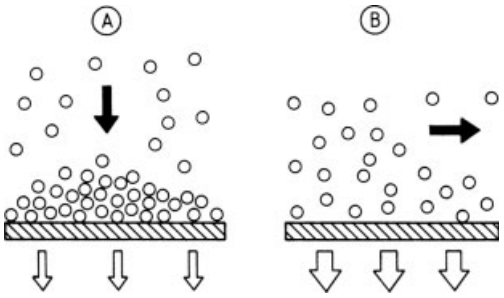


Fig. 19 Principles of conventional, dead end filtration (A) and cross-flow filtration (B)

rates of 30–50 $Lm^{-2}\ h^{-1}$ can be attained. Indeed, in many cases the use of a separator (see Section 3.2.2.2) is more economical [199]. The future of this method depends on the development of suitable membranes, but cross-flow filtration can be conveniently used in recombinant DNA techniques to separate organisms in a closed system.

3.2.2.2 **Centrifugation**

The sedimentation rate of a bacterial cell with a diameter of 0.5 µm is less than 1 mm/h. An economical separation can be achieved only by sedimentation in a centrifugal field. The range of applications of centrifuges depends on the particle size and the solids content (Table 3).

Table 3. Utilization of different centrifuges

Type of centrifuge	Solids content, %	Particle size, μm
Multichamber separator	0–5	0.5–500
Desludging disk separator	3–10	0.5–500
Nozzle separator	5–25	0.5–500
Decanter	5–40	5–50 000
Sieve centrifuge	5–60	5–10 000
Pusher centrifuge	20–75	100–50 000

The Σ value of a centrifuge is a good criterion for the comparison of centrifuges:

$$\Sigma = F \cdot Z$$

where $F = V/S$, with V the total volume of liquid in the centrifuge and S the thickness of the liquid layer in the centrifuge; i.e., F has the units of an area. $Z = r \cdot \omega^2/g$, where r is the radius of the centrifuge drum, ω the angular rotation speed, and g the gravitational constant.

Both sieve centrifuges and solid-wall centrifuges are available. Typical solid-wall centrifuges are shown in Figure 20.

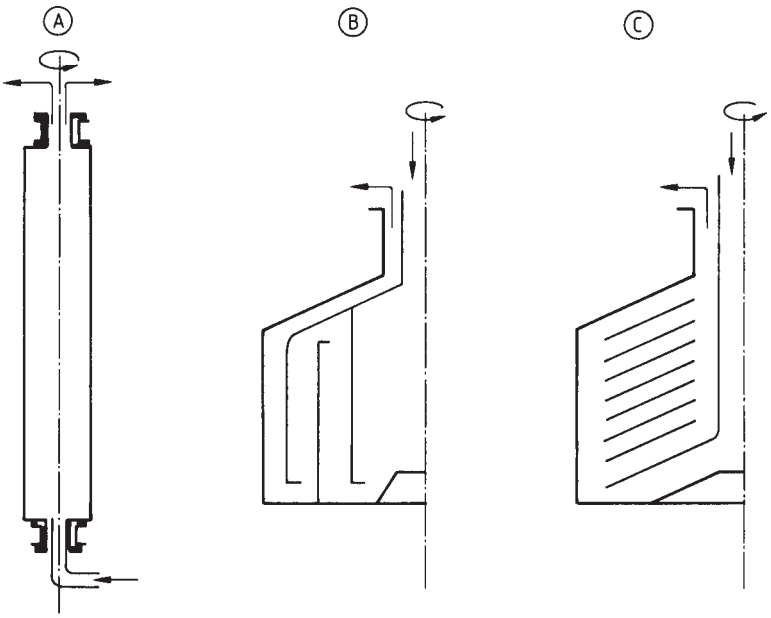


Fig. 20 Solid-wall centrifuges
A) Tubular bowl;
B) Multichamber solid bowl;
C) Disk stack

Decanters (scroll-type centrifuges) work with low centrifugal forces and are used in the separation of large cells or protein precipitates. Solid matter is discharged continuously by a screw conveyer moving at a differential rotational speed.

Tubular bowl centrifuges are built for very high centrifugal forces and can be used to sediment very small particles. However, these centrifuges cannot be operated in a continuous process. Moreover, solid matter must be removed by hand after the centrifuge has come to a stop. A further disadvantage is the appearance of aerosols.

Separators (disk stack centrifuges) can be used in the continuous removal of solid matter from suspensions. Solids are discharged by a hydraulically operated discharge port (intermittent discharge) or by an arrangement of nozzles (continuous discharge). Bacteria and cellular fragments can be separated by a combination of high centrifugal forces, up to 15 000 × gravity, presently attainable, and short sedimentation distances. Disk stack centrifuges that can be sterilized with steam are used for recombinant DNA techniques in a closed system [201], [202].

3.2.2.3 Extraction

An elegant method used to isolate intracellular enzymes is liquid–liquid extraction in an aqueous two-phase system [203–207]. This method is based on the incomplete mixing of different polymers, e.g., dextran [9004-54-0] and poly(ethylene glycol) [25322-68-3], or a polymer and a salt in an aqueous solution [208]. The first extraction step separates cellular fragments. Subsequent purification can be accomplished by extraction or, if high purity is required, by other methods. The extractability can be improved by using affinity ligands [209], [210] or modified chromatography gels, e.g., phenyl-Sepharose [211].

3.2.2.4 Flocculation and Flotation

Flocculation Separation of bacterial cells or cell debris by filtration or centrifugation can involve considerable difficulties due to their small size and physical properties. The compressible nature of the cells is the primary limiting factor for using filtration as a separation step to remove them. The low permeability of a typical cell cake results in a filtration rate that is often too slow to be practical. In cell removal by centrifugation, the small size and low density difference between the cells or cell debris and the medium results in a low sedimentation rate. Flocculation of cell suspensions has been reported to aid cell separation by both filtration and centrifugation [212–218].

Flocculation is the process whereby destabilized particles are induced to come together, make contact, and subsequently form larger aggregates. Flocculating agents are additives capable of increasing the degree of flocculation of a suspension. They can be organic or inorganic, and natural or synthetic. A comprehensive review of various categories of flocculating agents can be found in [219], [220].

Synthetic organic flocculating agents are by far the most commonly used agents for cell flocculation in industrial processes. They are typically water-soluble, charged polymeric substances with average molecular weight ranging from about 10^3 to greater than 5×10^6 and are generally referred to as polyelectrolytes. The positively and negatively charged polymers are referred to as cationic and anionic polyelectrolytes,

respectively. Polyelectrolytes containing both positive and negative charges are termed polyampholytes.

Flocculation of cells by polyelectrolytes is a two-step process. The first step is the neutralization of the surface charge on the suspended cells or cell debris. The second step involves the linkage of these particles to form large aggregates. The various mechanisms and theories of flocculation have been summarized [219–222].

Flocculant selection for a specific cell separation process is a challenge as many factors can impact flocculation. These factors can have their origin in the broth (cell surface charge and size, ionic strength, pH, cell concentration, and the presence of other charged matter), the polymer (molecular weight, charge and charge density, structure, type), and engineering parameters (mixing and mode and order of addition). The final criteria for flocculant selection should take into consideration all aspects of the flocculation process. These include the cost of the added flocculant, subsequent separation performance, process robustness, and yield. In some cases, flocculation can also provide purification by selectively removing unwanted proteins, nucleic acids, lipids and endotoxin from the cell broth [223–226].

Flotation If no stable agglomerates are formed, cells can be separated by flotation. Here, cells are adsorbed onto gas bubbles, rise to the top, and accumulate in a froth. An example is the separation of single cell protein [227].

3.2.3 Concentration

The enzyme concentration in starting material is often very low. The volume of material to be processed is generally very large, and substantial amounts of waste material must be removed. Thus, if economic purification is to be achieved, the volume of starting material must be decreased by concentration. Only mild concentration procedures that do not inactivate enzymes can be employed. These include *thermal methods, precipitation,* and to an increasing extent, *membrane filtration.*

3.2.3.1 Thermal Methods

Only brief heat treatment can be used for concentration because enzymes are thermolabile. Evaporators with rotating components that achieve a thin liquid film (thin-layer evaporator, centrifugal thin-layer evaporator) or circulation evaporators (long-tube evaporator) can be employed.

3.2.3.2 Precipitation

Enzymes are very complex protein molecules possessing both ionizable and hydrophobic groups which interact with the solvent. Indeed, proteins can be made to agglomerate and, finally, precipitate by changing their environment. Precipitation is actually a simple procedure for concentrating enzymes [228].

Precipitation with Salts High salt concentrations act on the water molecules surrounding the protein and change the electrostatic forces responsible for solubility.

Ammonium sulfate [7783-20-2] is commonly used for precipitation; hence, it is an effective agent for concentrating enzymes. Enzymes can also be fractionated, to a limited extent, by using different concentrations of ammonium sulfate. The corrosion of stainless steel and cement by ammonium sulfate is a disadvantage, which causes additional problems in wastewater treatment. Sodium sulfate [7757-82-6] is more efficient from this point of view, but it is less soluble and must be used at temperatures of 35–40 °C. The optimal concentration of salt required for precipitation must be determined experimentally, and generally ranges from 20 to 80 % saturation.

Precipitation with Organic Solvents Organic solvents influence the solubility of enzymes by reducing the dielectric constant of the medium. The solvation effect of water molecules surrounding the enzyme is changed; the interaction of protein molecules is increased; and therefore, agglomeration and precipitation occur. Commonly used solvents are ethanol [64-17-5] and acetone [67-64-1]. Satisfactory results are obtained only if the concentration of solvent and the temperature are carefully controlled because enzymes can be inactivated easily by organic solvents.

Precipitation with Polymers The polymers generally used are polyethylenimines and poly(ethylene glycols) of different molecular masses. The mechanism of this precipitation is similar to that of organic solvents and results from a change in the solvation effect of the water molecules surrounding the enzyme. Most enzymes precipitate at polymer concentrations ranging from 15 to 20 %.

Precipitation at the Isoelectric Point Proteins are ampholytes and carry both acidic and basic groups. The solubility of proteins is markedly influenced by pH and is minimal at the isoelectric point at which the net charge is zero. Because most proteins have isoelectric points in the acidic range, this process is also called *acid precipitation*.

Precipitation is usually carried out on a small scale. Problems can arise in scaling-up this process [229]. The mixing time, the residence time in the reactor (which affects agglomerate formation and enzyme activity), and the shearing forces generated by stirring (which affect the aggregates formed) are critical parameters. When the volume being processed is large, the mixing time is appropriately long and, especially with organic solvents, protein denaturation can occur. Experiments have been conducted to overcome difficulties of this kind by using a continuous process [230].

3.2.3.3 Ultrafiltration

A semipermeable membrane permits the separation of solvent molecules from larger enzyme molecules because only the smaller molecules can penetrate the membrane when the osmotic pressure is exceeded. This is the principle of all membrane separation processes (Table 4), including ultrafiltration. In reverse osmosis, used to separate materials with low molecular mass, solubility and diffusion phenomena influence the process, whereas ultrafiltration and cross-flow filtration are based solely on the sieve effect. In processing enzymes, cross-flow filtration is used to harvest cells, whereas ultrafiltration is employed for concentrating and desalting.

Table 4. Membrane separation processes

Process	Application	Separation range, M_r
Cross-flow microfiltration	Concentration of bacteria, removal of cell debris	>1 000 000 (or particles)
Ultrafiltration	Concentration of enzymes, dialysis, fractionation	>10 000 (macromolecules)
Reverse osmosis	Concentration of small molecules, desalting	>200

Difficulties arise from *concentration polarization*. The semipermeable membrane excludes larger molecules, which tend to accumulate near the surface of the membrane because back-diffusion into the solution is limited. As a result of the different rates of diffusion of molecules of different sizes, the separating ability of the membrane changes. Thus, the membrane holds back small molecules more strongly than would be expected from its pore size. This effect limits the applicability of membrane separation. The formation of *gel layers* on the membrane is reduced by maintaining a turbulent flow or a laminar flow with high flow rate. Loss of permeability is also caused by *membrane fouling* or *deposition* on the membrane. In particular, antifoaming agents from fermentation solutions are deposited on the membrane and make concentration of enzymes more difficult.

Membranes are available for ultrafiltration which exclude molecules ranging from 1000 to 300 000 dalton [231], [232]. Anisotropic membranes consisting of a very thin membrane layer (0.1–0.5 μm) and a thicker, porous support layer [233] are generally used. The different membranes employed are flat membranes (plate and frame, cassette type, or spiral winding module), hollow fibers, and tubular modules (Fig. 21). Cellulose acetate [9004-35-7] and organic polymers such as polysulfone [25135-51-7], poly(vinylidene fluoride) [24937-79-9], and polypropylene [9003-07-0] have proved to be useful as membrane materials. With the exception of cellulose acetate, these membranes can be cleaned easily with alkali or acid and steam sterilized.

Desalting The desalting of enzyme solutions can be carried out conveniently by diafiltration. The small salt molecules are driven through a membrane with the water molecules. The permeate is continuously replaced by fresh water. In fact, the concentration of salt decreases according to the following formula:

$$\ln \frac{c_0}{c} = \frac{V}{V_0}$$

where

c_0 = starting salt concentration
c = final salt concentration
V_0 = starting volume
V = volume exchanged

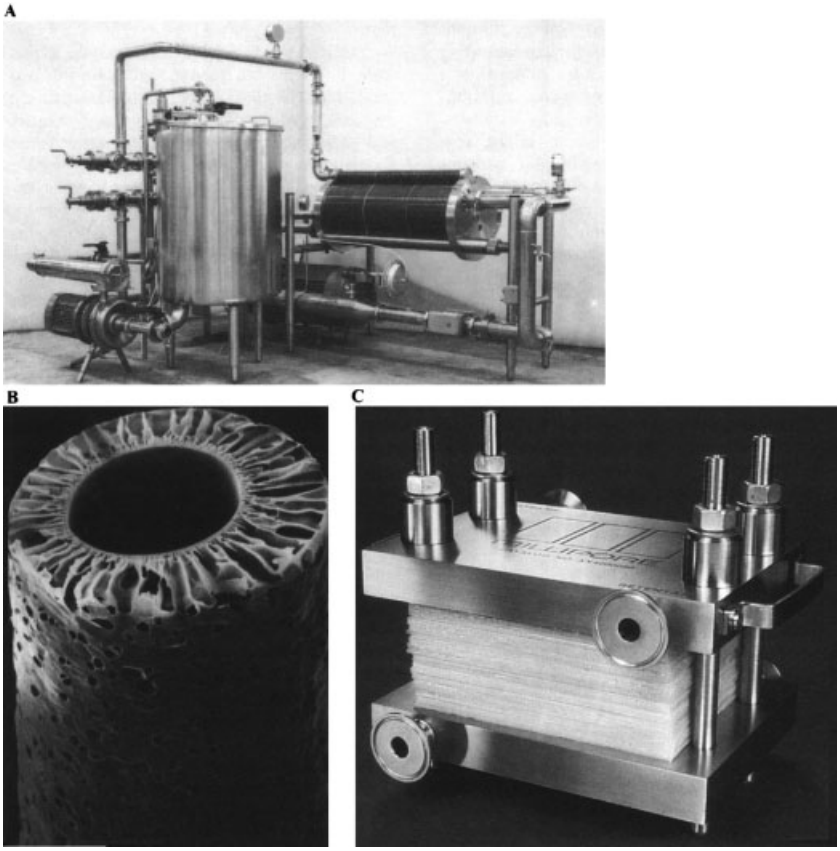


Fig. 21 Different ultrafiltration modules
A) Plate-and-frame module (by courtesy of DDS, Ro-Division);
B) Hollow-fiber module (by courtesy of Amicon);
C) Cassette module (by courtesy of Millipore)

Hence, a 99 % salt exchange can be achieved when the permeated volume is $5 \cdot V_0$, independent of the starting concentration.

3.2.4 Purification

For many industrial applications, partially purified enzyme preparations will suffice; however, enzymes for analytical purposes and for medical use must be highly purified. Special procedures employed for enzyme purification are crystallization, electrophoresis, and chromatography.

3.2.4.1 Crystallization

The rapid growth in the utilization of enzymes in commercial sectors such as agriculture and consumer products requires a cost-effective, industrial-scale purification method. Crystallization, one of the oldest chemical purification technologies, has the potential to fulfill these requirements. Enzyme crystallization is the formation of solid enzyme particles of defined shape and size. An enzyme can be induced to crystallize or form protein-protein interactions by creating solvent conditions that result in enzyme supersaturation. The theory and history of protein crystallization are well documented [234–237]. Much of the emphasis in enzyme crystallization has focused on obtaining crystals for X-ray diffraction analysis rather than as a purification process. However, crystallization is attracting increasing interest as a purification process.

The challenge remains to develop both the theory and screening techniques needed to establish a robust zone of high-yield crystallization without excessive empirical experimentation. Recent advances towards this include the use of self-interaction chromatography for measuring protein osmotic second virial coefficient for predicting protein crystallization behavior [238] and the development of high-throughput screening and analysis systems for establishing robust protein crystallization conditions [239]. Protein crystallization theory and scale-up practice have been recently reviewed [240], [241].

Many reports of enzyme crystallization from bulk fermentation for the purpose of purification have been published [242–244]. Enzymes that have been crystallized for commercial production include a cellulase [245], [246], glucose isomerase [247] subtilisin [247], [248], and alcohol oxidase [249].

Figure 22 shows the steps involved in the crystallization process for an industrial enzyme [248]. Typical crystallization process volumes can be as large as several thousands of liters of impure enzyme solution as the starting material. The desired characteristics of industrial scale enzyme crystallization are product purity, process yield, ease of crystal recovery, and short overall process time. To achieve these aims, the crystallization process must be carefully designed and developed to produce crystals with relatively large size and desired morphology.

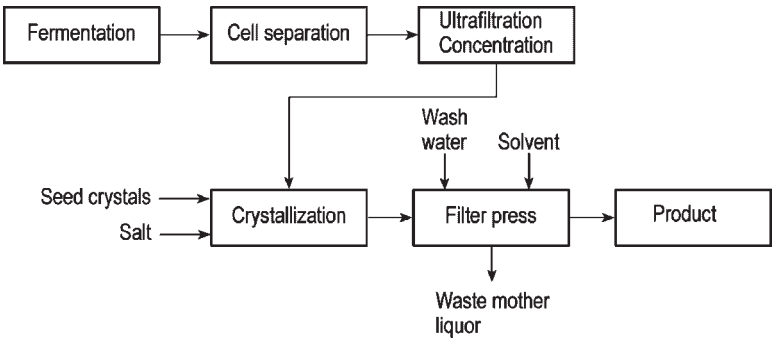


Fig. 22 Industrial-scale halide salt crystallization of subtisilin [248]

Many factors, including salt type and concentration, pH, temperature, the presence of variable amounts and types of impurities, mixing, and crystal seeding, can affect enzyme crystallization. Controlling the level of supersaturation throughout the crystallization process is essential for optimization of crystal size, which can be controlled by use of precipitants such as salt, pH, and temperature [235], [250]. Temperature plays a key role in the rate of enzyme crystallization. Cellulase [245], [248] and subtilisin [251], [252] were both reported to crystallize at a much higher rate with increasing temperature.

Some enzymes can be made to grow in a variety of different morphologies by simply changing the crystallization conditions during the course of crystallization. Subtilisin was shown to form rectangular plate-shaped crystals by first nucleating at low temperature followed by growing the crystals at elevated temperature. The rectangular plate-shaped crystal is a hybrid morphology between its high- and low-temperature morphologies, rods and square plates, respectively [252].

3.2.4.2 **Electrophoresis**

Electrophoresis is used to isolate pure enzymes on a laboratory scale. Depending on the conditions, the following procedures can be used: *zone electrophoresis*, *isotachophoresis*, or *porosity gradients*. The heat generated in electrophoresis and the interference caused by convection are problems associated with a scale-up of this method. An interesting contribution to the industrial application of electrophoresis is a continuous process in which the electrical field is stabilized by rotation [253], [254].

3.2.4.3 **Chromatography**

Chromatography is of fundamental importance to enzyme purification (Table 5). Molecules are separated according to their physical properties (size, shape, charge, hydrophobic interactions), chemical properties (covalent binding), or biological properties (biospecific affinity).

In *gel chromatography* (also called *gel filtration*), hydrophilic, cross-linked gels with pores of finite size are used in columns to separate biomolecules. Concentrated solutions are necessary for separation because the sample volume that can be applied to a column is limited to ca. 10 % of the column volume. In gel filtration, molecules are

Table 5. Chromatographic methods

Type of chromatography	Principle	Separation according to
Adsorption	surface binding	surface affinity
Distribution	distribution equilibrium	polarity
Ion exchange	ion binding	charge
Gel filtration	pore diffusion	molecular size, molecular shape
Affinity	specific adsorption	molecular structure
Hydrophobic	hydrophobic chelation	molecular structure
Covalent	covalent binding	polarity
Metal chelate	complex formation	molecular structure

separated according to size and shape. Molecules larger than the largest pores in the gel beads, i.e., above the exclusion limit, cannot enter the gel and are eluted first. Smaller molecules, which enter the gel beads to varying extent depending on their size and shape, are retarded in their passage through the column and eluted in order of decreasing molecular mass. The eluation volume of a globular protein is proportional to the logarithm of its molecular mass [255]. By varying the degree of cross-linking, gels of different porosities and with different fractionation ranges are obtained. Media that collectively cover all molecular sizes are available (Table 6). These include different types of Sephadex, which can be obtained by cross-linking dextran [9004-54-0] with epichlorohydrin [106-89-8], and Sephacryl, which is prepared by cross-linking allyldextran with *N,N'*-methylene-bisacrylamide [110-26-9]. Gel filtration is used commercially for both separation and desalting of enzyme solutions.

Ion-exchange chromatography is a separation technique based on the charge of protein molecules. Enzyme molecules possess positive and negative charges. The net charge is influenced by pH, and this property is used to separate proteins by chromatography on anion exchangers (positively charged) or cation exchangers (negatively charged) (Table 7). The sample is applied in aqueous solution at low ionic strength, and elution is best carried out with a salt gradient of increasing concentration. Because of the concentrating effect, samples can be applied in dilute form.

The ability to process large volumes and the elution of dilute sample components in concentrated form make ion exchange very useful. The matrix used to produce ion-exchange resins should (1) be sufficiently hydrophilic to prevent enzyme denaturation and (2) have a high capacity for large molecules at fast equilibration. In addition, industrial applications require ion exchangers that give good resolution, allow high flow rates to be used, suffer small changes in volume with salt gradients or pH changes, and can be completely regenerated.

Table 6. Gel filtration media

Trade name (manufacturer)	Matrix	Fractionation range, M_r
Biogel (Bio-Rad)	polyacylamide (P-type)	100–400 000
	agarose (A-type)	1000–150 × 10^6
Ultrogele (LKB)	agarose/polyacrylamide	60 000–1.3 × 10^6
	agarose	25 000–20 × 10^6
Fractogel (Merck)	vinyl polymer (various types)	100–5 × 10^6
Sephadex (Pharmacia)	dextran (various types)	50–600 000
Sepharose (Pharmacia)	agarose	10 000–40 × 10^6
	cross-linked agarose	10 000–40 × 10^6
Sephacryl (Pharmacia)	sephacryl/bisacrylamide	5000–1 × 10^6
Glycophase (Pierce)	surface-modified glass (1,2-dihydroxypropyl-substituted)	1000–350 000

Table 7. Ion-exchange resins

Trade name (manufacturer)	Matrix	Anion-exchange	Cation-groups**
Cellex (Bio-Rad)	cellulose	DEAE ECTEOLA QAE TEAE	CM phosphoryl
Bio-Gel A (Bio-Rad)	agarose	DEAE	CM
Trisacryl (LKB)	synthetic polymer	DEAE	CM
Fractogel (Merck)	vinyl polymer	DEAE	CM SP
Sephadex (Pharmacia)	dextran	DEAE QAE	CM SP
Sepharose (Pharmacia) Sepharose FF * Q-Sepharose S-Sepharose	agarose	DEAE	CM
Sephacel (Pharmacia)	cellulose	DEAE	

* FF = fast flow.

** CM = carboxymethyl: $-O-CH_2-COO^- Na^+$; DEAE = diethylaminoethyl: $-O-CH_2-CH_2-N^+(C_2H_5)_2H\ OH^-$; ECTEOLA = mixture of different amines; QAE = 2-hydroxypropylamine: $-O-CH_2-CH_2-N^+(C_2H_5)_2-CH(OH)-CH_3\ Br^-$; SP = sulfopropyl: $-O-CH_2-CH_2-CH_2-SO_3^-$; TEAE = triethylaminoethyl: $-O-CH_2-CH_2-N^+(C_2H_5)_3\ Br^-$.

For *hydrophobic chromatography,* media derived from the reaction of CNBr-activated Sepharose with aminoalkanes of varying chain length are suitable [256]. This method is based on the interaction of hydrophobic areas of protein molecules with hydrophobic groups on the matrix. Adsorption occurs at high salt concentrations, and fractionation of bound substances is achieved by eluting with a negative salt gradient. This method is ideally suited for further purification of enzymes after concentration by precipitation with such salts as ammonium sulfate.

In *affinity chromatography,* the enzyme to be purified is specifically and reversibly adsorbed on an effector attached to an insoluble support matrix. Suitable effectors are substrate analogues, enzyme inhibitors, dyes, metal chelates, or antibodies. The principle of affinity chromatography is shown in Figure 23. The insoluble matrix (C) is contained in a column. The biospecific effector, e.g., an enzyme inhibitor (I), is attached to the matrix. A mixture of different enzymes (E_1, E_2, E_3, E_4) is applied to the column. The immobilized effector specifically binds the complementary enzyme. Unbound substances are washed out, and the enzyme of interest (E_1) is recovered by changing the experimental conditions, for example by altering pH or ionic strength.

Metal ions can also serve as effectors. They are attached to the matrix with the help of complexing agents, e.g., iminodiacetic acid [142-73-4]. Here, enzymes are separated on the basis of differing strengths of interaction with metal ions [257].

Immunoaffinity chromatography occupies a unique place in purification technology. In this procedure, monoclonal antibodies are used as effectors. Hence, the isolation of a

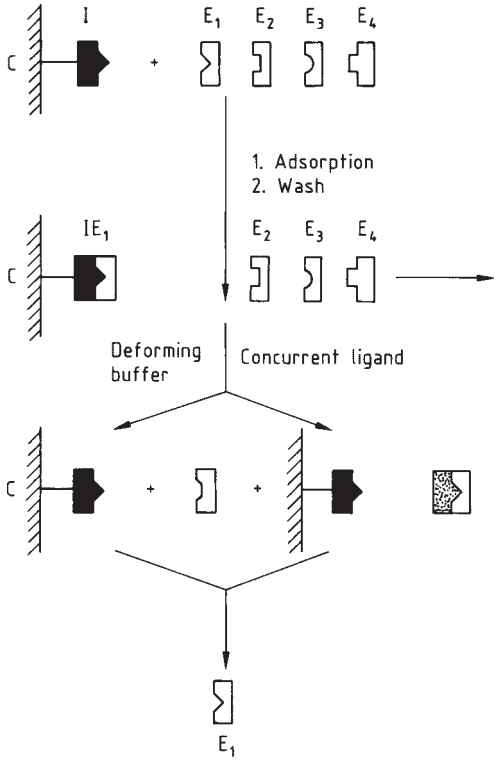


Fig. 23 Affinity chromatography. For explanation of abbreviations, see text

specific substance from a complex biological mixture in one step is possible. In this procedure, enzymes can be purified by immobilizing antibodies specific for the desired enzyme. A more general method offers the synthesis of a fusion protein with protein A by "protein engineering". Protein A is a *Staphylococcus* protein with a high affinity for many immunoglobulins, especially of the IgG class of antibodies. In this way, enzymes that usually do not bind to an antibody can be purified by immunoaffinity chromatography.

Covalent chromatography differs from other types of chromatography in that a covalent bond is formed between the required protein and the stationary phases. The principle is illustrated in Figure 24 for an enzyme containing a reactive mercapto group, such as urease (E.C. 3.5.1.5) [9002-13-5] [258].

Industrial-Scale Chromatography The main applications of industrial-scale chromatography in the 1960s were the desalination of enzyme solutions by use of highly cross-linked gels such as Sephadex G-25 and batch separations by means of ion exchangers such as DEAE-Sephadex A-50. The stability and hydraulic properties of chromatographic media have been improved so that these techniques are now used on a

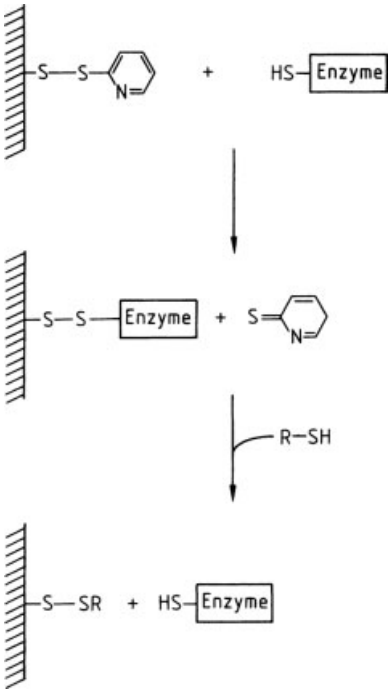


Fig. 24 Covalent chromatography

production scale. Important parameters for the scale-up of chromatographic systems are the height of the column, the linear flow rate, and the ratio of sample volume to bed volume [259]. Zone spreading interferes with the performance of the column. Factors that contribute to zone spreading are longitudinal diffusion in the column, insufficient equilibration, and inadequate column packing. Longitudinal diffusion can be minimized by using a high flow rate. On the other hand, equilibration between the stationary and the mobile phases is optimal at low flow rates. Because good process economy depends to a large extent on the flow rate, a compromise must be made. In addition, the flow rate is also dependent on particle size; the decisive factor is usually the pressure drop along large columns. Although optimal resolution is obtained only with the smallest particles, the gel must have a particle size that favors a good throughput and reduces processing times. The use of *segmented columns* prevents a large pressure drop in the column (Fig. 25) [260]. Above all, the column must be uniformly packed so that the particle-size distribution, porosity, and resistance to flow are the same throughout the column. If this is not done, viscous protein solutions can give an uneven elution profile, which would lead to zone bleeding. The design of the column head is important for uniform distribution of the applied sample. This is generally achieved by symmetrical arrangement of several inlets and perforated inserts for good liquid distribution. The outlet of the column must have minimal volume to prevent back-mixing of the separated components.

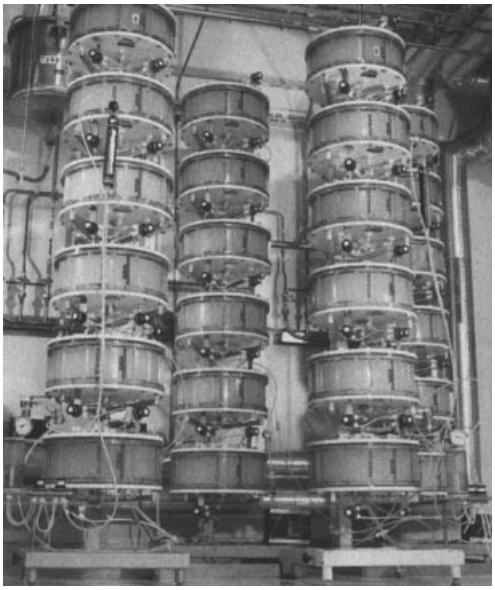


Fig. 25 Chromatographic KS 370 stack columns (by courtesy of Pharmacia)

Columns of glass, plastic, or in the case of large columns, metal (stainless steel) can be used. Metal columns are usually highly polished because a smooth surface is more resistant to corrosion. Viton or poly(tetrafluoroethylene) (PTFE) is applied as sealing material. Polyethylene tubing can be used to connect small columns. Larger tubing, with a diameter of more than 4 mm, must be made of metal, with special screw connections. No dead angles should be present in which liquid can collect and cause contamination by microbial growth.

Figure 26 shows a typical industrial chromatographic column. To avoid contamination of the column, sterile filters are placed at the entrance and exit. Optimal and efficient production systems not only must meet official standards such as those of the Food and Drug Administration (FDA) or Bundesgesundheitsamt (BGA), but must also comply with rules of good manufacturing practice (GMP), which makes cleaning-in-place (CIP) necessary. The system must be operated so that impurities from the process or from microbial growth can be removed completely. Chromatographic columns can be cleaned only by washing with chemicals. The reagents used for this purpose are described in Table 8. Figure 27 shows the steps involved in processing extracellular enzymes.

3.2.5 Product Formulation

Enzymes are sold as stabilized liquid concentrates or as particulate solids. The primary task of formulation is to minimize losses in enzymatic activity during transport,

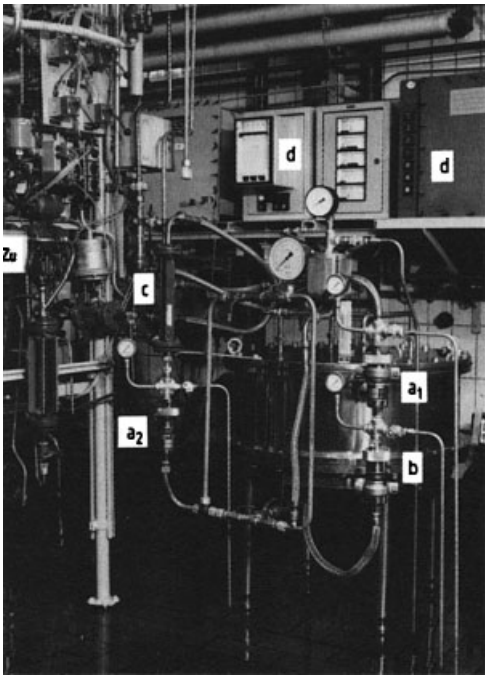


Fig. 26 Production-scale chromatography column (Pharmacia)

storage, and use. Enzymes are often exposed to humid, hot, or oxidative environments in industrial applications such as detergents, textile formulations, and food and beverage processing. Much can be done at the screening or research stage to identify enzymes that are structurally more stable or resistant to oxidation; chemical stabilizers can do little to protect thermally or chemically labile enzymes [261].

Formulations enhance stability by counteracting the primary forces of deactivation: denaturation, catalytic-site deactivation, and proteolysis [262]. Denaturation occurs by physical unfolding of an enzyme's tertiary protein structure under thermal or chemical stress. Once an enzyme begins to unfold it becomes dramatically more vulnerable to deactivation and proteolysis. To minimize unfolding, the formulator can alter the protein's environment so as to induce a compact protein structure; this is done most

Table 8. Reagents for cleaning-in-place (CIP) of chromatographic media

Substance	Purification power (protein and fat)	Sterilization and removal of pyrogens
Sodium hydroxide	++	+
Acids	–	+
Sodium phosphate	+	–
Surfactants	++	–

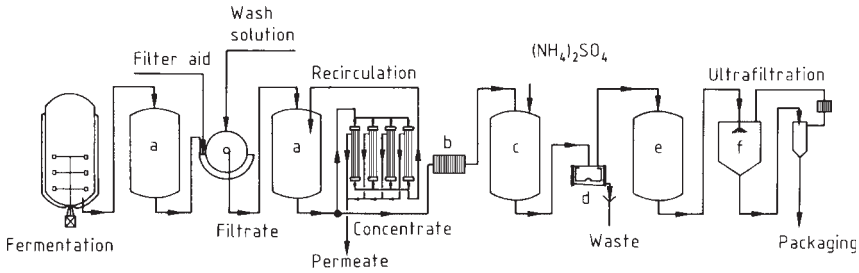


Fig. 27 Isolation of extracellular enzymes
a) Hold tank;
b) Filtration;
c) Precipitation vessel;
d) Centrifuge;
e) Suspension vessel;
f) Spray drier

effectively by ''preferential exclusion'' of water from the protein surface by adding water-associating compounds such as sugars, polyhydric alcohols, and lyotropic salts [263]. The best ways to combat active site inactivation are to ensure sufficient levels of any required cofactors, to add reversible inhibitors, and to exclude oxidizing or reactive species from the formulation.

Besides enzymatic stability, a formulation should meet several key secondary requirements, including preservation against microbial contamination, avoidance of physical precipitation or haze formation, minimizing the formation of sensitizing dusts or aerosols, and the optimization of esthetic criteria such as color and odor. Many of these problems are best addressed by focusing as far ''upstream'' as possible, including the choice of raw materials in the fermentation or enzyme recovery process. Downstream operations such as diafiltration, adsorption, chromatography, crystallization, and extraction can be used to remove impurities responsible for color, odor, and precipitation [264]. The risk of physical precipitation is minimized by formulating near the isoelectric point of the enzyme with hydrophilic solvents such as glycerol or propylene glycol. One can effectively also add moderate levels of solvating salts to avoid either salting-out or ''reverse salting-in''. To prevent microbial contamination, one can use a combination of filtration, acidification, and the minimization of free water; biocides can be effective, but the range of acceptable chemicals for controlling or killing microbes is increasingly circumscribed by health and safety regulations.

Worker safety has been a primary driver in the development of dry granular enzyme formulations for powdered laundry detergents and textile formulations. Since the 1970s, enzyme granules have become increasingly resistant to physical breakage and formation of airborne dusts upon handling. Two processes producing the most attrition-resistant granules to date are high-shear granulation and fluidized-bed spray coating. These processes use various binders, coatings, and particle morphologies to produce nonfriable particles which still protect enzymes during storage but allow for their ready release in solution during application.

3.2.6
Waste Disposal

Because of the generally low concentration of enzyme in the starting material, the volume of material that must be processed is large, and substantial amounts of waste accumulate. The spent fermentation medium can still contain large amounts of unused nutrients. However, recycling is generally not possible because of the presence of metabolites in the medium. Solid organ remains and mycelium, which are used as animal feed, can be separated. The latter must be carefully checked for undesired metabolites, e.g., antibiotics, before being fed to animals.

In recombinant DNA techniques, the need to maintain absolute containment is of great concern. Waste must be chemically or thermally inactivated before disposal, to ensure that no live organisms escape into the environment.

3.3
Immobilization

Synthesis, decomposition, and partial conversion of different compounds in biological systems are catalyzed by enzymes, water-soluble globular proteins. The catalytic activity of enzymes is brought about by their tertiary and quaternary (oligomeric enzymes) structures. All enzymes have a catalytic center, one or several substrate binding sites, and one or several regulatory sites.

The characteristics of enzymes as catalysts are (1) enzymes are able to catalyze reactions at ambient temperature and pressure and in a pH range around neutral, and (2) enzymes have strict substrate specificity, stereospecificity, regiospecificity, and reaction specificity. These facts suggest that energy-saving, resource-saving, and low-pollution processes could be designed by using enzymes, because such processes would operate at relatively low temperature and atmospheric pressure with little byproduct formation. However, the molecular structure of enzymes that is essential for their catalytic activity is liable to be destroyed under conditions such as high temperature, high or low pH, presence of organic solvents, or even conditions suitable for catalysis. The recovery of active enzymes from spent reaction mixtures is another problem when free (nonimmobilized) enzymes are used. Immobilization is one way of eliminating some of the disadvantages inherent to enzymes.

Therefore, the immobilization of biocatalysts–not only enzymes but also cellular organelles, microbial cells, plant cells, and animal cells–is attracting worldwide attention in the practical application of bioprocesses. In general, immobilized biocatalysts are stable and easy to handle compared to their free counterparts. One of their most important features is that they can be used repeatedly in a long-term series of batchwise reactions or continuously in flow systems. At present, applications of immobilized biocatalysts include (1) the production of useful compounds by stereospecific or regiospecific bioconversion, (2) the production of energy by biological processes, (3) the selective treatment of specific pollutants to solve environmental problems, (4) continuous analyses of various compounds with a high sensitivity and a high specificity, and (5) medical uses such as new types of drugs for enzyme therapy or artificial organs.

These processes require the immobilization not only of single enzymes but also of several different enzymes, organelles, or cells that catalyze more complex reactions. Various methods have been developed for the immobilization of biocatalysts, and they are being used extensively today.

3.3.1 Definitions

Immobilized enzymes are defined as "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, which can be used repeatedly and continuously" [265]. This definition is applicable to enzymes, cellular organelles, microbial cells, plant cells, and animal cells, that is, to all types of biocatalysts. In some cases, these biocatalysts are bound to or within insoluble supporting materials (*carriers*) by chemical or physical binding. In other cases, biocatalysts are free, but confined to limited domains or spaces of supporting materials (*entrapment*).

The European Federation of Biotechnology, Working Party on Applied Biocatalysis (formerly, the Working Party on Immobilized Biocatalysts) has proposed that immobilized cells should be classified as *viable* and *nonviable*, where viable includes *growing* and *nongrowing* or *respiring*. According to the same definition, the terms *dead, resting*, and *living* should not be used [266]. However, in this section, the terms *treated, resting*, and *growing* — and in some cases, *living* — are used for classifying the condition of immobilized cells. Treated cells are subjected to chemical or physical treatment before or after immobilization, and resting cells do not show growth during utilization.

3.3.2 History

In 1916, Nelson and Griffin observed that yeast invertase (E.C. 3.2.1.26) [9001-57-4] adsorbed on charcoal was able to catalyze the hydrolysis of sucrose [267]. After that, several reports were published on the immobilization of physiologically active proteins by covalent binding on several supports. However, immobilized enzymes were not used in practice until 1953, when Grubhofer and Schleith immobilized several enzymes, such as carboxypeptidase, diastase, pepsin, and ribonuclease, on diazotized polyaminostyrene resin by covalent binding [268]. Thereafter, Mitz reported the ionic binding of catalase (E.C. 1.11.1.6) [9001-05-2] on DEAE-cullulose in 1956 [269]. Bernfeld and Wan described the entrapment of trypsin (E.C. 3.4.21.4) [9002-07-7], papain (E.C. 3.4.22.2) [9001-73-4], amylase, and ribonuclease in polyacrylamide gel in 1963 [270], and Quiocho and Richards demonstrated the cross-linking of carboxypeptidase A (E.C. 3.4.17.1) [11075-17-5] with glutaraldehyde in 1964 [271]. Microencapsulation of carbonic anhydrase (E.C. 4.2.1.1) [9001-03-0] was reported by Chang in 1964 [272] and the preparation of liposomes containing amyloglucosidase (E.C. 3.2.1.3) [9032-08-0] by Gregoriadis in 1971 [273]; both were used in enzyme therapy. During this pioneering period, Katchalski-Katzir and co-workers made extensive contributions to the theoretical understanding of immobilized enzymes [274].

In 1969, CHIBATA and co-workers of Tanabe Seiyaku Co., Japan, were successful for the first time in the industrial application of immobilized enzymes. Fungal aminoacylase (E.C. 3.5.1.14) [9012-37-7] was immobilized on DEAE-Sephadex through ionic binding and used for the stereoselective hydrolysis of *N*-acyl-D,L-amino acids to yield L-amino acids and *N*-acyl-D-amino acids [275]. The first industrial application of immobilized microbial cells was also performed by CHIBATA and co-workers in 1973 to produce L-aspartate from ammonium fumarate by polyacrylamide gel-entrapped *Escherichia coli* cells containing a high activity of aspartase (E.C. 4.3.1.1) [9027-30-9]. Other processes that have been industrialized use immobilized enzymes and microbial cells. They are discussed briefly in Section 3.3.5.

3.3.3 Methods

For the application of immobilized biocatalysts, their screening to the desired activity and characteristics is most important. In addition, selection of the appropriate combination of supporting material and immobilization method, both of which should be suitable for each biocatalyst, is necessary. No systematic concept is available at present for design of the most appropriate method of immobilization for various biocatalysts. Optimization is carried out in general by trial and error.

At present, various immobilization techniques are available [276], and Figure 31 illustrates the principles of these methods. Because each method has its own merits and demerits, selection should be based on the intended purpose, including type of biocatalyst, type of reaction, and type of reactor.

Supporting materials should have adequate functional groups to immobilize the biocatalysts, as well as sufficient mechanical strength; physical, chemical, and biological stability; and nontoxicity. Easy shaping is also important for applying immobilized biocatalysts to different types of reactors. Furthermore, the economic feasibility should be examined. Some supporting materials are described in the following sections.

3.3.3.1 Carrier Binding

Enzyme proteins have amino acid residues containing chemically reactive groups, ionic groups, and/or hydrophobic groups, as well as hydrophobic domains. These amino acid residues and the hydrophobic domains can participate in the immobilization of enzymes through covalent linkage, ionic binding, or physical adsorption. Various types of insoluble supports (carriers) are utilized as is or after proper modification or activation.

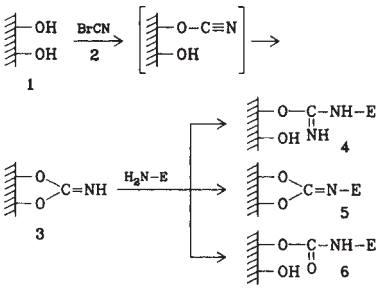
Covalent Binding Amino acid residues that are not involved in the active site or substrate-binding site of the enzymes to be immobilized can be used for covalent binding with supports. These are the ϵ-amino group of lysine, the mercapto group of cysteine, the β-carboxyl group of aspartic acid, the γ-carboxyl group of glutamic acid, the phenolic hydroxyl group of tyrosine, or the hydroxyl groups of serine and threonine. Hydroxyl, carboxyl, and amino groups are especially excellent targets because of their relative abundance in enzyme molecules.

Enzymes immobilized by covalent binding have the following advantages: (1) because of the tight binding, they do not leak or detach from supports during utilization; (2) immobilized enzymes can easily come into contact with substrates because the enzymes are localized on the surface of supports; and (3) an increase in heat stability is often observed because of the strong interaction between enzyme molecules and supports.

On the other hand, disadvantages of covalent binding are: (1) active structures of enzyme molecules are liable to be destroyed by partial modification; (2) strong interaction between enzyme molecules and supports often hinders the free movement of enzyme molecules, resulting in decreased enzyme activity; (3) optimal conditions of immobilization are difficult to find; (4) this method is not suitable for immobilization of cells; and (5) supports, in general, are not renewable. Hence, this principle is well-suited to expensive enzymes whose stability is significantly improved by covalent binding.

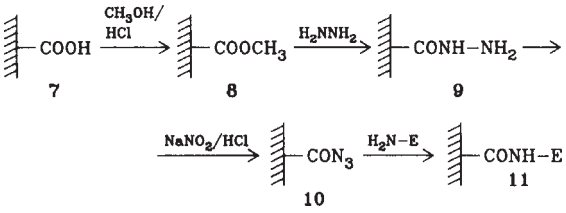
Despite these disadvantages, covalent binding is often applied to the preparation of immobilized enzymes for analytical purposes. Some typical examples of covalent binding methods are described below, but many other techniques have also been reported.

Cyanogen Bromide The cyanogen bromide method was first demonstrated by Axén and his co-workers [277]. It involves the activation of supports having vicinal hydroxyl groups (**1**), such as polysaccharides, glass beads, or ceramics, with cyanogen bromide [506-68-3] (**2**), to yield reactive imidocarbonate derivatives (**3**). The subsequent reaction between the activated supports and enzyme molecules gives *N*-substituted isourea (**4**), *N*-substituted imidocarbonate (**5**), and *N*-substituted carbamate derivatives (**6**).



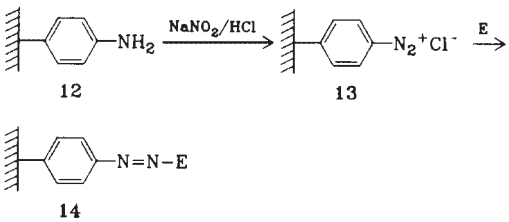
This method has been widely used for the immobilization of various enzymes, and CNBr-activated supports, such as CNBr-activated Sepharose, are available. Aminated glass beads and aminated polyacrylamide gel are also used as supports in this method. The insertion of spacers such as hexamethylenediamine [124-09-4] is also possible, to avoid strong interaction between enzyme molecules and supports.

Acid Azide Derivatives The acid azide method, which is used for peptide synthesis, is also applicable to the immobilization of various enzymes [278]. For example, carboxymethyl cellulose [9004-42-6] (**7**) is converted to the methyl ester (**8**) and then to the hydrazide (**9**) with hydrazine. The hydrazide reacts with nitrous acid to form the azide derivative (**10**), which can then react with enzyme molecules at low temperature to yield immobilized enzymes (**11**). Several supports containing carboxyl groups can be used for this method.

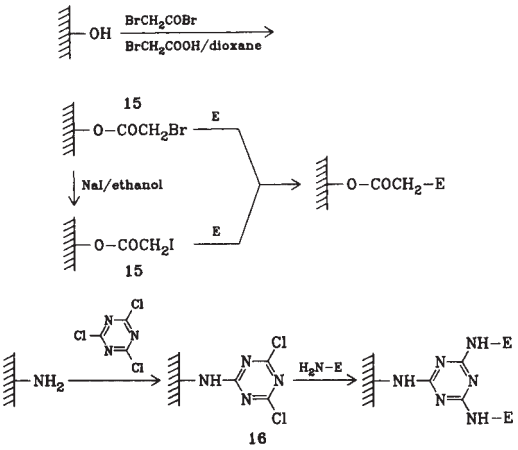


Condensing Reagents Carboxyl groups or amino groups of supports and amino groups or carboxyl groups of enzyme molecules can be condensed directly through the formation of peptide linkages by the action of carbodiimide reagents or Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3′-sulfonate) [4156-16-5]. Carboxymethyl cellulose is one of the supports used for this method [279].

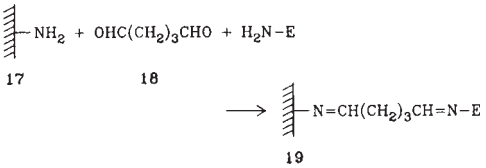
Diazo Coupling Supports having aromatic amino groups (**12**) are diazotized with nitrous acid to form the diazonium derivatives (**13**), which react with enzyme molecules to yield immobilized enzymes (**14**). 4-Aminobenzyl cellulose [9032-51-3] [280] and polyaminostyrene [9060-90-6] [281] are typical supports used for this method.



Alkylation Alkylation groups on supports can easily react with amino groups, phenolic hydroxyl groups, and sulfhydryl groups of the enzyme molecules. For example, halogenated acetyl cellulose (**15**) [282] and triazinyl derivatives of ion-exchange resins (**16**) or cellulose [283] may be used for this method.



Carrier Cross-linking Supports and enzyme molecules can be cross-linked with bi- or multifunctional reagents. For example, aminoethyl cellulose [9032-36-4] (**17**) and amino groups of enzyme molecules are combined through Schiff's base linkage (**19**) with glutaraldehyde [111-30-8] (**18**) [284]. Diisocyanates represent another group of cross-linking reagents. Several supports containing amino groups are also employed.



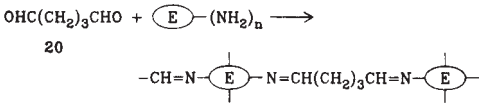
Ionic Binding Since catalase was found to be bound to ion-exchange cellulose [269], this method has been applied for the immobilization of many enzymes, because the procedure is very simple, the supports are renewable, and the enzymes are not modified. The most notable example is the production of L-amino acids by aminoacylase (E.C. 3.5.1.14) [9012-37-7] immobilized on DEAE-Sephadex [275]. Binding of enzymes on supports is affected by the kind of buffers used, pH, ionic strength, and temperature. Several derivatives of cellulose and Sephadex, as well as various ion-exchange resins, can be utilized for immobilization.

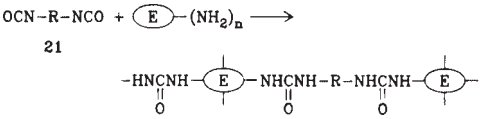
Physical Adsorption Biocatalysts often bind to carriers by physical interaction such as hydrogen bonding, hydrophobic interaction, van der Waal's forces, or their combined action. Although biocatalysts are immobilized without any modification, interaction between biocatalyst and support is generally weak and affected by such environmental conditions as temperature or concentration of reactants. Various inorganic supports are often used. Several synthetic resin beads and natural materials (e.g., chitosan beads with micropores of controlled size) having strong adsorption capacities are available. Adsorption followed by cross-linking with glutaraldehyde sometimes stabilizes the

activity of immobilized enzymes. Phenoxyacetylated cellulose and glass beads are used as specific supports of a hydrophobic nature. Tannins, which interact strongly with proteins, are also applied as ligands after appropriate immobilization. Cellular organelles and various types of cells can be immobilized by physical adsorption. Supports are renewable under appropriate conditions.

3.3.3.2 Cross-linking

Bi- and multifunctional compounds serve as reagents for intermolecular cross-linking of enzymes. Cross-linked enzymes are then insoluble macromolecules. In addition to glutaraldehyde [111-30-8] (**20**) [271] which is the most popular cross-linking reagent, several compounds such as toluene diisocyanate [1321-38-6] or hexamethylene diisocyanate [822-06-0] (**21**) are used. The yield of enzyme activity is usually low. Microbial cells are also cross-linked with glutaraldehyde to yield cell pellets.





3.3.3.3 Entrapment

Entrapped biocatalysts are classified according to the following different types:

Lattice type: biocatalysts entrapped in gel matrices prepared from polysaccharides, proteins, or synthetic polymers.

Microcapsule type: biocatalysts entrapped in microcapsules of semipermeable synthetic polymers.

Liposome type: biocatalysts entrapped within liquid membranes prepared from phospholipids.

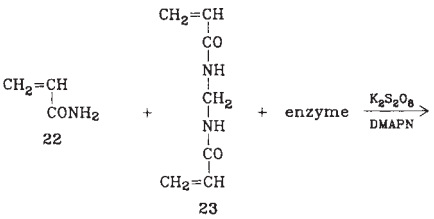
Hollow-fiber type: biocatalysts separated from the environment by hollow fibers.

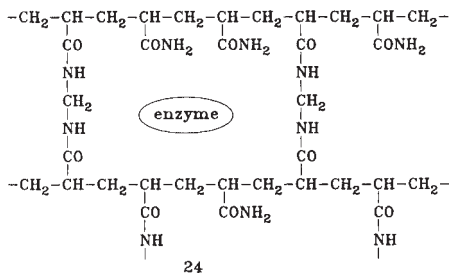
Membrane type: biocatalysts separated from the spent reaction solution by ultrafiltration membranes.

The advantages of entrapping methods are that not only single enzymes but also several different enzymes, cellular organelles, and cells can be immobilized with essentially the same procedures. Biocatalysts are not subjected to serious modification, and immobilization eliminates the effect of proteases and enzyme inhibitors of high molecular mass. However, disadvantages are (1) substrates of high molecular

mass can hardly gain access to the entrapped biocatalysts and (2) supports are not renewable. Entrapment within ultrafiltration membranes can avoid the disadvantages inherent in entrapping methods, although inactivated enzyme molecules often precipitate on the membrane surface, which results in decreased permeability to reaction solutions. The lattice-type method is most widely applied for preparing immobilized biocatalysts. Several examples of this technique are mentioned in the following paragraphs.

Polyacrylamide Gel Since BERNFELD and WAN [270] reported the entrapment of several enzymes in polyacrylamide gels (**24**), different types of biocatalysts including cellular organelles and microbial cells have been immobilized by this method. This method was also applied to the industrial production of L-aspartate, L-malate, and acrylamide by immobilized microbial cells. In a typical procedure, acrylamide [79-06-1] (**22**) and *N,N′*-methylenebisacrylamide [110-26-9] (**23**) (cross-linking reagent) are mixed with biocatalysts and polymerized in the presence of an initiator (potassium persulfate [7727-21-1]) and a stimulator (3-dimethylaminopropionitrile [1738-25-6] (DMAPN) or *N,N,N′N′*-tetramethylenediamine [110-18-9]). Although this technique is used for various purposes, acrylamide monomer sometimes inactivates enzymes. Several analogues or derivatives of acrylamide can also be used in this method.





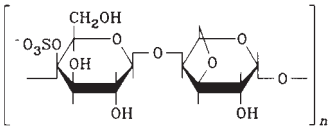
Alginate Gel Several natural polysaccharides, such as alginate, agar, and κ-carrageenan, are excellent gel materials and used widely for the entrapment of various biocatalysts.

Sodium alginate I [9005-38-3], which is soluble in water, is mixed with a solution or suspension of the biocatalysts and then dropped into a calcium chloride solution to form water-insoluble calcium alginate gels that immobilize enzymes, cellular organelles, or microbial cells [285]. However, gels are gradually solubilized in the

presence of calcium ion-trapping reagents such as phosphate ions. Aluminum ions or several divalent metal ions can be substituted for calcium.

This method is used widely for immobilization of various biocatalysts because of its simplicity and the availability of sodium alginate.

κ-Carrageenan Gel CHIBATA and co-workers [286] have extensively screened gel materials for the immobilization of enzymes and microbial cells in industrial applications and have found κ-carrageenan (**25**) to be the best.



25 (n = 250–2000)

κ-Carrageenan in saline is mixed with a solution or suspension of biocatalysts and dropped into a solution of gelling reagent, such as potassium chloride [7447-40-7] [287]. Various cations, such as ammonium, calcium, and aluminum, also serve as good gelling reagents. Hardening of gels with glutaraldehyde and hexamethylenediamine often stabilizes the biocatalysts entrapped in κ-carrageenan gels.

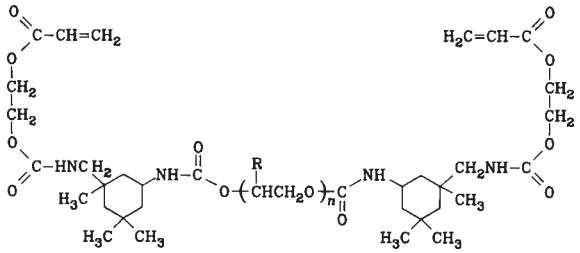
This method has replaced polyacrylamide gels in the industrial production of L-aspartate [288] and L-malate [289] by means of immobilized microbial cells.

κ-Carrageenan gels can be solubilized in saline or in water, which enables the number of cells in the gel to be counted.

Synthetic Resin Prepolymers With the application of a variety of bioreactions, including synthesis, transformation, degradation, or assay of various compounds having different chemical properties, entrapment of biocatalysts in gels of controlled physicochemical properties has become desirable. Selection of suitable gels and modification of natural polymers to meet each purpose are usually difficult. For these reasons, a new entrapping method using synthetic resin prepolymers has been developed by FUKUI and co-workers [290].

Specific features and advantages of the prepolymer method are (1) entrapment procedures are very simple and proceed under very mild conditions; (2) prepolymers do not contain monomers that may have unfavorable effects on enzyme molecules; (3) the network structure of gels can easily be controlled by using prepolymers of optional chain length; and (4) the physicochemical properties of gels, such as the hydrophilicity–hydrophobicity balance and the ionic nature, can be changed by selecting suitable prepolymers synthesized in advance in the absence of biocatalysts.

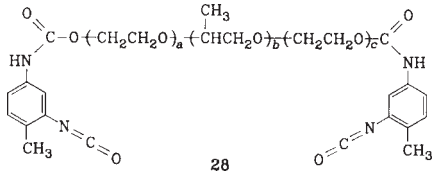
Photo-cross-linkable resin prepolymers having hydrophilic (**26**) or hydrophobic (**27**) properties, cationic or anionic nature, and different chain lengths have been developed [290], [291].



26: R = H
27: R = CH_3

Mixtures of prepolymers and biocatalysts are gelled by irradiation with long-wavelength UV light for several minutes in the presence of a proper sensitizer such as benzoin ethyl ether [574-09-4]. This method has been applied for the pilot-scale production of ethanol by immobilized growing yeast cells.

Entrapment by urethane prepolymers (**28**) with different hydrophilicity or hydrophobicity and chain length is much simpler [290], [292]. When the liquid prepolymers are mixed with an aqueous solution or suspension of the biocatalysts, the prepolymers react with each other to form urea bonds and liberate carbon dioxide.



28

These prepolymers can immobilize not only enzymes but cellular organelles and microbial cells as well. The hydrophilicity–hydrophobicity balance of these gels has been demonstrated to affect especially the bioconversion of lipophilic compounds in organic solvent systems [293].

3.3.4 Characterization

The characteristics of immobilized biocatalysts should be described in the literature because different methods and preparations must be compared for their evaluation. However, determination of what kinds of description and what types of parameters should be given is difficult. Methods for estimating these parameters vary from group to group. Therefore, at present, the results described in the literature cannot be compared. The Working Party on Immobilized Biocatalysts within the European Federation of Biotechnology has made a proposal for the description of several properties of immobilized biocatalysts [294]. Although the proposal will be difficult to follow completely, it will be useful for the characterization of immobilized biocatalysts.

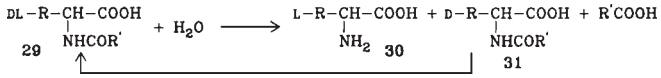
The minimum requirements recommended for characterization of an immobilized biocatalyst are as follows:

1. *General description*
   reaction scheme
   enzyme and microorganism
   carrier type
   method of immobilization
2. *Preparation of the immobilized biocatalyst*
   method of immobilization, reaction conditions, dry mass yield, activity left in supernatant
3. *Physicochemical characterization*
   biocatalyst shape, mean wet particle size, swelling behavior
   compression behavior in column systems, abrasion in stirred vessels, or minimum fluidization velocity and abrasion in fluidized beds
4. *Immobilized biocatalyst kinetics*
   Initial rates vs.
   - substrate concentration for free and immobilized biocatalyst, effect of pH and buffer
   - diffusional limitations in the immobilized biocatalyst system (effect of particle size or enzyme load on activity)
   - degree of conversion vs. residence time (points on a curve)
     storage stability (residual initial rate after storage for different periods)
   - operational stability (residual initial rate or transforming capacity of reaction system) after operation for different periods

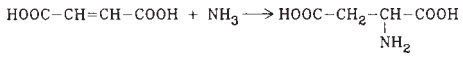
3.3.5 Application

Immobilized biocatalysts — enzymes, cellular organelles, microbial cells, plant cells, and animal cells — have been applied to the production or conversion of various compounds such as amino acids, peptides and enzymes, sugars, organic acids, antibiotics, steroids, nucleosides and nucleotides, lipids, terpenoids, fuels, or commodity chemicals. Some typical examples are described below.

A major industrial application of immobilized enzymes is the production of various L-amino acids, such as L-alanine, L-isoleucine, L-methionine, L-phenylalanine, L-tryptophan, and L-valine, by fungal aminoacylase (E.C. 3.5.1.14) [9012-37-7] immobilized on DEAE-Sephadex by ionic binding [275]. This enzyme hydrolyzes stereoselectively the L-isomer of *N*-acyl-D,L-amino acids (**29**) to yield L-amino acids (**30**) and unreacted *N*-acyl-D-amino acids (**31**); the latter are subsequently converted into the racemic form by heating. In this way, both the L- and D-isomers are converted completely to L-amino acids.



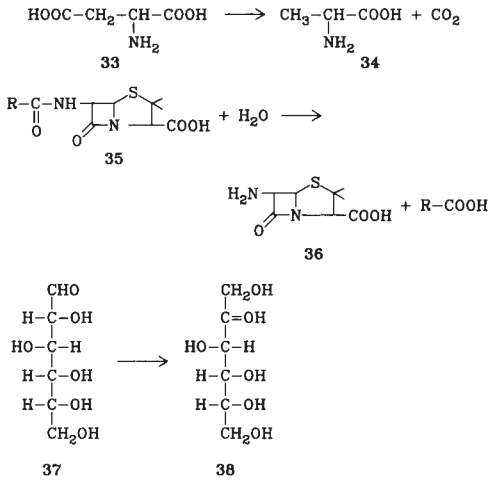
CHIBATA and co-workers also succeeded in producing L-aspartic acid [56-84-8] (**33**) from ammonium fumarate [14548-85-7] (**32**) by polyacrylamide gel-entrapped *Escherichia coli* cells containing a high activity of aspartase (E.C. 4.3.1.1) [9027-30-9].



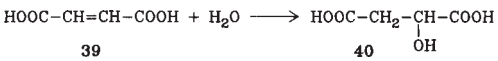
An active and stable preparation was obtained (half-life, 120 d) when the immobilized cells were incubated with a substrate solution for 48 h at 30 °C [295]. This process is the first example of an industrial application of immobilized cells and the enzyme partially isolated from *E. coli* cells is not as stable even after immobilization. At present, the process can be improved by entrapping the cells in κ-carrageenan gels, followed by hardening with glutaraldehyde and hexamethylenediamine; these immobilized cells have a half-life of 680 d [288].

L-Alanine [56-41-7] (**34**) is produced from L-aspartic acid [56-84-8] (**33**) by κ-carrageenan gel-entrapped cells of *Pseudomonas dacunhae* having a high activity of L-aspartate 4-decarboxylase (E.C. 4.1.1.12) [9024-57-1] [296]. This process was commercialized in 1982 by the Tanabe Seiyaku Co. in Japan.

Immobilized penicillin acylase (amidase) (E.C. 3.5.1.11) [9014-06-6] is now widely used in the production of 6-aminopenicillanic acid [551-16-6] (**36**) from penicillin G [61-33-6] (**35**). Glucose isomerase (E.C. 5.3.1.18) [9055-00-9] is used in the production of high-fructose syrup (**38**) from glucose [50-99-7] (**37**).

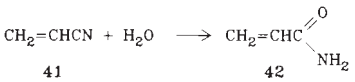


A process for production of L-malate (**40**) from fumarate (**39**) has been developed by CHIBATA and co-workers by using *Brevibacterium ammoniagenes* cells containing fumarase (E.C. 4.2.1.2) [9032-88-6] entrapped in polyacrylamide gels [297].



The entrapped cells were treated with bile extract to suppress the formation of byproduct succinate, which leads to the industrial application of this system in 1974. Later *B. flavum* was substituted for *B. ammoniagenes* and κ-carrageenan gels for polyacrylamide gels. κ-Carrageenan gel-entrapped cells showed a half-life of 160 d [289].

The latest use for immobilized biocatalysts is the production of cheap commodity chemicals such as acrylamide (**42**) from acrylonitrile (**41**).



Polyacrylamide gel-entrapped cells of *Corynebacterium* species are used for the production of acrylamide at low temperature by Nitto Chemical Industry in Japan. YAMADA and his co-workers have found that in *Pseudomonas chlororaphis*, nitrile hydratase [82391-37-5] participates in this reaction [298].

In addition to the immobilized enzymes and treated cells mentioned above, other immobilized living or growing cells are now being investigated extensively for the production of more complex compounds by using the metabolic activities of these cells. Plant and animal cells as well as microbial cells are targets of immobilization because these cells have self-regenerating and self-proliferating catalytic systems [299]. Genetically improved cells are also useful biocatalysts.

Bioconversion of lipophilic compounds in organic solvent systems is another subject of interest industrially. Immobilization in or on proper supports stabilizes biocatalysts even in the presence of organic solvents [293].

Thus, the applications of immobilized biocatalysts are now expanding along with the development of immobilization techniques and the improvement of biocatalysts.

4
Discovery and Development of Enzymes

4.1
Enzyme Screening

4.1.1
Overview

Screening and selection are early processes in the research and development cycle of a product. In essence this means searching for a particular gene coding for the target enzyme. In the past this involved exclusively the screening of living microorganisms (classical microbial screening), but with the application of the modern tools of molecular biology, screening can be performed without the need to culture the organisms involved. As with all methods, there are limitations, and a combination of techniques (combinatorial screening) often provides the greatest success.

The most rewarding starting point for the process of search and discovery is the rich diversity of microorganisms in nature [300]. It is seldom appreciated that for 85 % of the Earth's history, life was restricted to microbial forms. The metabolic diversity this has created is truly immense. During more than four billion years of evolution, enzymes have emerged that are perfectly adapted for maintaining cellular processes. However, many enzymes are required to perform in a milieu that is far removed from the natural physiological conditions and may even involve exposure to chemicals known to degrade or denature enzymes, which leads to loss of activity. Usually, enzyme screening and selection strategies are based on knowledge of the application and the physical and chemical conditions under which the enzyme must operate. Therefore, selection for enzyme performance under application conditions is an essential early step in the screening process. Access to a large gene pool is another prerequisite for a successful screening program. The approaches can involve the indiscriminate examination of heterogeneous material (e.g., soil) gathered from the environment, or an ecological approach with a targeted examination of specific habitats where a particular activity is likely to be found. Strategies can be adopted to search specifically in poorly explored environments in order to access a greater variation in genomes from novel microbes. In the search for new genes, exploration of extreme environments such as volcanic hot springs, hypersaline lakes, and polar soils has yielded many novel microorganisms with peculiar properties [301–303]. Alternatively, a taxonomy-based approach involving a

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systematic study of known organisms can also be rewarding. By combining enzyme screening with modern techniques such as protein engineering and directed evolution, hybrid molecules with an improved performance under specific application conditions can be found. This chapter will describe the different screening methods currently used in industry to obtain enzymes with desired properties.

4.1.2 Natural Isolate Screening

The source material for enzyme discovery can be plant or animal matter and microbes, both prokaryotes (e.g., bacteria and archaea) and eukaryotes (e.g., yeasts and fungi). Microorganisms are the major source for industrial enzymes. The classical method of screening natural microbial isolates is a well-established process having its roots in antibiotic discovery (e.g., penicillins, streptomycin) in the decades after 1945. It involves the examination of thousands of samples of soils, plant material, etc., and the random isolation and screening of the resident microbial flora. Although capable of significant automation, the throughput capacity largely determines the speed of progress. In the past, screening strategies using soil samples as a starting point were highly empirical. But much can be done to reduce the need for extensive experimentation. These processes involve subjecting an environmental sample (e.g., soil) to selective pressure using the principles of enrichment culture by manipulating the growth conditions so that only microorganisms expressing a particular enzyme are able to grow and survive. For example, by providing only xylan as a source of carbon and energy, only microbes producing a xylanase constitutively or by induction will grow, and the culture becomes enriched in these organisms. The system is capable of considerable refinement; for example, if an acidic xylanase is required, then the pH of the enrichment culture can be designed to permit growth of microbes producing the enzyme at pH 4–6. A successful outcome can be improved by a judicious choice of environment for examination, for example, acidic soils, peat bogs, and forest litter.

An ecological approach takes advantage of the natural selective pressure of the environment. For example, an alkaline environment such as a soda lake at pH 9 is inhabited only by alkaliphilic microorganisms. It is likely that these organisms have extracellular enzymes that are stable and perform optimally at high pH, and this is indeed the case [304]. For screening of feed enzymes such considerations are often less clear-cut. What, for example, is the natural habitat for a phytase-producing microorganism? Phytate, a storage phosphate, is often a major component of some seeds, and screening microorganisms in intimate association with the roots of germinating seeds (rhizosphere) would be an appropriate niche to examine.

The time between collection of the environmental sample and its examination in the laboratory can be critical, since changes will inevitably occur. Even in the best-preserved samples, chemical and physical changes may lead to the death of some organisms and reduced viability of others. The longer the time lapse, the more serious these changes can be. There are many advantages in treating the sample immediately in the field, since this can lead to the isolation of a totally different population of microbes. It can be useful to provide ''bait'' for specific microbes or types of microbial activities. An example of

this in situ enrichment is placing cotton linters in an environment such as lake sediment and retrieving the sample at a later date. The sample can then be taken back to the laboratory and screened for cellulolytic activity. Many other enrichment techniques have been developed empirically, such as the use of water- or chitin-agar for isolation of slow-growing actinomycetes, and such ideas are limitless [305].

In addition to enrichment for desired microorganisms, the numbers of unwanted microbes can be reduced. In fact, the first problem in processing of samples is often one of numbers. Composts and soils rich in organic matter contain enormous numbers of microbes. Some form of dilution is required, or targeting of specific groups. Selective techniques can involve, for instance, careful air-drying of the (soil) sample, which reduces the numbers of Gram-negative bacteria, or a brief heat treatment at 80 °C, which kills most microorganisms but leaves the resistant spores of bacteria such as *Bacillus* intact. Antibiotics may be incorporated into the growth medium. For example, Gram-positive bacteria are more sensitive towards penicillin than Gram-negative bacteria.

Whatever the screening strategy adopted, all these approaches rely on some form of selection criteria for the organism-activity combination being sought. The most widely applied methods involve growing the microbes on an agar plate containing the appropriate enzyme substrate for detecting the desired enzyme activity. Often the methods employed are proprietary and frequently constitute more of an art than an exact science. Some methods, however, are well understood. For instance, incorporating casein as sole carbon source into an agar medium with an opaque surface will reveal protease-secreting organisms as a clear halo of hydrolyzed casein around the growing colony. Similarly, lipase activity can be monitored by a change in color of an indicator that visualizes the drop in pH due to the release of fatty acids from a lipid substrate. Specific cleavage reactions can also be detected by release of a fluorescent product or a strongly absorbing (chromogenic) product from an appropriate substrate [306–308]. An interesting possibility is provided by display of a quenched fluorescent substrate on the cell surface of a recombinant host that after cleavage gives a fluorescent product which remains associated with the cell [309].

The selection of microorganisms is a very critical stage in the screening process and can be quite subjective, especially if the numbers involved are very large. Even though the screening has followed some form of selection process it is often not easy to distinguish the different types of microorganism growing on an agar medium. This is particularly true of bacteria, which, unless they are clearly colored, all look much the same. This gives rise to one of the biggest drawbacks of the random approach to isolation and screening of naturally occurring strains: the problem of redundancy. The same microbe will recur time and again from different samples collected from different locations, and this may explain why so many products come from a very limited range of microbes. Until relatively recently most screening strategies were fairly conservative. The tendency to use rich media that favor fast-growing, nutritionally undemanding organisms has lead to an overemphasis on particular groups of microorganisms. This is coupled to the fact that certain fungal genera such as *Aspergillus* and *Trichoderma*, and *Bacillus* and *Pseudomonas* species among the bacteria, have traditionally been a rich and proven source of commercial enzymes. To ensure greater diversity and as a measure of how extensive a screening has been, some estimate of the biodiversity of the population

screened is required. A decade ago this was a daunting task involving a detailed examination of each organism, but nowadays fast molecular methods are available. A range of techniques rapidly group collections of unknown strains into clusters of related organisms. Total cell protein patterns, amplified fragment length polymorphism, ribotyping, amplified ribosomal DNA restriction analysis, internally transcribed spacer region PCR, single-strand conformational polymorphism combined with sophisticated electrophoresis methods, and image analysis for data acquisition and computation can provide information on the diversity of a strain collection. Today, the ease with which small subunit ribosomal RNA (ssu rRNA) gene sequencing can be performed, combined with a large and growing database for comparison, means that specific organisms can often be quickly assigned to known taxonomic groups, or novel microorganisms can be recognized. The clustering of organisms into related groups of strains can be combined with screening of selection data, thus permitting the identification of specific attributes with recognized groups of organisms. This information allows one to devise particular procedures for the isolation of a specific target group and the imposition of more stringent selection criteria. Even so, it is still too easy to overlook potential novel isolates, especially when confronted by many hundreds of culture dishes, each one containing several hundred similar-looking microbial colonies. Nowadays, the task can be performed by colony-picking robots and visual imaging systems coupled to computers.

One problem often encountered early in the screening process is that natural microbial isolates usually produce commercially important enzymes in exceedingly low concentrations. Environmental manipulation that optimizes the growth and production conditions is limited by the intrinsic maximum ability of the wild-type organism. Classical genetic manipulation by selecting mutants with improved properties can lead to an increase of potential yield. The classical techniques of mutation and selection for microorganisms with improved attributes has been applied for many decades. Modern methods of molecular biology have accelerated the possibilities of creating in the laboratory microorganisms with new or improved activities.

4.1.3
Molecular Screening

If a suitable lead enzyme, for example, an acid xylanase, has been identified in a particular species then a search can be made for homologous enzymes in related species. The process requires the application of reverse genetics. Enzymes are polymers of amino acids covalently bonded in a defined sequence. The order in which the amino acids are arranged in the enzyme can be analyzed by a process known as N-terminal sequencing. Since the genetic code is universal it is possible to predict the probable sequence of nucleotide bases in the gene coding for the specific enzyme. Using this information a probe can be constructed consisting of 15 to 20 nucleotides (primer set) in the correct sequence, which, because of the double helix nature of DNA, will bind to the complementary strand. By using the primer set in the polymerase chain reaction (PCR) on chromosomal DNA from related organisms, a homologous gene can be amplified and transferred to a host organism such as *E. coli* for expression of the gene

product, i.e., a homologous enzyme [310]. In practice the procedure can be a little more complex than is described here, but it is possible to track down enzymes with identical functionality (e.g., a xylanase) but with a significantly different sequence of amino acids, whereby only the crucial elements of the enzyme such as the active site have been conserved through evolution. In application tests these "new" enzymes may have significantly different properties, such as improved temperature or pH stability compared with the lead molecule. In this way whole families of related organisms can be screened. Alternatively, the microbial DNA can be probed with DNA carrying a radioactive or fluorescent label by a technique called hybridization. However, all molecular methods based on similarity screening have one big drawback. They rely on a comparison with data that has already been recorded.

4.1.4 Environmental Gene Screening

Even though molecular screening is a very powerful technique it seems limited by the ability to grow and extract suitable DNA from the target organisms. It is also confined to known microorganisms that have been isolated, described, and deposited in culture collections. However, it has been estimated that fewer than 1 % of all microorganisms that exist in nature have been isolated, and even fewer have been adequately characterized and described [311]. It is thought that fewer than 25 % of the microbes in a sample taken from the environment can actually be cultured in the laboratory. There may be many reasons for this "unculturable" portion of the microbial community. Some cells may be in a resting phase, damaged, or moribund; some may not be true residents and not form part of the active population; others may be opportunists waiting for a shift in environmental conditions in their favor; while others may have unknown requirements for culture that remain unmet in the laboratory.

Cultivation in the laboratory has a strong selective bias; the techniques are very conservative and have hardly changed for 100 years. Yet we are imprisoned within these constraints since we have no intellectual answers to this problem [312]. The main biodiversity of life is microbial, distributed among three primary relatedness groups or domains: Archaea, Bacteria, and Eukarya. The number of validly described species of prokaryotes is about 4500 and increases at a rate of 100–200 per year. In 1987, Woese described the bacterial domain as comprised of about 12 naturally related groups (divisions) based on ssRNA sequence analysis of familiar cultivated organisms and showed that they displayed greater evolutionary depth (genetic biodiversity) than plants, animals, and fungi [313]. Phylogenetic studies of cultured and environmental sequences have since expanded substantially our appreciation of the scope of bacterial biodiversity. Three trail-blazing, seminal research reports, all published in 1990, indicated that bacterial biodiversity based on molecular studies was significantly larger than that known from pure cultures. From a DNA-DNA reassociation study it was estimated that a single sample of forest soil contains about 12 000 bacterial species [314]. The other two studies reported cultivation-independent retrieval of partial 16S rDNA sequences from marine bacterioplankton [315] and hot springs [316]. The retrieved 16S rRNA sequences did not match those of known cultured bacteria and

thereby provided evidence for numerous as yet unknown bacteria. A more recent examination of environmental DNA derived from diverse habitats revealed, in addition to a vast biodiversity of yet to be cultivated microorganisms, that the cultivated microorganisms may not be the dominant flora in these environments, and this provides a further challenge to the screening by enrichment culture methods [317, 318]. Now, after nearly a decade of direct rRNA sequence retrieval from the natural environment, nearly 40 distinct bacterial divisions (kingdom-level phylogenetic divergences) are resolved. Just four of these divisions account for 90 % of all cultivated bacteria (Proteobacteria, Cytophaga-Flavobacterium-Bacteroides, and the high and low G+C Gram-positives). It is small wonder that most microbial biotechnology is devoted to organisms that fall into these groups. For over 15 of the bacterial divisions, not a single organism in culture is known, and for a further 15 divisions one or two cultured representatives are supported only by the environmental sequence footprint [319]. The phylogenetic differences between the bacterial divisions are probably reflected in substantial physiological differences [320].

Fortunately recent technological developments have permitted access to at least a portion of these uncultivated microbes [317, 321]. It is possible to extract DNA or even RNA directly from the microbial community present in a sample taken from the environment. Although there are many techniques for extracting chromosomal DNA from natural microbial communities, not all cells are equally susceptible to lysis, and the DNAis never recovered to 100 %. Another concern is the detection of organisms that form only a minor proportion of the total community. Their DNA will be underrepresented unless efforts are made to normalize the sample. The environmental DNA can be tested for known genes by using the molecular techniques described above. This requires good quality DNA, since PCR screening is particularly sensitive to disturbance by contaminating substances in the environment. Alternatively, the environmental DNA can be cut into smaller fragments by using restriction enzymes, and the fragments cloned into an expression host such as *E. coli*. Likewise, environmental RNA can be used to construct cDNA expression libraries. The clones can then be screened for new enzyme-encoding genes in a manner similar to many of the techniques described for the screening of natural microbial isolates thus allowing the detection of desired activities encoded by genes that might show only limited or no similarity at all to known genes.

4.1.5 Genomic Screening

Spectacular progress has been made in the direct sequencing of the nucleotides of a DNA strand. Already there are millions of gene sequences in public databases. The whole genomes of over 800 organisms can be found in public databases such as Entrez Genomes [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome]. The genomes represent both completely sequenced organisms and those for which sequencing is in progress. All three main domains of life — bacteria, archaea, and eukaryota — are represented, as well as many viruses and organelles. At present (2006) 302 prokaryotic (279 bacteria and 23 archaea) genomes and several microbial eukaryotic genomes have

been sequenced in their entirety, and the sequencing of more than 20 microbial genomes is in progress. Random shotgun sequencing allows rapid elucidation of microbial genomes [322], and technical advances are expected to further accelerate genome sequencing. Originally, pathogenic species were the main focus of microbial sequencing projects, but many microbes with industrial potential are being sequenced as well. For example, the genomes of thermophilic, alkaliphilic, acidophilic, and salt-tolerant microbes have been sequenced [323–327]. This illustrates the general belief that genome analysis will reveal the mechanisms underlying the respective lifestyles of these microbes, aid in identifying industrially relevant extremophilic enzymes, and possibly allow the recognition of general protein rules that result in enzymatic performance under extreme conditions. Global analysis of thermophilic proteins shows, for instance, a tendency to reduced protein size; increased hydrogen bonding, beta-strand content, and helix stabilization through ion pairs; and preference for charged residues over uncharged polar residues [323], [328], [329].

When a microbial genome sequence is available, protein-coding genes can be identified computationally by essentially two different methods [330]. Ab initio searches use a template that integrates general knowledge about gene composition and signals to find novel genes. A more reliable computational method uses similarity to a protein-coding gene in another organism to identify novel genes. The latter method depends, however, on the availability of homologous genes, but it has as added benefit the ability to indicate gene function. Since prokaryotic genes frequently overlap with each other, exact prediction is notoriously difficult, and as a result the true number of protein-coding genes for sequenced microbial genomes remains debatable [331], [332]. A further complication that adds to the discrepancy between gene predictions and expressed proteins is presented by alternative splicing, which can occur in microbial eukaryotes [333]. Several experimental approaches, such as EST sequencing and scanning-tiling microarray analysis, allow validation of computationally identified genes [334].

The enormous amount of sequence data in the public domain has driven the emergence of bioinformatics, which can be defined as the interface between biological and computational sciences. Bioinformatics uses computational tools to organize and analyze biological data and aims to extract biological information and knowledge. Typical examples include identification of homologous genes or specific protein domains and transfer of functional knowledge associated with these homologues or domains to predict function for the new genes [335–337]. For example, similarity searching at the gene level allows in silico screening for enzymes [338]. Note, however, that sequence data are certainly not flawless, and a considerable error rate should therefore be considered in the analysis [335], [339]. Error frequency can be reduced by integration of additional information that can be extracted from genomic or experimental data [340]. Other computational methods that make use of available genomic sequences to infer function rely on, e.g., genomic clustering of functionally related genes [341], [342], phylogenetics [343], [344], metabolic pathway alignment [345], [346], and combined approaches [347]. Despite such powerful tools, about 25 % of the putative genes revealed by total genome sequencing projects cannot currently be assigned a function because they only show homology to other unannotated genes. Another 25 %

of the genes are of unknown function and seem to be unique to their particular species [348]. Conversely, almost 40 % of the well-defined enzyme activities that have been assigned specific E.C. numbers are not yet associated with published gene sequences [349]. These data indicate that the resources for screening of novel enzymes are still largely untapped. Sparked by these findings several new high-throughput experimental technologies have been developed that aim to identify functions for these orphan genes. Amongst others, expression and structural studies have been initiated to determine whether such genes encode "hypothetical" proteins to which a specific function can be assigned [350], [351].

Many methods have been developed that use loss of function, cellular localization, protein interaction, or timing of expression to predict function for novel proteins and can be claimed as tools for so-called functional genomics. Essentially, most of these methods infer function indirectly as "guilt by association" in time or space with characterized proteins. For example, a wide variety of techniques are available to disrupt genes, random or directed, on a large scale in virtually any organism [352–357]. Subsequent phenotypic analysis, quite similar to natural isolate screening, allows determination of putative biological functions of the inactivated proteins. Some of the gene disruption methods also allow analysis of protein localization [358], which can aid functional annotation. When specifically screening for extracellular enzymes, secretion can also be predicted on the basis of sequence information [359] or transcript analysis [360]. Alternatively, the use of yeast-based signal sequence traps allows selection of secreted proteins from both eukaryotes and prokaryotes without prior sequence information [361]. Large-scale analysis of protein interactions by genetic 2-hybrid or biochemical/mass spectrometric approaches has also been used to assign function [362]. In addition, computational methods have proven successful in predicting functional interaction between proteins [364].

So far, however, genome-wide transcript analysis has emerged as most used tool for functional genomics. Based on the assumption that proteins with similar functions show similar regulation, transcript data can be used to functionally classify genes [363], [365], [366]. Microarrays of oligonucleotides or entire genes representing the complete genome can be hybridized with differentially labeled cDNAs obtained from separate cells or cell states. Comparison of transcript profiles indicates regulation of gene expression as a result of changes in the cell's conditions. Alternatively, differential expression of proteins can also be followed directly at the level of protein — albeit still at lower throughput — instead of indirect assessment via the analysis of transcription [367]. Co-regulation of genes (or proteins) can then be used to identify genes that are relevant to a particular cellular process. For example, global transcript analysis in *Thermotoga maritima* is used to identify genes involved in the biodegradation of simple and complex plant polymers with potential as renewable energy sources, such as xylan and cellulose [368]. Designing a whole-genome DNA microarray for a fully sequenced microbe is conceptually straightforward [359]. Arraying multiple DNA arrays simultaneously can further increase throughput of expression analysis. For example, expression levels of more than 6000 human genes in 49 different cell types were recently detected in a single hybridization experiment [370]. Many other applications exist for the use of DNA microarrays in addition to transcript profiling [366].

4.1.6 Proteomic Screening

The above-mentioned functional genomics approaches are all clearly very valuable, but they still infer protein function indirectly. Direct assays for function require high-throughput expression of the identified genes, followed by functional analysis of purified proteins. This approach introduces new challenges to protein expression, including the need for high-throughput cloning and purification [371]. Many new technologies that facilitate such large-scale approaches are not limited to the full complement of sequenced genomes, the proteome, but can be adapted for the functional analysis of large numbers of recombinant proteins, for instance, those encoded by cDNA or environmental libraries.

Although any gene can be engineered to alter specific restriction sites and facilitate cloning into a suitable vector, this process cannot efficiently be performed when dealing with large sets of genes. To circumvent this, several recombination-mediated cloning strategies have been developed that allow flexible cloning into many different vectors [372–374]. High-throughput protein purification requires fusion with specific affinity tags that facilitate purification, immuno-localization, and/or immuno-precipitation [375], [376]. For example, genome-wide fusion to different tags has allowed parallel purification and characterization of thousands of different proteins. Protein fusions have also been used to improve secretion and solubility of small or low-solubility proteins. Although addition of tags might affect protein function, they strongly facilitate detection and purification, and can allow cell surface display or immobilization on beads or any other support [377–379].

Some exciting examples of proteomic screening were reported in the late 1990s and early 2000s. More than 6000 yeast genes fused to glutathione S-transferase (GST) were expressed and purified [377]. Pools of purified fusion proteins were assayed for different enzymatic activities, and after deconvolution specified activities could be assigned to orphan genes. In a related approach nearly all protein kinases from baker's yeast were arrayed in microwells and simultaneously analyzed by using 17 different substrates [380]. The same authors also expressed most yeast genes with a 6xHIS tag, which allowed binding of the fusion proteins to nickel-coated slides. The purified proteins were printed on these slides and screened for their ability to interact with proteins and phospholipids. Probing the proteome chips yielded many new functional interactions [378]. In two independent studies fusion proteins were also used to purify and characterize multi-protein complexes on an unprecedented scale [379], [381]. In a related study it was shown that an affinity purified TAP-tagged multiprotein complex retained its activity [382].

Many ideas to construct protein chips are being pursued [383] but from an enzyme screening perspective attention should focus on methods that are most likely to retain enzymatic activity. In an interesting alternative [384], instead of printing proteins, an expression library is printed and overlayed with competent cells, resulting in localized expression by transformants. The cell clusters can then be screened for clone-dependent properties. Cell surface display of protein libraries has been used to select novel or improved binders but can also be used to screen for enzymatic activity [385]. For example, mutants with improved catalytic activity have been identified by surface

display of cellulase [386] and subtiligase [387]. Combined display of a quenched fluorescent substrate and a library of protease variants has been used to screen for novel substrate specificity [309]. Such approaches should also make it feasible to screen expression libraries of natural diversity.

4.2 Protein Engineering

4.2.1 Introduction

Protein engineering employs a combination of tool sets that allow any amino acid in the amino acid sequence of a protein to be replaced by one of the other 19 naturally occurring amino acids of proteins in order to change their properties. The genetic code can also be expanded by the introduction of nonnatural amino acids into a protein [388–390]. These changes can be either directed to one position or randomly spread along the whole primary sequence.

The establishment of protein engineering as a new technology has been enabled by the advent of recombinant DNA technology, accompanied by the continuous improvement of 3D structure determination by X-ray crystallography and NMR spectroscopy.

Until the 1980s the possibilities for changing an enzyme were limited to chemical modifications, which are restricted to the reactive amino acid side chains and are neither regio- nor site-specific.

Recombinant DNA technology has revolutionized protein modification in a number of ways. It is now not only possible to replace one particular amino acid by one of the nineteen others, but these changes are also permanent. New material of the modified protein of the same quality is available simply by repeated fermentation of the production organism, followed by purification. In addition, enzymes produced in recombinant systems are available in larger quantities and higher purity. This in turn facilitates the determination of protein 3D structures and thus increases the knowledge base for further protein-engineering experiments.

Both technologies for protein structure determination, X-ray crystallography and NMR spectroscopy, have benefited from the availability of larger quantities of pure proteins, but without the simultaneous development of the physical methods the huge amount of structural data would not be available today (see Chap. 1). The quality of X-ray data has been improved by hardware developments of the traditional X-ray beam sources and, in particular, by the availability of coherent X-ray beams generated by synchroton radiation, which allow the resolution of crystallographic data to be brought down to down to even less than 1 Å (Fig. 28). At this level hydrogen atoms and hydrogen bonds in protein 3D structures can be directly observed [391], [392].

At ultra-high resolution of 0.54 Å it is even possible to study the valence electron density of proteins [393].

NMR spectroscopy has gained from the development of better magnets that allow better separation of signals and finer determination of the chemical shifts of the atoms.

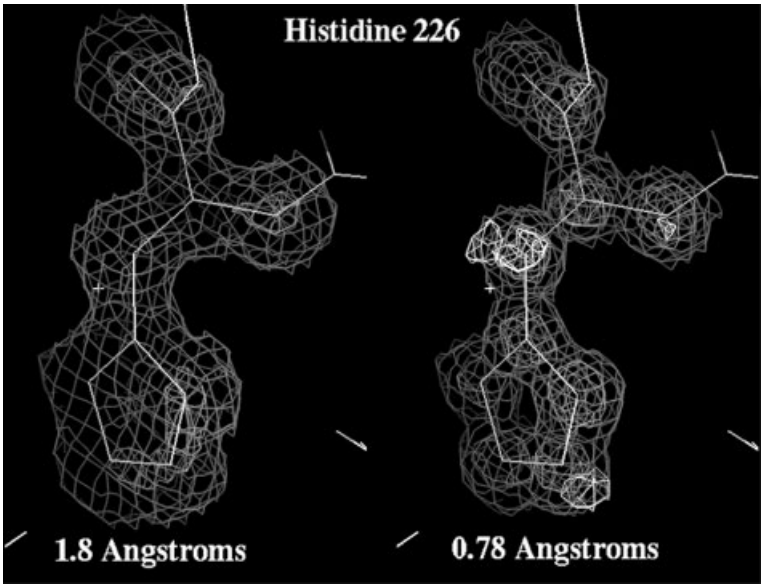

Fig. 28 Information content of electron-density maps at different resolutions. The figure compares information available at 1.8 Å (left) with 0.78 Å (atomic resolution, right). Both maps were calculated from the same refined data set and phases derived from a refined model at 0.78 Å resolution. In both maps a standard $2F_o-F_c$ electron density was contoured at σ and 4 σ. At 0.78 Å, the 4 σ contour delineates individual atoms, and the relative dimensions indicate whether the atom is carbon, nitrogen, or oxygen having 6, 7, or 8 electrons, respectively. Contoring at 4 σ in the 1.8 Å plot does not convey this information. In the 0.78 Å map positive difference electron density is observed for hydrogen electrons, which will be valence electrons participating in covalent bonds. As such, the positive electron density will not be centered about the hydrogen atom nucleus but rather about the covalent bond.

NMR spectroscopy is also influenced by the general developments in biotechnology that allow the effective production of the necessary ^{13}C- and/or ^{15}N-labeled proteins.

Both structure determination methods, however, were most influenced in the 1980s by the sudden availability of continuously increasing computational power. Since protein X-ray crystallography and NMR spectroscopy are indirect methods, they heavily depend upon computational power to process the data, and they require visual inspection of the results on high-end graphical computers.

The graphical computer systems paved the way for the establishment of the important knowledge base for protein engineering, the establishment of the structure-function relationship. Until then it was only possible to study the events around enzymatic catalysis by interpretation of the kinetic data under different conditions, but with the availability of structural data and cheaper graphics computers for visualization, it became possible to rationalize the observation at atomic detail by combining experimental and structural data. A very good example for this is the structural explanation for the reduction in the catalytic activity of subtilisin due to oxidation. Bott *et al.* [394] determined the structure of the oxidized form of subtilisin

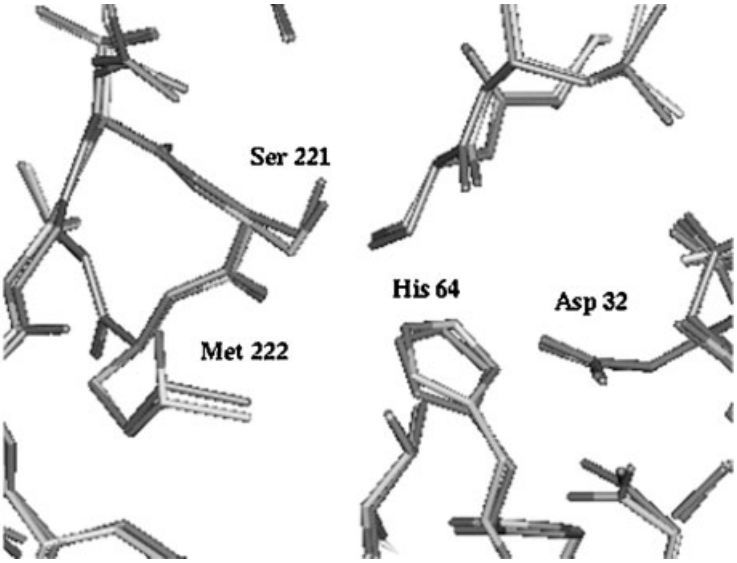

Fig. 29 Comparison of the structure of native subtilisin with the structure of the enzyme with peroxide-oxidized met 222

(EC 3.4.21.62) from *Bacillus amyloliquefaciens* and compared it to the nonoxidized native form (Fig. 29). In the oxidized form, the oxygen atom of the oxidized methionine 222 projects into the oxyanion hole. Thus, the substrate carbonyl atom will not bind in an identical manner as in the native enzyme. This may not only affect the relative binding energy of the substrate K_m, but the catalytic rate as well, since the oxyanion hole has been shown to be critical for catalysis. Substitution of Asn 155, for example, has been shown to reduce catalytic activity as measured by k_{cat} by 100–1000-fold [395].

Besides structural knowledge, the generation of mutants is a major aspect of protein-engineering experiments. In older days, the majority of mutational experiments were performed by so-called cassette mutagenesis [396], [397], a technique with which all nineteen possible natural amino acids could be substituted at a given site. From these studies variants having altered substrate specificity were found that displayed increased specificity by as much as a 1000-fold by introduction of a single mutation. Moreover, the pK values of catalytic residues could be modified and the pH rate profiles could be tailored to the desired performance pattern. This technique allowed the generation of site-directed mutations and of small random mutagenesis banks in defined regions of the molecules. Using this technique, numerous sites near critical subsites were determined and evaluated in subtilisin. Three amino acid changes were sufficient to interchange specificities between related subtilisins [398], while in trypsin numerous changes in addition to the position 189 side chains were needed to interchange trypsin and chymotrypsin specificities [399].

These efforts are indicative of another recurring theme in modern protein engineering, namely, the exploitation of natural diversity for guidance in selecting sites and

substitutions. Natural diversity is the result of millions of years of adaptation to various extremes of conditions such as temperature, pH, and substrates. Within a certain class of enzyme the overall tertiary folding pattern is often highly conserved, and thus it is possible to associate changes that can often be introduced at particular positions and associate specific substitutions with particular alterations in performance profiles and substrate specificities. The theme of exploiting natural diversity reappears in most commonly used techniques, either for rational selection of sites and substitutions or by recombination as a means of extending diversity.

Mutagenesis Techniques After the recognition that recombinant DNA technology could be used to modify the sequence and thereby the function of an enzyme by altering the DNA sequence encoding for the protein, the next advancement came with the advent of cassette mutagenesis [396]. Complete sampling of all natural amino acid substitutions became realizable. With this tool, the full repertoire of diversity was now available for molecular evolution, at least one site at a time.

Around the same time, the idea of alanine scanning was introduced. In this model, many sites could be examined by replacement of existing amino acid residues with alanine. In this case, the diversity looks to identify sites that may play an important role for functioning of the enzyme. In this approach potential sites are screened that can later be explored by structural analysis and/or diversity generation.

The discovery of error-prone polymerase chain reaction (PCR) was the first revolution in enzyme-variant generation. In fact, PCR allows both the production of site-directed protein variants and the generation of random variant libraries over the whole gene or defined parts of it. Mutation frequency of the error-prone PCR experiments can be driven by the reaction conditions and the choice of the polymerase.

Another method for the generation of molecular diversity at the gene level is gene shuffling [400–402]. In gene shuffling experiments a set of homologous genes is pooled and cut into pieces. Finally the fragments are recombined randomly in a subsequent PCR. As a result an enzyme variant library is obtained that allows the combinations of all natural variations that were distributed across the genes in the input set to be explored. The variations not only allow variations of individual amino acids to be tested, but also the effect of amino acid insertions or deletions among the different proteins.

The ability to identify the right variants for a given purpose determines the success of a protein-engineering experiment. A site-directed variant is of course easy to characterize, but the screening of a random library requires excellent knowledge about the assay that is used for screening.

Phage display of proteins has become an important tool for screening and selection of proteins. In a phage-display approach, the gene which encodes for the protein to be engineered is linked to one of the genes which encode for the phage's surface proteins. As a consequence the protein is expressed at the N- or C-terminus of one of the surface proteins. Since it is on the surface, the displayed protein can be selected for binding to a certain target. Phage display allows selection from more than 10^9 variants for binding in a single experiment. In addition the selected protein is always isolated in combination with the gene that codes for it. The gene can be multiplied by infection of a host organism with the phage, followed by either sequencing or a subsequent mutagenesis/

selection cycle. Phage display has now been proven to be a good tool for selection of proteins that bind selectively to a target molecule.

Although active enzymes have been expressed on phages, phage display is not a suitable technique to change the catalytic properties of enzymes. Because the binding to a transition state analogue, which can be used for a selective binding screen, does not correlate well with improved catalysis [403], [404], it is necessary to design special capturing systems for the detection of phage-displayed enzymes with high catalytic activity [405].

For the improvement of an enzyme for technical application it is of outmost importance that the selective activity assay closely resembles the final application. Several examples have clearly demonstrated that activity assays with artificial substrates show no correlation with performance in real-world applications. This was shown by correlating wash performance of a protease with the enzyme's activity on casein [406]. The directed-evolution experiment for the improvement of the activity of galactose oxidase (EC 1.1.3.9) revealed a reasonable correlation between the activity of the different galactose oxidase variants on the model substrate methylgalactose and their performance on the technical substrate guar. Ironically the best performer in the technical application had to be excluded from the dataset to obtain the good correlation coefficient of r^2=0.92 (with the best performer included the correlation coefficient was r^2=0.72) [407].

It must be clear that none of the protein engineering tools is able to improve enzymes alone. The strength of protein-engineering projects lies in the combination of structural/functional knowledge with the right molecular biology approach for the generation of variants.

Finally, the importance of screening technology has grown during the last few years. Mainly, automation in combination with miniaturization of assays down to microscopic scales has lead to a shift from pure site-directed mutations to regio-directed random mutagenesis experiments followed by intensive variant library screening.

The most comprehensive use of protein engineering is known under the names directed evolution, molecular evolution, and molecular breeding. Common to these approaches is the iterative follow up of randomization and selection steps. Directed evolution mimics the natural evolutionary process in the laboratory. It can be very successful, because the laboratory allows a nonnatural selective pressure to be applied to direct the successive improvement of a particular enzyme to improve its performance in a technical environment. Directed evolution proved to be very successful. Often three or fewer iterative cycles of randomization and selection for a desired property can achieve enormous improvements. This success can mainly be explained by the fact that the directive influences of natural evolution do not force a molecule to perform well in, e.g., a laundry washing machine. In addition, the researcher can introduce a much higher mutation frequency than occurs in Nature.

4.2.2
Application of Protein Engineering in Academia and Industry

Both the academic scientific community and industrial scientists embraced the new protein engineering technologies when they became available in the second half of the 1980s. In academia, protein engineering is used extensively for elucidating intrinsic

protein and enzyme properties such as stability, catalytic mechanism, and activity under different conditions. Industrial scientists focus on engineering for improved performance of enzymes in industrial applications.

Deciphering the Catalytic Mechanism Protein engineering can be used to identify amino acids which participate in the catalytic mechanism, before or even after the availability of 3D structural information. The most comprehensive studies on enzyme mechanisms by protein engineering were performed on tyrosyl-tRNA synthetase starting in the 1980s [408]. The complete investigation has been summarized elsewhere [409].

Protein engineering has also been used for the identification of the active sites in proteins, for example, in lipases. The principle nucleophile in hormone-sensitive lipase was identified by sequence analysis of the characteristic GXSXG segment of the α/β-hydrolase fold and subsequent replacement of serine 423, by several other amino acids. Since all variants had no activity at all, the authors could conclude that serine 423 is indeed the active site serine of this lipase [410]. Other groups identified in similar experiments the whole catalytic triad of different lipases [411], [412]. The investigations of Martinelle, Holmquist *et al.* [413], [414] expand this concept by investigating the role of amino acids in the proximity of the active site of *Humicola lanuginosa* lipase in substrate specificity. They combined mutagenesis results of interesting mutants with careful kinetic investigations of the variants under different conditions.

Stability of Enzymes *Fundamental Research* The intrinsic stability of proteins and the influence of single amino acids on the stability of proteins have been investigated with protein-engineering. Since the effect of exchanging an amino acid on protein structure depends on its position in the protein structure, it is necessary to use the right model system and to plan the mutation on the basis of the 3D structure of that protein.

One route to a more stable protein is the introduction of disulfide bonds by site-directed mutagenesis. In subtilisin, an enzyme having no cysteine residues, the introduction of cysteine for serine or alanine at positions 22 and 87 or 24 and 87 resulted in the formation of disulfide bridges [415] but yielded enzymes with little or no improvement in overall stability. Clarke *et al.* showed in a study on barnase that engineered disulfide bonds can stabilize and destabilize proteins. The most stabilizing disulfide bridge does not change the overall structure of the molecule and leads to local stabilization according to hydrogen-exchange experiments. The destabilizing disulfide bridge induced considerable structural changes, including the displacement of a loop in the vicinity of the new disulfide bond [416], [417].

The influence of single amino acid replacements has been studied in detail with several model proteins. The studies clearly show that the influence of an amino acid on helix stability depends on its intrahelical position, the position of the helix in the globular protein structure, and the solvent accessibility of the mutation site [418–420].

The complexity of interaction between stabilizing and destabilizing mutations during evolution has been demonstrated in a comparative mutagenesis study with barnase and binase. Both are RNAses with 85 % sequence identity. Only 17 amino acids, scattered over the whole sequence, differ in these proteins, which have the same 3D

structure but differ in stability. One-by-one replacement and combinations of the single mutations showed that each mutation has an effect on stability and that the effects are additive. However, a combination of only six of these mutations created a protein more stable than either of the parent molecules without affecting the activity [421]. This study shows that a simple comparison of sequences and 3D structures of enzymes from mesophilic and thermophilic organisms will not lead to the identification of the stabilizing amino acid changes, because nature balances several effects during evolution to obtain a set of enzymes which is good enough for its purpose.

Industrial Application Enzymes have been successfully incorporated into industrial starch processing. In the first stage α-amylase is used to break down starch into short oligosaccharides. In the commercial process the enzyme must survive passage through a steam jet at 105 °C, which is necessary to explode the starch granule, and function at 90 °C for 1–2 h. With the natural amylase of choice, *B. licheniformis* α-amylase (BLA), the pH must be adjusted to 6 and then readjusted in subsequent stages. An engineered α-amylase that would operate at lower pH would save considerable time and money by eliminating the need for pH adjustment. Analysis of BLA as a function of pH indicated that stability of the enzyme was reduced at lower pH. Initially, random mutagenesis and screening was performed in the absence of a reliable model of its three-dimensional structure, and several variants were identified [422]. Once a structure became available it was noticed that variants represented substitutions found to exist between related α-amylases. Surprisingly, the related α-amylases had lower overall stability than BLA. By using the three-dimensional structure as a guide it was realized that all of the variant substitutions were located on the surface and filled a cavity present on the surface of BLA. An additional five residues were identified, and each individually increased the stability of the enzyme and could be combined with a cumulative increase in stability by a factor of 23.

Subtilisin operates in an environment quite different from its natural milieu. As a detergent additive, subtilisin must perform at highly alkaline pH, high surfactant concentration, and high temperature. It must operate on a wide variety of proteinaceous soils and may encounter other agents such as oxidants like hydrogen peroxide, added as a bleaching agent. Thus the enzyme was engineered to be more active and stable under these conditions. One of the most beneficial results has been mutations from which susceptibility to oxidants found in commercial bleaching agents has been eliminated. This was primarily achieved by replacing a bleach-sensitive methionine residue [423].

Investigations on Protein Folding The application of protein engineering for the dissection of protein folding pathways has been reviewed [424]. The number of different experimental techniques that have been used for this purpose clearly emphasizes the interdisciplinary nature of protein engineering.

Changing the Substrate Specifity of Enzymes The use of enzymes as biocatalysts for chemical reactions has recently regained interest (see Section 6.1). Several protein-engineering projects have been successfully performed. They mainly applied different

directed-evolution approaches to demonstrate their capabilities for the development and improvement of new biocatalysts.

The activity of subtilisin E in aqueous DMF was improved by protein engineering [425], [426]. Subtilisins have only low activity and reduced stability in DMF. The authors were able to enhance the stability in 40 % DMF more than twofold. But even more important is the activity enhancement that was achieved by subsequent evolution cycles. The final molecule showed a 471-fold activity improvement in 60 % DMF on a test substrate compared to the wild-type molecule.

The ability to change the substrate range and selectivity by protein engineering methods has been demonstrated by several authors. Zhang *et al.* [427] used DNA shuffling to modify the substrate specificity of β-galactosidase (EC 3.2.1.23). Whereas the wild-type molecule showed no activity on test substrates with fucose moieties, the final enzyme was active against these substrates. The activity against galactose substrates dropped significantly.

The success of engineering of enantioselective enzymes finally demonstrates that protein-engineering technologies nowadays can even address the subtlest problems in chemical synthesis. In several studies on various enzymes it has been demonstrated that it is possible to protein-engineer enzymes to obtain improved enantioselectivity, mainly for hydrolysis reactions [428–430].

4.2.3 Outlook

There are similarities between natural and directed evolution, the difference being largely in the management of diversity. Protein engineering has always been directed evolution where screening and diversity management intervene in place of natural selection. Even now knowledge of the three-dimensional structure and its relation to function is often pivotal in locating critical sites and regions for focused diversity and recruitment. DNA shuffling techniques again use screening strategies to combine features between natural selected genes in an effort to recombine or recruit the desirable features in a family of enzymes.

Now with the knowledge of whole genomes and the expected comparison of the genomes of closely related species, it may be possible to perform in silico shuffling and perhaps understand inherent preferences on individual molecular evolution imposed by the organisms' metabolome — the ensemble of molecular functions necessary for the maintenance of metabolism and reproduction in the whole organism. Another element of in silico directed evolution is the requirement to be able to predict the three-dimensional structure and with this further be able to recognize the catalytic potentials and even to deduce the probable physiological role of that molecule within the metabolome.

5
Industrial Enzymes

5.1
Enzymes in Food Applications

5.1.1
Enzymes in Baking

5.1.1.1 Introduction

Bread and baked products are among the main nutritional sources today. Over 600×10^6 t of wheat are grown in the world each year, making it the single most important crop [431]. Europeans are estimated to obtain approximately half their required carbohydrates and about one-third of their protein from bread [432].

Throughout the history of bread making, enzymes have always played an important role. Even the ancient Egyptians made use of enzymes present endogenously in the flour, although they may not have been aware of the effect. However, not until the 20th century were enzymes used as flour improvers [433]. The first application of enzymes in baked goods was supplementation of α-amylase by addition of malt to correct the concentration of endogenous α-amylase in the flour. Later malt was substituted by microbial α-amylases having a more suitable thermostability for baking. This was the first enzyme industrially produced as a tool to compensate for variations in natural flour. Today, a whole range of enzymes is available for end users of flour. These make it possible to correct suboptimal concentrations of endogenous flour enzymes. Furthermore, these biological catalysts are able to produce value-adding ingredients in situ from compounds present in the flour, and thus it is possible to upgrade lower grades of flour.

In many cases the beneficial effects of using enzymes in the baking industry are also obtainable by using chemical agents such as sodium metabisulfite, cysteine, azodicarbonamide (ADA), potassium bromate, etc. However, the food market today shows a clear trend towards more ''green'' products, and this has clearly favored the use of the biological catalysts enzymes.

Baking enzymes are summarized in Table 9.

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Table 9. E.C. numbers; reactions; and recommended, systematic, and other names of baking enzymes according to IUBMB (http://www.chem.qmul.ac.uk/iubmb/)

Recommended name	E.C. number	Systematic name	Reaction	Other name(s)
α-Amylase	E.C. 3.2.1.1	1,4-α-D-glucan glucanohydrolase	endohydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units	glycogenase; α amylase, alpha-amylase; endoamylase; Taka-amylase A
Glucan 1,4-α-maltotetraohydrolase	EC 3.2.1.60	glucan 1,4-α-maltotetraohydrolase	hydrolysis of 1,4-α-D-glucosidic linkages in amylaceous polysaccharides, to remove successive maltotetraose residues from the nonreducing chain ends	exo-maltotetraohydrolase; G4-amylase; maltotetraose-forming amylase
Glucan 1,4-α-maltohydrolase	E.C. 3.2.1.133	1,4-α-D-glucan α-maltohydrolase	hydrolysis of (1 → 4)-α-D-glucosidic linkages in polysaccharides so as to remove successive α-maltose residues from the nonreducing ends of the chains	maltogenic α-amylase
Endo-1,4-β-xylanase	E.C. 3.2.1.8	1,4-β-D-xylan xylanohydrolase	Endohydrolysis of 1,4-β-D-xylosidic linkages in xylans	endo-1,4-xylanase; xylanase; β-1,4-xylanase; endo-β-1,4-xylanase; endo-1,4-β-D-xylanase; 1,4-β-xylan xylanohydrolase; β-xylanase; β-1,4-xylan xylanohydrolase; β-D-xylanase
Glucose oxidase	E.C. 1.1.3.4	β-D-glucose:oxygen 1-oxidoreductase	β-D-glucose + O_2 D-glucono-1,5-lactone + H_2O_2	glucose oxyhydrase; corylophyline; penatin; glucose aerodehydrogenase; microcid; β-D-glucose oxidase; D-glucose oxidase; D-glucose-1-oxidase; β-D-glucose:quinone oxidoreductase; glucose oxyhydrase; deoxin-1; GOD
Hexose oxidase	E.C. 1.1.3.5	D-hexose:oxygen 1-oxidoreductase	β-D-glucose + O_2 → D-glucono-1,5-lactone + H_2O_2	

Galactose oxidase	E.C. 1.1.3.9	D-galactose:oxygen 6-oxidoreductase	D-galactose + O_2 → D-galacto-hexodialdose + H_2O_2	D-galactose oxidase; β-galactose oxidase
Pyranose oxidase	E.C. 1.1.3.10	pyranose:oxygen 2-oxidoreductase	D-glucose + O_2 → 2-dehydro-D-glucose + H_2O_2	glucose 2-oxidase; pyranose-2-oxidase
Thiol oxidase	E.C. 1.8.3.2	thiol:oxygen oxidoreductase	4 R′C(R)SH + O_2 → 2 R′C(R)S-S(R)CR′ + 2 H_2O	sulfhydryl oxidase
L-Amino-acid oxidase	E.C. 1.4.3.2	L-amino-acid:oxygen oxidoreductase (deaminating)	L-amino acid + H_2O + O_2 → a 2-oxo acid + NH_3 + H_2O_2	ophio-amino-acid oxidase
Lipoxygenase	E.C. 1.13.11.12	linoleate:oxygen 13-oxidoreductase	linoleate + O_2 → (9*Z*,11*E*)-(13*S*)-13-hydroperoxyoctadeca-9,11-dienoate	lipoxidase; carotene oxidase; lipoperoxidase; fat oxidase; lipoxydase; lionoleate: O_2 oxidoreductase
Peroxidase	E.C. 1.11.1.7	donor:hydrogen-peroxide oxidoreductase	donor + H_2O_2 → oxidized donor + 2 H_2O	myeloperoxidase; lactoperoxidase; verdoperoxidase; guaiacol peroxidase; thiocyanate peroxidase; eosinophil peroxidase; Japanese radish peroxidase; horseradish peroxidase (HRP); extensin peroxidase; heme peroxidase; MPO; oxyperoxidase; protoheme peroxidase; pyrocatecholperoxidase; scopoletin peroxidase
Catechol oxidase	E.C. 1.10.3.1	1,2-benzenediol:oxygen oxidoreductase	2 catechol + O_2 → 2 1,2-benzoquinone + 2 H_2O	diphenol oxidase; *o*-diphenolase; phenolase; polyphenol oxidase; tyrosinase; pyrocatechol oxidase; Dopa oxidase; catecholase; *o*-diphenol:oxygen oxidoreductase; *o*-diphenol oxidoreductase
Laccase	E.C. 1.10.3.2	benzenediol:oxygen oxidoreductase	4 benzenediol + O_2 → 4 benzosemiquinone + 2 H_2O	urishiol oxidase; urushiol oxidase; *p*-diphenol oxidase

Table 9. (continued)

Recommended name	E.C. number	Systematic name	Reaction	Other name(s)
Monophenol monooxygenase	E.C. 1.14.18.1	monophenol, L-dopa:oxygen oxidoreductase	L-tyrosine + L-dopa + O_2 → L-dopa + dopaquinone + H_2O	tyrosinase; phenolase; monophenol oxidase; cresolase; catechol oxidase; polyphenolase; pyrocatechol oxidase; dopa oxidase; chlorogenic oxidase; catecholase; polyphenol oxidase; monophenolase; o-diphenol oxidase; chlorogenic acid oxidase; diphenol oxidase; o-diphenolase; tyrosine-dopa oxidase; o-diphenol:oxygen oxidoreductase; polyaromatic oxidase; monophenol monooxidase; o-diphenol oxidoreductase; monophenol dihydroxyphenylalanine:oxygen oxidoreductase; *N*-acetyl-6-hydroxytryptophan oxidase; monophenol, dihydroxy-L-phenylalanine oxygen oxidoreductase; *o*-diphenol:O_2 oxidoreductase; phenol oxidase
Triacylglycerol lipase	E.C. 3.1.1.3	triacylglycerol acylhydrolase	triacylglycerol + H_2O → diacylglycerol + a carboxylate	lipase; triglyceride lipase; tributyrase; butyrinase; glycerol ester hydrolase; tributyrinase; Tween hydrolase; steapsin; triacylglycerol lipase; triacetinase; tributyrin esterase; Tweenase; amno *N*-AP; Takedo 1969-4-9; Meito MY 30; Tweenesterase; GA 56; capalase L; triglyceride hydrolase; triolein hydrolase; tween-hydrolyzing esterase; amano CE; cacordase; triglyceridase; triacylglycerol ester hydrolase; amano P; amano AP; PPL; glycerol-ester hydrolase; GEH; meito Sangyo OF lipase; hepatic lipase; lipazin; post-heparin plasma protamine-resistant lipase; salt-resistant post-heparin lipase; heparin releasable hepatic lipase; amano CES; amano B; tributyrase; triglyceride lipase; liver lipase; hepatic monoacylglycerol acyltransferase

Phospholipase A2	E.C. 3.1.1.4	phosphatidylcholine 2-acylhydrolase	phosphatidylcholine + H_2O → 1-acylglycerophosphocholine + a carboxylate	lecithinase A; phosphatidase; phosphatidolipase; phospholipase A
Galactolipase	E.C. 3.1.1.26	galactolipase	1,2-diacyl-3-β-D-galactosyn-sn-glycerol + 2 H_2O → 3-β-D-galactosyl-sn-glycerol + 2 carboxylate	
Phospholipase A1	E.C. 3.1.1.32	phosphatidylcholine 1-acylhydrolase	phosphatidylcholine + H_2O → 2-acylglycerophosphocholine + a carboxylate	
Phospholipase C	E.C. 3.1.4.3	phosphatidylcholine cholinephosphohydrolase	A phosphatidylcholine + H_2O → 1,2-diacylglycerol + choline phosphate	lipophosphodiesterase I; lecithinase C; *Clostridium welchii* α-toxin; *Clostridium oedematiens* β- and γ-toxins; lipophosphodiesterase C; phosphatidase C; heat-labile hemolysin; α-toxin
Phospholipase D	E.C. 3.1.4.4	phosphatidylcholine phosphatidohydrolase	A phosphatidylcholine + H_2O → choline + a phosphatidate	lipophosphodiesterase II; lecithinase D; choline phosphatase
Peptidase	E.C. 3.4.x.xx	Depending on specific peptidase	Endo- or exo- hydrolysis of peptide bonds	proteases
Protein-glutamine γ-glutamyl-transferase	E.C. 2.3.2.13	protein-glutamine:amine γ-glutamyltransferase	protein glutamine + alkylamine → protein N^5-alkylglutamine + NH_3	transglutaminase; Factor XIIIa; fibrinoligase; fibrin stabilizing factor; glutaminylpeptide γ-glutamyltransferase; polyamine transglutaminase; tissue transglutaminase; *R*-glutaminyl-peptide:amine γ-glutamyl transferase

5.1.1.2 Amylases

Amylases for Flour Standardization The most widely used enzyme in bread baking in terms of amount dosed and value is so-called fungal α-amylase or Taka-amylase from *Aspergillus oryzae* (E.C. 3.2.1.1). This is supplemented to sound flours to optimize their α-amylase activity with regard to final volume and crumb structure of the baked product. The primary effect of amylase supplementation was previously believed to be securing adequate gassing power by degradation of damaged starch granules in the dough, which facilitates maltose production by endogenous β-amylase. The resulting maltose can serve as fermentable sugar for yeast fermentation [434]. This mechanism may be important in some very lean dough systems without added sugars, but generally the primary effect is reduction of dough viscosity during initial starch gelatinization [435], which provides a prolonged oven spring leading to an increased volume and a more homogenous crumb structure.

Taka-amylase is named after Takamine, who in 1894 obtained US patent 525 823 on the use of mold fungi for production of amylase. The use of fungal amylase in bread baking was patented in 1928 [436] but first approved in 1955 for use in bread baking in the USA. The advantage of Taka-amylase is a very low protease side activity and an adequate thermostability, so that inactivation of the enzyme occurs after initial starch gelatinization (70–80 °C). The most common alternatives — malt amylases from barley or wheat malt — have higher protease side activities and higher thermostability, and this makes them more prone to negative side effects when overdosed [437].

Antistaling Amylases The second important goal for use of amylases in baking is antistaling, i.e., improving the fresh keeping of baked goods. Staling is a highly complex phenomenon [438, 440–442], but it is generally accepted that retrogradation of amylopectin is the main contributor to bread staling. The original model for the role of starch was developed by Schoch in 1965 [438]. Upon cooling in the first hours after baking the initial crumb structure is set by amylose gelation, which creates a network in which the gelatinized starch granules are embedded. Recrystallization of amylopectin side chains leads to rigidification of the starch granules and to an overall strengthening of the crumb structure, measured as an increase in crumb firmness. A refinement of this model adds inter- and intragranular amylose recrystallization to the staling process [439].

Different types of amylases used for antistaling provide different functionalities. First, a limited effect of fungal α-amylase is caused indirectly by volume increase. Secondly, endo-amylases weaken primarily the intergranular amylose network, leading to reduced crumb firmness immediately after baking and during storage. This effect is more pronounced when endo-amylases with high thermostability are used. An example of the most effective type is the *B. amyloliquefaciens* α-amylase, which has been used in the USA since the 1950s [443]. This amylase survives baking and is probably active during cooling of the bread and during storage [444]. Consequently, only a very narrow window of optimal dosage exists, and even moderate overdosing can lead to keyholing of the bread and a gummy crumb structure with strongly reduced elasticity (resilience). As an alternative, intermediately thermostable amylases have been

developed [444, 445]. They have lower risks of overdosing, but may also be limited in their antistaling effects [444].

Compared to classical endo-amylases, exo-amylases such as G4-amylase (E.C. 3.2.1.60) and maltogenic amylase (E.C. 3.2.1.133) offer clear improvements for antistaling applications. By shortening amylopectin side chains and releasing maltooligosaccharides, they efficiently reduce the rate of amylopectin retrogradation, leading to substantial crumb softening and improved elasticity without risk of excessive weakening of the amylose network [446, 447]. In a model system the shortening of amylopectin side chains has been shown to reduce retrogradation substantially [448]. Furthermore, balanced fragmentation of amylose by exo-amylases leads to accelerated amylose recrystallisation prior to amylopectin retrogradation, thus reducing overall connectivity and crumb firming [439].

5.1.1.3 **Xylanases**

Xylanases (E.C. 3.2.1.8, β-1,4-D-xylan xylanohydrolase), traditionally named pentosanases or hemicellulases, catalyze the endo-hydrolysis of β-1,4-D-xylosidic linkages in xylans. In dough, xylanases reduce problems associated with variations in flour quality, improve dough handling and dough stability, and give improved crumb structure and bread volume [449], [450].

The target substrates in cereals used for baking are arabinoxylan (AX; Fig. 30), one of the major nonstarch polysaccharides in cereals, making up 60-70 % of wheat cell walls [451] and 2–3 % of cereal flour [450].

Traditionally, the arabinoxylans have been divided into two fractions, a not extractable with water arabinoxylan fraction (WU-AX) and a water-extractable arabinoxylan fraction (WE-AX). Why some AX are water-extractable and others are not has been the topic of many studies. Early hypotheses explaining the differences were based on structure analysis of the two fractions, which showed a difference in the degree of substitution [452]. However, recent literature points more in the direction of both fractions having a similar degree of substitution [453], but the molecular weights of the two AX fractions differ, with WU-AX having the higher molecular weight. Furthermore, the concentrations of diferulates in the two fractions differ, and this may at least partly explain the

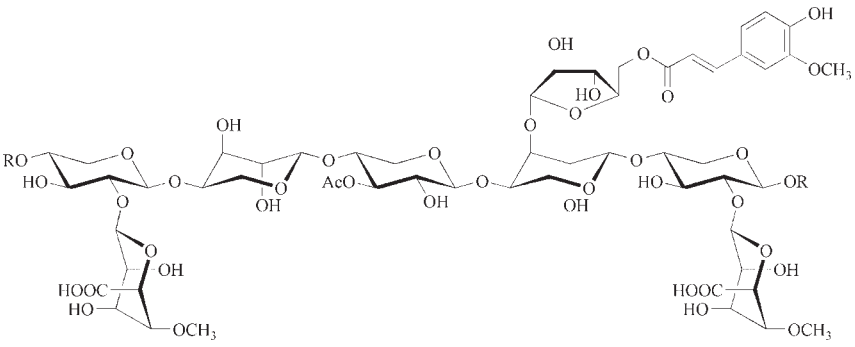

Fig. 30 Model structure of arabinoxylan

difference in molecular weight and extractability of the two AX populations. The WU-AX has a much higher concentration of diferulates than the WE-AX [454]; this higher concentration of cross-linking between AX molecules will increase the molecular weight and probably make part of the AX water-unextractable.

Some of the best known characteristics of cereal AX are the water-holding capacity of WU-AX and the ability of AXs to produce viscosity. Girhammer [455] showed that WU-AX could absorb water corresponding to 10 times its own weight, and Izydorczyk and Biliaderis [456] showed that AX has a very high intrinsic viscosity. These characteristics are probably the main factors determining the functionality of xylanases in the baking industry. In some baked products these phenomena are beneficial, but in others they are not.

Depending on the nature of the baked product, the flour AX may influence, in different ways, dough processing, dough characteristics, and the quality of the final product. This calls for the use of different, specific xylanases. In proofed and raised bread types, literature characterizes the WU-AX as detrimental for gluten strength and hence also for the stability of the dough [457], [458]. By influencing the dough stability, WU-AX also influences dough machinability and the quality of the final bread. Without xylanases the final products will have a more open crumb, and the volume will be smaller compared to dough in which the WU-AX fraction has been modified by a xylanase. Solubilizing the WU-AX into high molecular weight solubilized arabinoxylan (S-AX) by using a specific xylanase opens the possibility of converting WU-AX into a functional polymer. The high molecular weight S-AX will act as a hydrocolloid and increase the viscosity in the dough, giving a more stable dough system. Several xylanases capable of this modification of AX have been reported [459–462]. The use of WU-AX-specific xylanases in such baked products will typically improve the dough stability, dough machinability, and improve crumb structure and volume (10–30 %) of the final product [449].

In dry cereal products such as crispbread, crackers, etc., AX influences the dough or batter characteristics through its viscosity-enhancing properties [463]. Furthermore, AX influences the evaporation of water during baking of these products, due to its high water-binding capacity, which prevents even and fast evaporation of water from the product. This may result in longer baking time, checking, and darker products. In contrast to the specificity of the xylanase mentioned above, the xylanase used in dry cereal products should be specific for WE-AX and S-AX, and thus decrease dough resistance and dough liquid viscosity.

5.1.1.4 **Oxidoreductases**

Oxidative enzymes have a beneficial effect on dough development and dough quality. Dough quality influences other quality parameters such as volume, texture, and crumb structure of the baked product [464]. During dough mixing and molding, disulfide linkages are formed and broken between gluten proteins. When this reaction takes place optimally, it leads to a good dough development, that is, a strong gluten network is formed. Chemical oxidants such as ascorbic acid and bromate are used in breadmaking worldwide to support and promote the formation of disulfide linkages. Today consumers in the Western world prefer food without added chemical ingredients. Due to regulatory

problems with bromate in the USA and the E.C. countries, the wish to replace bromate exists. Hence, substitution with oxidative enzymes is a desirable alternative.

Glucose oxidase (E.C. 1.1.3.4) is usually obtained from *Aspergillus niger*. Glucose oxidase catalyzes the oxidation of glucose with consumption of oxygen to give gluconolactone and hydrogen peroxide. The generated hydrogen peroxide reacts with free thiol groups in the gluten with formation of disulfide linkages, which give increased gluten strength [465]. Glucose oxidase is known to give a dry and nonsticky dough, the latter characteristic being very useful in combination with xylanases, which may cause some stickiness when used alone [466].

Hexose oxidase (E.C. 1.1.3.5) is an enzyme originating from carrageenan seaweed, *Chondrus crispus*. This enzyme has broad substrate specificity, being able to oxidize glucose, galactose, maltose, etc. [467]. The enzyme oxidizes the endogenous substrates of the flour, mainly glucose and maltose, to the corresponding lactones with consumption of oxygen and formation of hydrogen peroxide. The hydrogen peroxide formed then oxidizes the thiol groups in the gluten proteins with formation of disulfide bonds [467].

Lipoxygenase (E.C. 1.13.11.12) enters bread production through the use of soybean flour or bean flour (France) that has not been heat-treated, i.e., is still enzymatically active. It improves the whitening of the breadcrumb. Lipoxygenase catalyses the oxidation of unsaturated fatty acids containing *cis-cis*-1,4-pentadiene groups. The resulting fatty acid hydroperoxide can react with carotenoids in the dough. According to Casey [468] the molecular basis of bread improvement through the action of lipoxygenase is likely to involve the formation of intermolecular disulfide bridges between the high molecular weight gluten proteins [468].

Sulfhydryl oxidase (E.C. 1.8.3.2), isolated from *A. niger*, catalyzes the oxidation of free sulfhydryl groups to disulfide groups in a variety of reduced thiol systems. A discussion continues as to whether sulfhydryl oxidase has only limited affinity for SH groups in high molecular weight substrates such as gluten [464]. Szalkucki wrote: ''further testing is necessary to demonstrate the commercial utility of sulfhydryl oxidase in baking'' [469].

Peroxidase (E.C. 1.11.1.7, horseradish peroxidase) is the general name for a group of enzymes with different substrate specificity. All peroxidases act on hydrogen peroxide as electron acceptor and oxidize a multitude of donor compounds [470].

During the peroxidase reaction a free radical is produced that reacts nonenzymatically with other compounds present in the dough [471]. It is well documented that certain peroxidases have a dough-improving effect [471], but different theories exist concerning the mechanism of function. The peroxidase might form diferulic acid bridges and thus lead to covalent cross-linking of arabinoxylans [472]. Coupling of arabinoxylans to proteins has also been demonstrated in model systems; this proceeds by oxidative cross-linking between ferulic acid and cysteine and/or tyrosine residues in proteins [471]. Finally, model studies also indicated the action of peroxidases in dough strengthening to be the result of protein–protein cross-linking through lysyl residues. Sulfhydryl groups may also be involved [471].

Polyphenol oxidase oxidizes diphenolic groups to quinones. Three types of enzymes are included in the group of polyphenol oxidases. Tyrosinase (monophenol mono-

oxygenase, E.C. 1.14.18.1), diphenol oxidase or catechol oxidase (E.C. 1.10.3.1), and laccase (E.C. 1.10.3.2). Two mechanisms for the action of polyphenol oxidases in dough are postulated. One involves oxidation of ferulic acid esterified to arabinoxylan, which induces their cross-linking, and the other assumes cross-linking of gluten proteins by oxidation of tyrosyl groups or by conjugation between tyrosyl and thiol groups [473, 474].

A number of enzymes that generate hydrogen peroxide by oxidation of various substrates with consumption of oxygen have been described as having dough-improving effects: glycerol oxidase [475], pyranose oxidase (E.C. 1.1.3.10) [476], L-amino acid oxidase (E.C. 1.4.3.2) [477], galactose oxidase (E.C. 1.1.3.9) [478], and glucooligosaccharide oxidase [2089].

5.1.1.5 Lipases

Lipases (E.C. 3.1.1.3) can be defined as carboxylesterases which catalyze the hydrolysis of long-chain acylglycerols to glycerol, free fatty acids, and mono- and diglycerides. The substrate for lipases in wheat flour is the 2–3 % of endogenous wheat lipids. The composition of the free lipids (extractable with water-saturated butanol at room temperature) is shown in Figure 31.

Lipids are probably the longest known and used bread improvers, whereas the use of lipase as a bread improver is relatively new. The understanding of lipase functionality is continuously being studied, but is mostly based on knowledge of the effects of lipids in dough and baking. The desirable effects obtained by lipases are improved dough stability and better dough handling properties, and improved stability of the gas cells in dough, yielding a nice, homogenous crumb structure and increased bread volume.

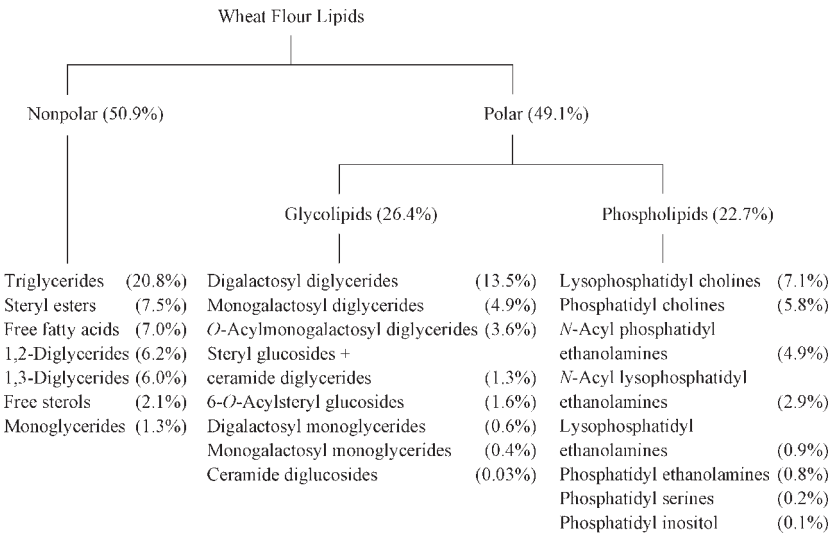

Fig. 31 Composition of total wheat flour lipids, extracted with water saturated butanol [480]

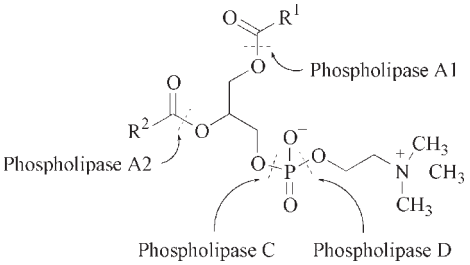

Fig. 32 Phospholipase specificity (modified from A. L. Lehninger, Biochemistry, Worth Publishers, Inc. 1970, p. 199, fig 10-4.)

Lipase modifies flour lipids or added lipids, and the beneficial effect of an added lipase is dependent on the effect of the hydrolysis products [481]. Some of these hydrolysis products are quite surface-active or more surface-active than the unmodified lipid and therefore better stabilize the gas cells in the dough. This again leads to better crumb structure, increased dough stability, and increased volume [482]. Among the potential reaction products, monoglycerides are well-known crumb softeners in baking, as they form a complex with starch and thus decrease retrogradation [466].

Phospholipases are a class of four enzymes hydrolyzing specific bonds in phospholipids (see Fig. 32): phospholipase A1 (E.C. 3.1.1.32), phospholipase A2 (E.C. 3.1.1.4), phospholipase C (E.C. 3.1.4.3), and phospholipase D (E.C. 3.1.4.4).

The release of one fatty acyl chain from phosphatidylcholine yields 1-acylglycerophosphorylcholine, more commonly known as lysolecithin, which is an excellent emulsifier with a very positive effect in baking [483].

Recently, a new class of lipases acting on polar lipids as well as triglycerides has become commercially available [484–486]. These new lipases have a higher activity on galactolipids and phospholipids than on triglycerides. By modifying phospholipids and galactolipids to the corresponding lysolipids (e.g., lecithin to lysolecithin and digalactosyl diglyceride to digalactosyl monoglyceride), very strong surfactants are generated. The new lipases therefore offer the opportunity to generate surface-active materials in situ and possibly to reduce or replace the use of added emulsifiers in bakery products [487]. The ability of the polar lipids to form lipid monolayers at the gas–liquid interface leads to stabilization of the gas cells in the dough and thus to better gas retention [484]. Furthermore, the interaction between polar flour lipids and gluten proteins may play an important role in gas retention [484].

5.1.1.6 **Proteases**

Proteases (E.C. 3.4.X.X) catalyze the hydrolysis of peptide bonds in proteins [488]. Depending on the protease used, this modification may lead to a minor modification that gives more extensible dough [489], [490] or to an extensive hydrolysis of the gluten structure with loss of the viscoelastic properties [490]. Apart from the impact on the physical and rheological properties of the dough, some proteases are known to influence the flavor and color of the final product as well.

As proteins are a highly heterogeneous population of molecules, due to the 20 amino from which they are composed, the population of enzymes capable of hydrolyzing proteins is also very diverse [491]. This population of enzymes is divided into two categories: the peptidases and the polypeptidases (synonymous with proteases). The peptidases are exo-acting enzymes that remove one amino acid or a peptide from the C- or N-terminus of the protein, and the proteases are endo-acting.

Wheat flour is unique in its ability to form a viscoelastic gluten matrix when mixed with water [492]. This gluten matrix can retain gas in the dough, giving wheat flour its unique baking performance. The quality and quantity of protein are therefore major quality parameters for wheat flour [431], [493].

In normal raised bread products too strong a flour may prohibit a good volume, as proper expansion of the dough will not be possible. Application of a moderate specific protease (typically a fungal protease) modifies the gluten, giving a better extensibility, faster development, and thereby better structure and volume of the final product [490]. The same functionality can be obtained by using chemical reducing agents, such as L-cysteine or sodium metabisulfite (Fig. 33). However, the biological, and labeling-free enzyme solution is often preferred.

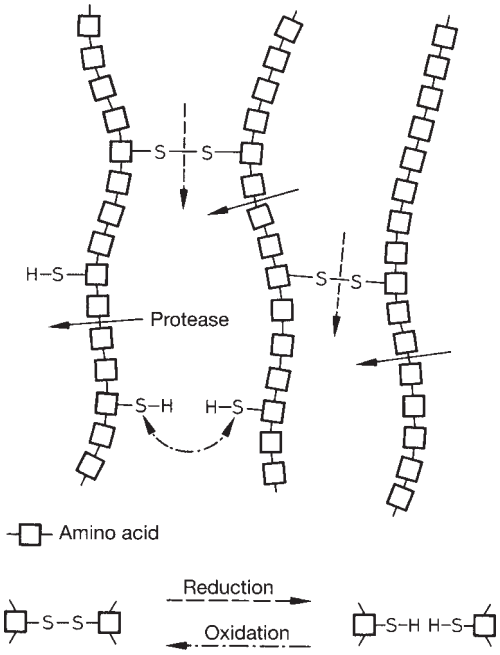

Fig. 33 Gluten protein structure and modification by proteolytic hydrolysis or chemical reduction, respectively [After [432] (H. Uhlig: ''Enzymes in flour processing and baking'', in: *Industrial Enzymes and their Application*. John Wiley & Sons, Inc. (1998). fig 5.36, pp 284)]

In cracker, biscuit, wafer, and pizza dough weaker flour having less viscoelastic properties is preferred [494]. A strong gluten network would prohibit the right shape and texture of the final product. Applying less specific proteases (typically a *Bacillus* protease) to these types of products allows the manufacturer to be more independent of flour quality. It is possible to obtain the desired dough rheological properties by using most flours in combination with protease. Again, the same solution is obtainable with chemical reducing agents, but the demand for biological and labeling-free solutions favor enzyme solutions.

Application of peptidases to bread might influence the flavor and crust color of the product [490]. The products of the peptidase, amino acids or peptides, influence the flavor. The amino acids are intermediate products in the production of aromatic components, and peptides are potential oxidants, taste enhancers, sweeteners, and bitter agents [495]. Furthermore, amino acids released from gluten proteins can react with sugar through the Maillard reaction, thereby contributing to flavor and crust color.

5.1.1.7 Transglutaminase

Transglutaminase (protein-glutamine γ-glutamyltransferase, E.C. 2.3.2.13) introduces covalent cross-links in proteins by forming a bond between lysine and glutamine residues. Based on its ability to cross-link gluten proteins it can be used as a dough strengthener for bread and other baked products [496]. As further advantages reduction of the needed work input as well as increased water binding have been reported [497]. Beneficial effects on baking performance of mixed nonwheat/wheat flours have also been claimed [498]. However, Gerrard and Sutton [499] have recommended discontinuing use of transglutaminase in cereal products containing wheat, barley, rye, or oats because of the risk of generating the epitope triggering coeliac disease.

5.1.2 Enzymes in Fruit Juice Production and Fruit Processing

5.1.2.1 Introduction

The first application of enzymes in the fruit juice industry was the use of pectinases for apple juice clarification in the 1930s [500]. The fast clarification of juice after breakdown of pectin by pectinases and the decrease in juice viscosity resulted in a shorter process and greatly improved the quality of industrial apple juice. Later, pectinases were applied to depectinization of red-berry juice. The use of pectinases and amylases to degrade apple pectin and starch during the hot clarification stage prevents post-bottling haze formation, and thus concentration of the juice by a factor of six has become possible. This results in a smaller storage volume, cheaper transportation, and better concentrate stability without spoilage. Treatment of apple pulp with pectinases and hemicellulases was introduced later: by lowering the viscosity of the pulp, the press capacity and the yield were significantly improved. Depectinization of juice with pectinases with high arabanase activity improved product quality further by preventing araban haze formation after concentration of the juice. In the production of clear and stable red-berry juice or concentrate, hot maceration with enzymes ensures a higher juice yield and a better

color extraction [501]. Nowadays, enzyme suppliers provide fruit juice producers with tailor-made enzyme preparations optimally blended on the basis of fruit composition for improved quality and stability of finished products, together with shorter process duration and larger plant capacity. In association with new equipment and processing technologies, industrial enzymes allow processors to add value to raw material for food and feed and to reduce waste quantity. Industrial processes are very diverse and numerous. Here the most common are described, but differences still exist depending on the company and plant.

5.1.2.2 **Biochemistry of Fruit Cell Walls**

The pulp of fruits and vegetables is composed of cells. They are surrounded by a cell wall, which resists internal pressure and external shock (Fig. 34). Polysaccharides constitute 90–100 % of the structural polymers of walls of growing plant cells, known as primary cell walls. Secondary cell walls develop from primary cell walls during cell growth. Cell wall composition depends on fruit species and evolves in dependence on agronomic and climatic conditions, fruit ripeness, the type and duration of storage of the fruit (cell growth and cell senescence). The composition of plant cell walls has been widely studied, and numerous models of the three-dimensional structure have been proposed [502–504]. Three major independent domains are distinguished: the xyloglucan network, the pectin matrix, and the structural proteins. The cellulose–xyloglucan network is embedded in the pectin matrix. Pectin is the major structural polysaccharide component of fruit lamellas and cell walls. Three pectic polysaccharides are present in all primary cell walls: homogalacturonan and rhamnogalacturonans I and II [505] (Fig. 35). Recent models divide pectin into so-called smooth regions of

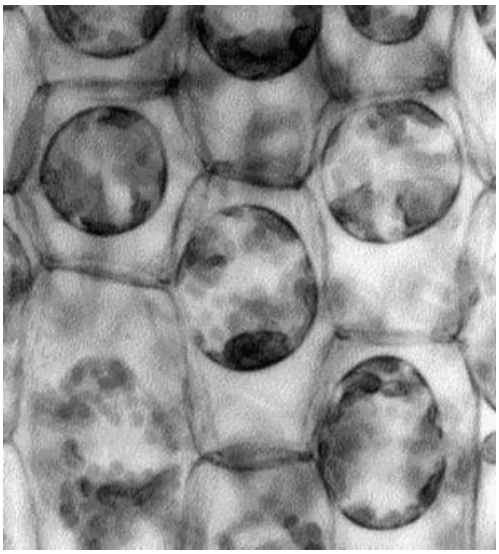

Fig. 34 Fruit cells (fc) and middle lamellae (ml) connecting the cell walls

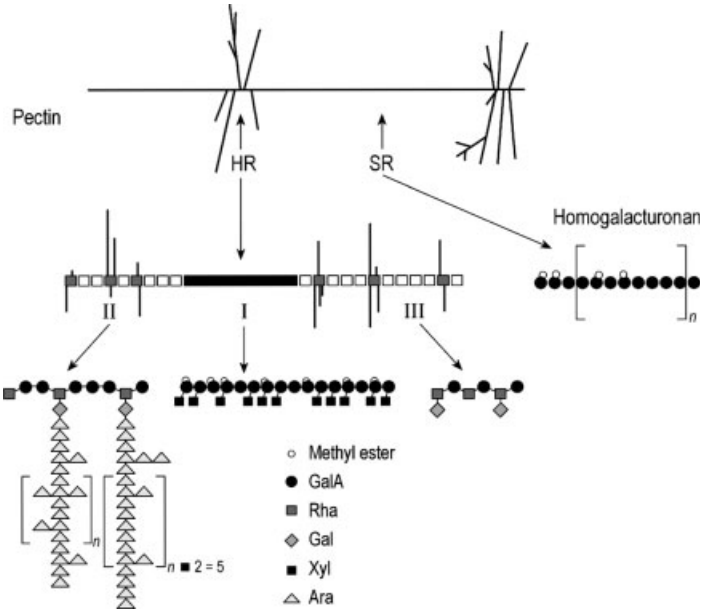

Fig. 35 Model of pectin smooth regions (SR) and hairy regions (HR) model [506]. Ara = arabinose, Gal = galactose, GalA = galacturonic acid, Rha = rhamnose, Xyl = xylose

unbranched homogalacturonan (60–90 %) and hairy regions of highly branched rhamnogalacturonan I (10–40 %) [507], [508]. Homogalacturonan is a homopolymer of (1→4)-α-D-galactosyluronic acid residues, capable of forming gels. Carboxyl groups of the galactosyluronic acid residues of primary cell wall homogalacturonan can be methyl-esterified at the C-6 position and acetyl-esterified at the C-2 or C-3 position. Helical chains of homogalacturonan that are less than 50 % methyl-esterified can form a gel-like structure and condense by cross-linking with calcium ions, which are present in primary cell walls. Degree of methylation, molecular weight, and pectin content are specific to fruit species (Table 10).

Table 10. Fruit pectin composition

Fruit	Pectin, wt %	Methylation wt %
Apple	0.5–1.6	80–92
Blackcurrant	1.0–1.2	50–80
Grape	0.1–0.4	50–65
Orange peel	3.5–5.5	65
Pear	0.7–0.9	50–70
Pineapple	0.04–0.1	22–40
Strawberry	0.5–0.7	20–60

These characteristics must be taken into consideration in choosing the right pectinase balance for best fruit processing. During ripening, the protopectin in primary cell wall is slowly transformed into soluble pectin by the action of endogenous pectinases present in the fruit. The decrease in molecular weight is due to pectin depolymerases, and the decrease in degree esterification mainly to pectin methylesterase. However, these activities are very low. Homogalacturonan contains chains of up to about 200 galacturonic acid units, 100 nm long, with some rhamnose. A xylogalacturonan subunit (XGalA), substituted with xylose at the C-3 position of the galacturonic acid residue, has been identified as part of the galacturonan backbone. It can be methylesterified. Rhamnogalacturonan I (RGI) has a backbone of up to 100 repeats of the disaccharide rhamnose-galactose. The side chain can vary in size from a single glycosyl residue to 50 or more glycosyl residues [505]. In general, about half of the rhamnosyl units of RGI have sidechains, but this can vary with cell type and physiological state. [509].

Arabinans are mostly 5-linked arabinofuranosyl units forming helical chains, but arabinosyl units can be interconnected at each free O-2, O-3, and O-5 position to form a diverse group of branched arabinans [510]. The RGI of primary cell walls is branched with (1→5)-α-L-arabinan, (1→3)- or (1→2)-α-L-arabinosyl residues, and arabinogalactan (AG) type II with a (1→3),(1→6)-β-D-galactan backbone, with (1→3)-α-L-arabinosyl residues [512, 511]. Other macromolecules such as cellulose, xyloglucan, and arabinogalactan proteins (AGP) are associated with the plasma membrane [513]. Rhamnogalacturonan II (RGII) is a low molecular weight (ca. 4.8 kdalton) complex polysaccharide with a backbone of nine (1→4)-α-D-galactosyluronic acid residues and four side chains attached to O-2 or O-3 of the backbone.

The side chains are composed of twelve different sugars [514] — apiose, 2-*O*-methyl-l-fucose, 2-*O*-methyl-D-xylose, aceric acid (3-*C*-carboxy-5-deoxy-l-xylose), Kdo (3-deoxy-D-manno-octulosonic acid), Dha (3-deoxy-D-lyxo-heptulosaric acid) — bound in more than 20 different linkages. The two main hemicelluloses of all primary cell walls are xyloglucan and arabinoxylan. Hemicelluloses bind tightly via hydrogen bonds to the surface of cellulose linking or cross-linking microfibrils to create a cellulose–hemicellulose network. Interconnections with the pectic polysaccharides are of primary importance for the integrity of the pectin network. Cellulose is a (1→4)-β-D-glucan which accounts for about 20–30 % of the dry matter of most primary cell walls and is particularly abundant in secondary cell walls. In 1993-1994, VINCKEN and VORAGEN demonstrated that xyloglucan was a key structure of apple cell walls for the degradation of cell-wall-embedded cellulose (around 57 % of the apple cell-wall matrix) [515]. The cellulose–xyloglucan network determines the strength of the cell wall and is embedded in an independent pectin matrix, hemicelluloses, and proteins. In apples the xyloglucan fraction makes up about 24 % of the total amount of sugar. About 5 wt % of some primary cell walls is made up of the hydroxyprolin-rich structural glycoprotein extensin, which binds some polysaccharides together. Fractions of the different macromolecules in fruit are given in Table 11.

Starch is present in unripe apples in amyloplasts. This is the largest biological molecule, with a molecular weight of 10^5–10^9 dalton. Starch is composed of two components: amylose and amylopectin [516]. Amylose, the minor component, is a

Table 11. Fruit cell wall composition in g/kg fresh matter

Fruit	EIR*	Pectin	Hemicellulose	Cellulose	Lignin	Protein	Total
Apple	20	272	169	349	2	76	868
Pear	15	281	148	267	69	82	847
Mango	25	408	91	236	27	127	889
Pineapple	13	163	267	210	85	94	819
Strawberry	12	411	66	232	11	255	975
Raspberry	20	168	89	177	73	277	784
Cherry	13	396	49	130	169	244	988
Papaya	26	364	165	124	4	127	784

*Ethanol-insoluble residue.

(1→4)-α-D-glucan and has a linear structure. Various degrees of polymerization have been ascribed to this fraction, with chain lengths in the range of 100–1000 glucose units. Amylopectin contains α-(1→6) and α-(1→4) glucose linkages. Amylopectin exhibits branching at the 1→6 position, and its degree of polymerization is far higher than that of amylose. The ratio amylose/amylopectin can vary in natural starches in the general range 1/3 to 1/4.

5.1.2.3 Cell-Wall-Degrading Enzymes

Pectinases Progress in enzymology has been so fast that certain activities are not yet described in the International Enzyme Classification (E.C. no.). Many microorganisms produce enzymes that degrade fruit cell walls. Commercial pectinases for the fruit juice industry come from selected strains of *Aspergillus* sp. Enzymes are produced during fungal growth, purified, and concentrated. Pectinases are defined and classified on the basis of their action toward pectin (Fig. 36). Pectin lyase (PL, E.C. 4.2.2.10) is a pectin

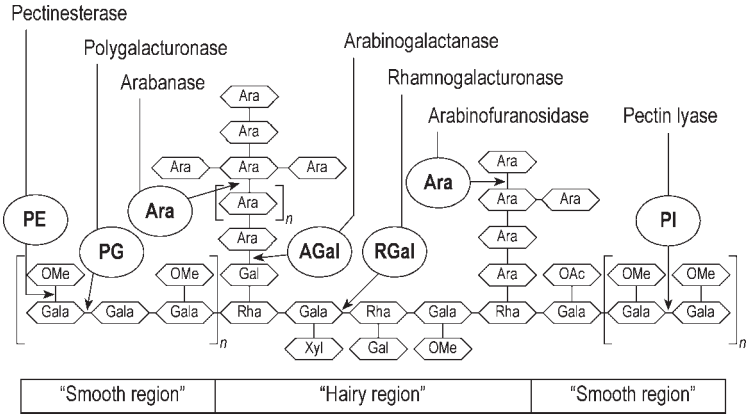

Fig. 36 Fruit pectin and pectin-degrading enzymes.
Ara = arabinose, Gal = galactose, GalA = galacturonic acid,
OMe = methyl ester, OAc = ethyl ester, Rha = rhamnose, Xyl = xylose.

depolymerase of the endo type which has a great affinity for long, highly methylated chains and acts by β-elimination of methylated α-1,4 homogalacturonan with the formation of C4-C5 unsaturated oligo-uronides [517]. Pectin methylesterase (PME, E.C. 3.1.1.11) removes methoxyl groups from pectin, and at the same time decreases the affinity of PL for this substrate. This results in the formation of methanol and less highly methylated pectin. PME from *Aspergillus* has a strong affinity for highly methoxylated pectin such as apple pectin and acts according to a multichain mechanism [518]. Demethylation with PME generates free carboxylic acid groups and the pectin becomes negatively charged. Polygalacturonase (PG, E.C. 3.2.1.15) exists in two forms: endo-PG and exo-PG. Both types act only on pectin with a degree of esterification of less than 50–60 %. Endo-PG acts randomly on the α-1,4-polygalacturonic backbone and results in a pronounced decrease in viscosity. Exo-PG acts at the nonreducing end of the chain. Exo-PG releases small fragments from the chain and does not significantly reduce the viscosity.

Seven endo-PGs, two exo-PGs, and seven PL isoenzymes from *Aspergillus niger* have been described [519], [520]. Different enzymes acting on rhamnogalacturonan I were identified and purified from *Aspergillus* sp. [521]. RGase A was identified as a hydrolase that splits the α-D-GalA*p*-(1→2)-α-L-Rha*p* linkage of RGI, while RGase B appeared to be a lyase that splits the α-*l*-Rha*p*-(1→4)-α-D-GalAp linkage by β-elimination. Two novel enzymes were also identified: a rhamnogalacturonan rhamnohydrolase and a rhamnogalacturonan galacturonohydrolase. As an accessory enzyme for the RGases, rhamnogalacturonan acetyl esterase (RGAE) was also described. Although the structure of RGII substrate has been described, enzymes able to hydrolyze it are still unknown and have not been described yet (2003).

Arabanases are pectinases, since they remove arabinose covalently bound to the homogalacturonan backbone. Three enzymes have been described: an endo-arabinanase (α-1→5; ABFA) and two arabinofuranosidases, namely, exo-arabinofuranosidase A (α-1→2;α-1→3) (ABFA) and exo-arabinofuranosidase B (α-1→3;α-1→5; ABFB; E.C. 3.2.1.55). All three are produced by *Aspergillus niger* [522]. High activities are required for apple and pear processing. In general, pectinase activity and ratio of the different pectinases can vary in different commercial preparations.

Hemicellulases Hemicellulases are enzymes that hydrolyze arabinogalactans, galactans, xyloglucans, and xylans. Arabinanases are classified as pectinases when they act on pectin side arabinans. They are also classified as hemicellulases when acting on arabinogalactans or arabinoxylans. *Aspergillus* sp. produces enzymes that hydrolyse arabino-(1→4)-β-D-galactans type I and arabino-(1→3)-(1→6)-β-D-galactans type II [512], [523], [524].

The exo-(1→3)-β-D-galactanase is able to release galactose and (1→6)-β-D-galactobiose. *Aspergillus* enzyme is able to bypass a branch point in a β-(1→3) backbone. The action of this enzyme is enhanced by the presence of ABFB. Xylanases hydrolyse the (1→4)-β-D-xylans in synergy with ABFs [525].

Amylases In fruit juice industry, fungal acid amylase and amyloglucosidase are used to process fruits which contain starch. This is the case for unripe apples at harvest time.

Aspergillus niger produces acid α-amylase and amyloglucosidase [526]. These exogenous enzymes are applied after starch gelatinization (occurring above 75 °C) for preventing post-bottling haze formation (starch retrogradation). Acid endoamylase (AA) acts on amylose and amylopectin. It produces dextrins, which are substrates for glucoamylase or amyloglucosidase (AG) α-1→4,1→6 exo-hydrolase, which release glucose from the non-reducing end of the chain.

5.1.2.4 Apple Processing

After oranges, apples are the most important raw material worldwide for the production of clear juice and clear concentrate. In 2004/2005 1.3×10^6 t of apple juice was produced [528]. The major producers of apple juice are China, Poland, and Argentina [527].

5.1.2.4.1 Apple Pulp Maceration

The current trend is to process apple juice from table varieties having defects or not sold for table consumption (Golden Delicious, Granny Smith, Jonagold, Red Delicious). Apples processed in the crop period are easily pressed with a relatively high yield. They are stored at low temperature in controlled atmosphere for several months and processed according to market demand. During storage, the insoluble protopectin is slowly transformed into soluble pectin by endogenous apple pectinases (protopectinase type), and starch is slowly degraded by endogenous apple amylases into glucose and consumed during post-harvest metabolism. Soluble pectin content can increase from 0.5 up to 5 g per kilogram of over-ripe apple [529]. Apples become difficult to press unless macerated with pectinases (Fig. 37). In the traditional process, enzymes are used at two different stages (Fig. 38). Application of commercial pectinases from *Aspergillus* sp. in apple mash is necessary because activity of endogenous enzymes is too low to cause an immediate noticeable effect. Because apple pectin is highly methylated, commercial enzyme preparations must contain a high concentration of pectinlyase or pectin methylesterase in association with polygalacturonase and arabanase, together with side activities such as rhamnogalacturonase and xylogalacturonase. Rapidase Press (DSM), Rapidase Smart (DSM), Pectinex UltraSP (Novozymes), and Rohapect MA+ (AB Enzymes) pectinases sold for apple pulp maceration contain enzymes necessary to obtain a good pressability and high yield throughout the entire season.

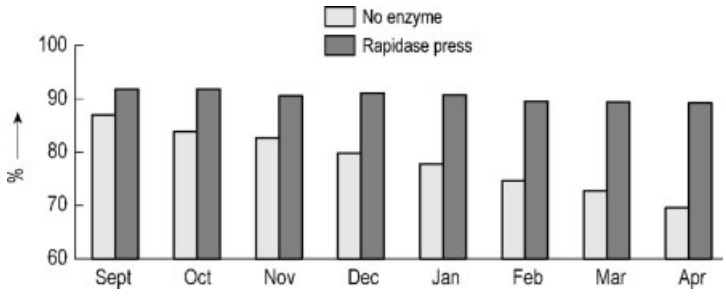

Fig. 37 Effect of pectinases on percentage apple juice yield during the processing season

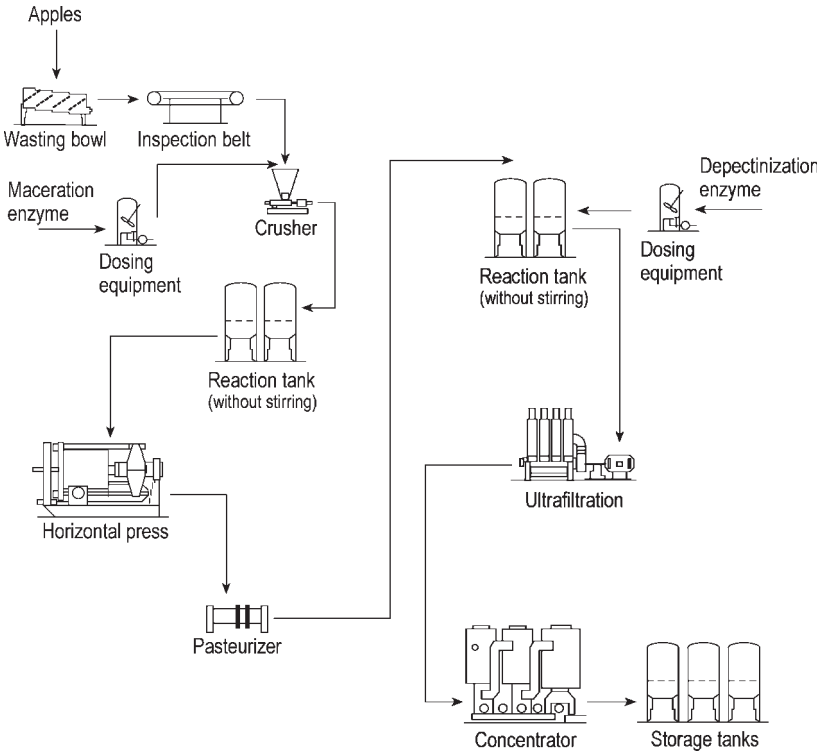

Fig. 38 Production of apple concentrate

Commercial products contain more or less the enzyme activities described below, but with different ratios from non-GM and GM organisms.

Pulp enzyming results in a fast decrease in pulp viscosity, a large volume of free-run juice, and fast pressing. The yield is over 90 % with hydraulic press and pomace leaching, compared to 75–80 % maximum without enzyme treatment.

5.1.2.4.2 **Apple Juice Depectinization**

Pectin is the main cause of juice turbidity. One liter of juice with 13 % dry matter can contain 2–5 g of pectin after pressing, depending on the ripeness of the fruit. Pectinase dosage is determined in the laboratory by performing the alcohol test. Acidified ethanol (2 volumes of ethanol containing 0.5 % HCl + 1 volume of apple juice) is used to precipitate residual pectin with a molecular weight of up to 3000 dalton. It is important to use acidified ethanol; otherwise, precipitation of organic acids or calcium pectate occurs because of the pH change after mixing ethanol with the juice, and thus a a false positive result is obtained. Pectinlyase or pectin methylesterase plus polygalacturonase and arabanase are the most important enzymes. Arabinose represents 55 % of neutral sugars in hairy regions of apple pectin. Arabanase activity prevents haze formation after juice concentration. The juice clarification is carried out after enzymatic depectinization [530]. The first stage consists of destabilization of the cloud by PL, which has no

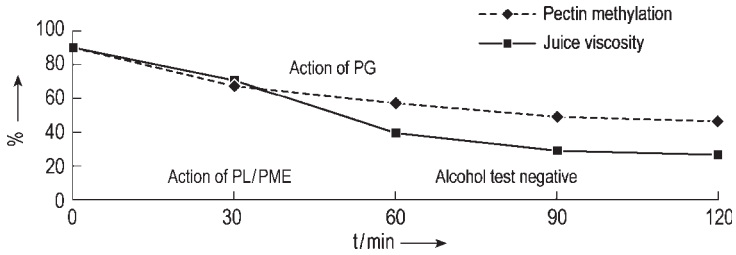

Fig. 39 Apple juice depectinization

visible effect but results in a strong decrease in the viscosity of the juice (Fig. 39). PL cuts pectin at random, and cutting 1–2 % of the linkages is enough to reduce apple juice viscosity by 50 %. PG becomes active only after the action of PME. Because of its high molecular weight, PG cannot hydrolyze pectin with a degree of methylation greater than 50–60 % due to steric hindrance. At this stage, PL is no longer active and pectin hydrolysis is due to the system PME/PG. The second stage is cloud flocculation. The sedimentation necessary for the clarification of apple juice occurs only after enzymatic degradation of pectin and starch. The cloud is composed of proteins that are positively charged at the pH of the juice (3.5–4.0) since their isoelectric point lies between pH 4.0 and 5.0. These proteins are bound to hemicelluloses that are surrounded by pectin as a negatively charged protective colloid layer. Pectinases such as Rapidase C80 Max (DSM), Pectinex C80 Max (Novozymes), and Rohapect DAL (AB Enzymes) partially hydrolyze the pectin gel, and thus results in the electrostatic aggregation of oppositely charged particles (positive proteins and negative tannins and pectin), flocculation of the cloud, and then clarification of the juice [530]. The optimal pH for this mechanism is 3.6. The alcohol test shows whether juice depectinization is complete.

At the beginning of the processing season, unripe apples contain 5–7 g of starch per liter of juice. At this point, the iodine test gives a dark blue color and starch can be precipitated with iodine. Starch is present as granules 2–13 μm in size, composed of 30 % amylose and 70 % amylopectin chains. The latter can fix 20 wt % iodine. Apples contain endogenous amylases, but the process is too short and activities are too low to degrade the starch. When the juice is heated to 75–80 °C, starch changes from an insoluble form to a soluble form (gelatinization with water). If no amylase is added, these molecules can subsequently undergo retrogradation and form large aggregates that are difficult to hydrolyze and clog filters. The addition of fungal amylase and amyloglucosidase results in the hydrolysis of soluble starch into glucose monomers. Retrogradation and post-bottling haze formation are prevented, and clarification and filterability are improved. Preparations such as Hazyme DCL (DSM) and AMG (Novozymes) contain α-amylase and amyloglucosidase from *Aspergillus niger*. The dosage is determined by the iodine test. The third stage is clarification with finings and sheet filtration, which is being replaced by micro- or ultrafiltration (UF). Inorganic membranes have cut-offs from 20 000 to 500 000 dalton. Insoluble particles and soluble undegraded molecules such as parts of RGI, RGII, proteins including enzymes, and polyphenols, remain to a greater or lesser extent in the retentate, depending on the cut-off of the filtration membrane. Recently, it

was found that undegraded RGI, RGII, and dextrins can foul UF membranes. Rapidase UF (DSM) and Novoferm 43 (Novozymes) contain rhamnogalacturonases and other side activities to improve ultrafiltration flow rate.

In conclusion, apple juice is easily produced after pulp maceration and juice depectinization with enzymes, with possible concentration of up to 70° brix (percentage by weight of soluble solids in a syrup at 68 °F), without risk of gel or haze formation. A few producers make "natural" or cloudy apple juice, e.g., in Germany and the USA. Until now, they did not add enzymes, because the existing commercial pectinases induce fast clarification of the juice. DSM sells a purified pectin methylesterase without pectin depolymerase activity. It can be used in the cloudy-juice process at the maceration stage to improve the yield, and also in the French cider process with calcium for defecation with flotation techniques.

5.1.2.5 **Red-Berry Processing**

The production of clear juice and concentrate from blackcurrant, raspberry, or strawberry requires enzymatic maceration and depectinization [531], [532]. Clarification, filtration, and concentration are difficult because these juices have high pectin content (typical content of residual low molecular weight pectin of 7 g L^{-1} compared to 0.5 g L^{-1} in apple juice). It is assumed that pectin hairy regions remain as soluble colloid in the juice and hemicelluloses tend to bind to phenolics and proteins during processing and storage. The result is the formation of irreversibly linked brown complexes that enzymes can no longer break down. An additional problem is related to the frequent contamination of red berries, mainly strawberry and raspberry, with *Botrytis cinerea*. This parasitic fungus, growing on rotten berries, secretes a β-1,3-1,6-linked glucan into the berries with a molecular weight of ca. 10^6 dalton [533]. This gum reduces the filterability and the clarity of the juice. It is possible to hydrolyze this glucan with β-glucanases with Filtrase (DSM)Glucanex (Novozymes).

Single-Stage Red-Berries Process The single-stage process consists of simultaneous blackcurrant or blackberry maceration and depectinization (Fig. 40). Pectinases are used to improve juice and color extraction while retaining the organoleptic properties of the fruit. However, the extracted color is sometimes partly destabilized by anthocyanases (side activities of pectinases) or by oxidation. Oxidation can be chemical or enzymatic, due to the endogenous polyphenol oxidase (PPO) of the fruit, and is catalyzed by metal ions. It is therefore recommended that the pulp be heated to 90 °C to inhibit fruit oxidases prior to maceration with enzymes. Some red berries are very acidid (pH 2.6–2.8) and have high contents of phenolics and anthocyanins, which are inhibitors of pectinases. Hence, red-berry processing requires commercial pectinases that are especially stable under these conditions. This is the case for Klerzyme 150 (DSM), Klerzyme Intense (DSM), and Pectinex BEXXL (Novozymes).

Two-Stage Red-Berries Process The two-stage process consists of enzymatic maceration of fruit pulp, followed by a second addition of enzyme for juice depectinization at low temperature. Raspberries or gooseberries are heated to 90 °C for at least two minutes to increase color extraction and to destroy fruit polyphenol oxidase. The pulp is then cooled to 20–25 °C for enzymatic maceration. After pressing, the extracted juice is

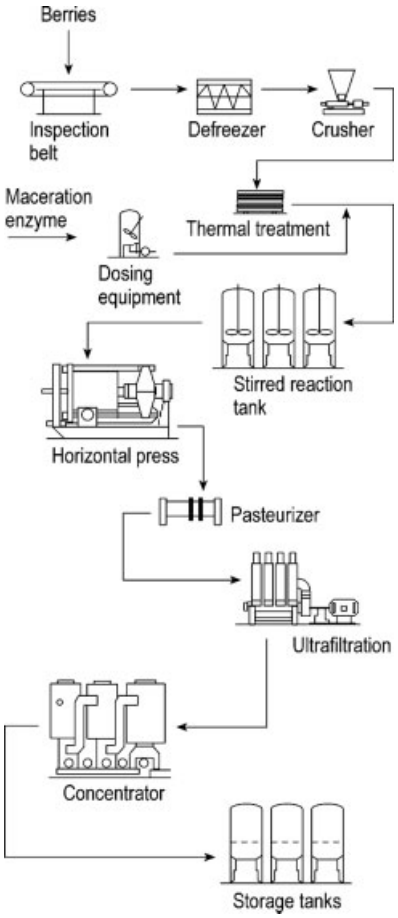

Fig. 40 Production of blackcurrant concentrate

depectinized with pectinases. The low processing temperature after heating prevents aroma losses, and high-quality juices and concentrates can be produced. In the case of strawberries, it is inappropriate to heat the pulp because it would create an unpressable purée, aromas loss, and juice browning. Hence, strawberries are processed at ambient temperature [532].

5.1.2.6 Tropical Fruit and Citrus Processing

Tropical Fruit Tropical fruit is mainly processed to purée and stored before further processing to cloudy or clear juice. Fruit purée, cloudy or clear juice from apricot, peach, kiwi, mango, guava, papaya, and banana are often processed without enzymes. The main problem is viscosity, which can be decreased with pectinases [528]. Pectinases and amylases can be later used for clear juice production.

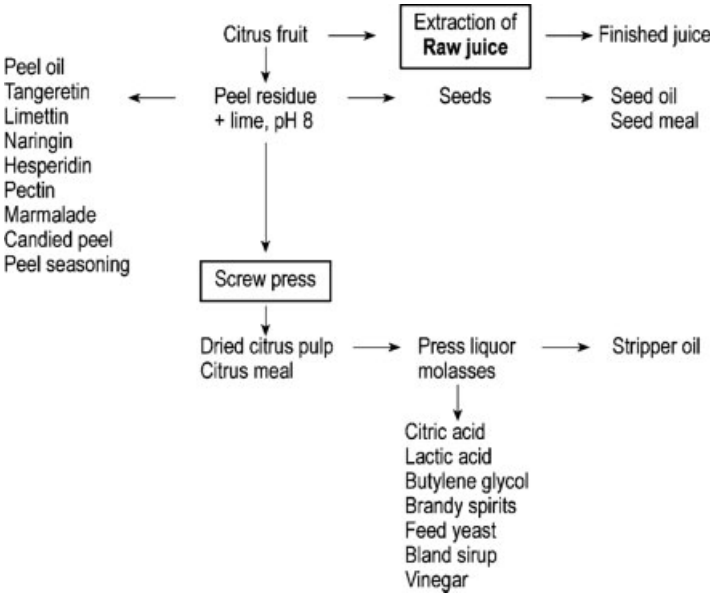

Fig. 41 Citrus processing

Citrus Fruits The development of frozen concentrated orange juice started in 1940. Orange juice is the most consumed fruit juice in the world. Citrus fruit processing includes exploitation of all byproducts, among which pectin and essential oils are the most significant (Fig. 41). Although in certain countries it is not permitted to use enzymes in the production of premium orange juice, they can be used in other applications. Enzymes can increase the yield of solids recovery during pulp washing, facilitate the production of highly concentrated citrus base, improve recovery of essential oil from peel, debitter juice, and clarify lemon juice [528]. An example of citrus processing using enzymes is fruit peeling. The whole orange or grapefruit is treated with pectinases for digestion of albedo (the white, inside part of the peel), which binds flavedo (the orange or yellow, outside part of the peel). The fruit peel is scored and whole fruits are treated with a 2 % pectinase solution by vacuum infusion technology. After vacuum break, fruits are maintained at 40 °C for 15–60 min for albedo digestion inside the fruit. Peeled fruits are then rinsed, cooled, and packed.

Another example is the production of clear lemon concentrate. Cloudy lemon juice coming from the extractor contains particles composed of pectin and proteins remaining in suspension and binding citral aroma (predominant lemon flavor). Clarification of lemon juice was achieved in the past by addition of large amounts of bentonite or sulfur dioxide. Today, clarification can be carried out with the very acidic pectinase Clarex Citrus 12XL (DSM), which degrades the pectin part of the cloud. Enzymes are added to the juice in amounts of 10 g hL^{-1} at 8–10 °C to avoid juice oxidation. The insoluble solids are removed by filtration or ultrafiltration. The brilliant lemon juice can be concentrated at 65° brix.

5.1.2.7 Conclusion

Nowadays, enzyme producers provide a wide range of pectinases for processing of fruit to give various products such as puree, cloudy juice, clear juice, and concentrate. The evolution of technology and processes including enzymes allow processors to obtain higher juice yield (productivity) together with higher quality of finished products. The use of specific pectinases adapted to the fruit process improves the shelf life of juices and concentrates (stability of color and freedom from turbidity). Apart from juice processing, the wide range and the high specificity of commercial enzymes open the way to new processes and new types of fruit-derived products. The trend is to process fruit under milder and more strictly controlled conditions to obtain new fruit products with sensory characteristics closer to those of fresh fruit.

However, because processing technology evolves faster than regulation, it is necessary to define food standard values and process references (equipment, process stages, type of enzymes. . .). In Europe, a Code of Practice has been developed to maintain quality and authenticity standards [534]. Members of Association of the Industry of Juices and Nectars from fruits and vegetables (AIJN) in parallel with the Association of Microbial Food Enzyme Producers (AMFEP) have established references for fruit juice composition and enzyme specifications, in line with commercial standards and regulations of different countries.

5.1.3 Enzymes in Brewing

5.1.3.1 Introduction

Brewing is an old and traditional process that uses a variety of raw materials; in centuries past, many brewing processes have evolved, and they show the versatility and craftmanship of historic brewmasters. By far the most important brewing style is the ''lager'' type, characterized by the use of very pure water, low-temperature fermentation, and a long maturation period. Even in this relatively young beer style, many variations have developed. The common denominator in the brewing of beer is that part of the starch of a cereal is converted into alcohol. In the first stage enzymes hydrolyze starch into fermentable sugars. In a second process stage, these sugars are converted to alcohol and carbon dioxide by yeast. (Not all beers use yeast for fermentation; e.g., in the case of gueuze beers and weissen, bacteria are involved.)

The traditional source of enzymes used for the conversion of cereals into beer is malted grain, and malted barley is a key ingredient in brewing. Malting depends on many factors, including the barley variety and quality, the malt kilning regime and the skill of the malter. The extent and success of the malting process depends on the development, distribution, and survival of indigenous barley enzymes, and in the case of the notoriously heat labile malt enzymes, quality varies from season to season and from region to region.

During the malting of barley a series of enzymes — proteases, amylases, glucanases, and cellulases — developes in the malt. Enzymes such as β-amylase, exo-peptidase, and carboxy-peptidase are present in the starchy endosperm of the barley, and are

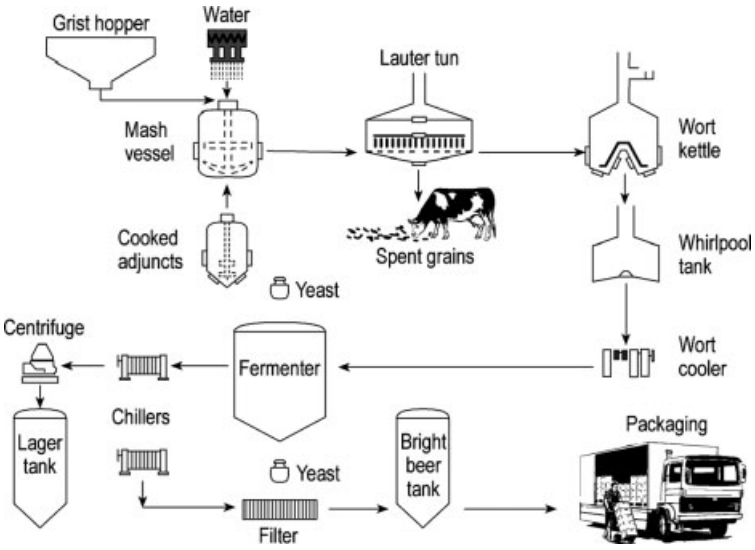

Fig. 42 Schematic of the brewing process

activated during malting. In the aleurone layer of the barley, β-glucanase, endoproteases, α-amylase and pentosanases are formed during malting. The formation and activation of the enzymes is promoted by increasing moisture and oxygen concentrations during the steeping process, and is then fixed by the kilning of the green malt. At this stage each enzyme with its individual properties is present at its individual concentration.

Detailed steps of the brewing process are never universal due to their wide variety in both starting material, brewing style, and technical preferences and possibilities. In this chapter a simplified brewing scheme is adopted (Fig. 42):

- Milling: reducing the size of the dry malt
- Mashing: adding water to the malt; controlled rise of temperature to allow enzymes to degrade proteins and carbohydrates
- Brewing: boiling the mash
- Filtration:separate spent grain and most (lautering)
- Cooling of the most
- Fermentation: convert sugars into alcohol and carbon dioxide
- Filtration: separate yeast from bright beer
- Maturation: settling of insolubles, reduction of vicinal diketones
- Packaging: in casks, kegs, bottles, cans
- Pasteurization: improving microbiological stability

Of the steps in this schemes, four are directly affected by enzymes (mashing, fermentation, maturation, and packaging); indirectly, the several filtration steps are strongly affected, too. Enzymes have an impact on several qualities of the final product, such as appearance, taste, and economics.

Starting from malt, the concentration of each enzyme will change to some extent during storage, milling, and steeping, i.e., before the mashing step. Each enzymes set of intrinsic properties (temperature stabilities, substrate affinities, pH dependences, etc.) does not change, but the expression of enzyme activity is dependent on the reaction conditions (time, temperature, pH [536], salts [537], other enzymes, etc.), i.e., the mashing conditions.

Since brewing, and therefore mashing, is a highly optimized process [538], there are several undesirable consequences if too little of an enzyme activity is expressed in the mash; for example:

- The extract yield is too low
- Wort separation takes too long
- Fermentation is too slow
- Too little alcohol is produced
- The beer filtration rate is reduced
- Off-flavour of the beer (diacetyl)
- Inferior stability of the beer

Auxiliary enzymes are used to supplement endogenous malt enzymes in order to solve or prevent these problems (see Section 5.1.3.3). Furthermore, enzymes can be used improve the brewing process (e.g., better adjunct liquefaction, shortening of the beer maturation time, production of beer from cheaper raw materials; see Section 5.1.3.4). Finally, by using new enzymes it has become feasible to produce ''nontraditional'' types of beer (low-calory ''light beer'', IMO beer, ''sparkling wine''; see Section 5.1.3.5).

5.1.3.2 Enzymes in Malting and Mashing

During the malting process, enzymes migrate from the aleurone layer into the starchy endosperm. Cell-wall material is degraded by hemicellulases and β-glucanase, and breakdown of proteinaceous material by proteases enables amylases to act on the starch granules. Each malt enzyme has its own set of intrinsic properties. The most notable property is the dependency of both activity and stability on a host of physicochemical parameters [temperature, water quality (pH, salts), malt and adjunct concentration and composition, etc.]. The dependency is different for each enzyme. Figure 43a shows enzyme initial activity and stability (half-life time) as functions of temperature at two pH values. The action of an enzyme is determined by its capability to convert a substrate into a product (starch to maltodextrins, or protein to oligopeptides), that is, the integrated enzyme action over a period of time during which the enzyme simultaneously loses its enzymic function due to (e.g., thermal) instability. As a consequence, somewhere between temperatures too low for the enzyme to be highly active, and temperatures too high for the enzyme to survive, an optimum for the enzymic effect will occur. It is dependent on many physicochemical parameters, for example, pH, as

illustrated by Figure 43b, or time (Figure 43c). Clearly, the optimum temperature is a derived variable and not an intrinsic property of an enzyme. In the end, it is the optimum overall process that will prevail over the fate of a single enzyme.

During mashing the situation is complicated, since some substrate molecules are formed by the action of one enzyme, and competed for by another enzyme, and these figures are based on a highly simplified model.

Figure 43d shows such a simulation of product formation with time by a typical enzyme at constant temperature, and Figure 43e over a temperature course typically used in a double-decoction mashing procedure. These graphs illustrate how conditions

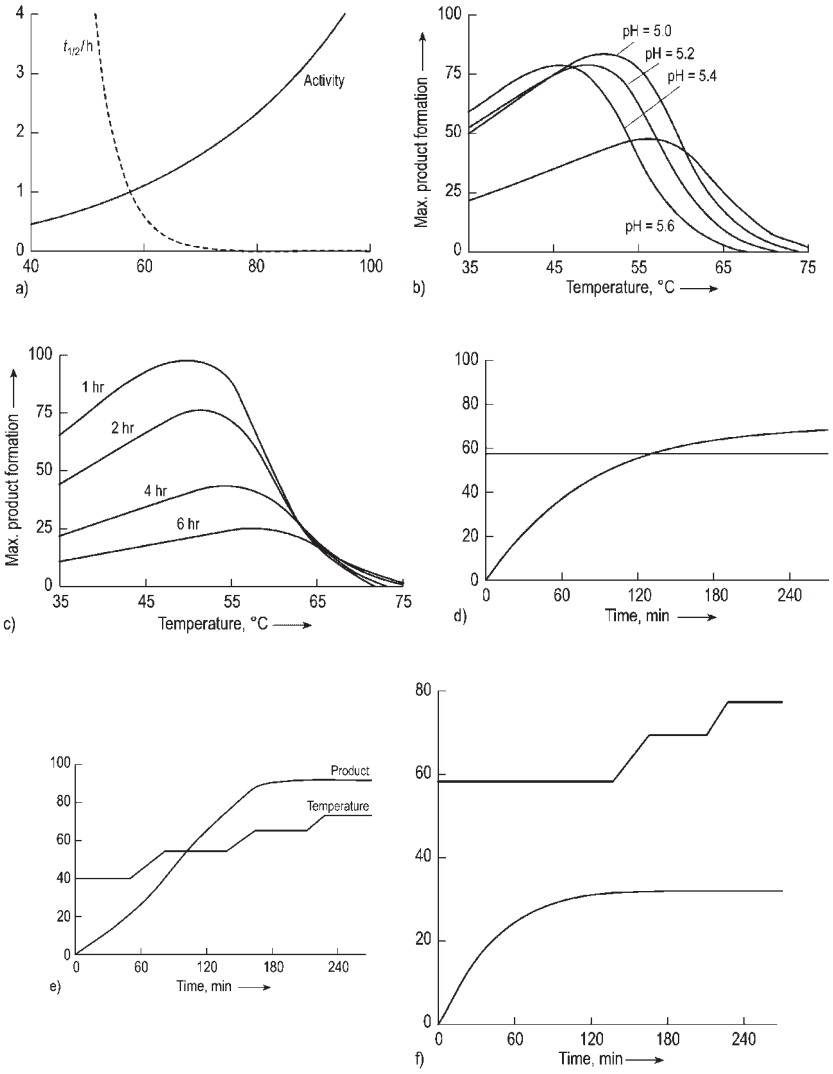

Fig. 43 Enzyme activity as a function of various parameters

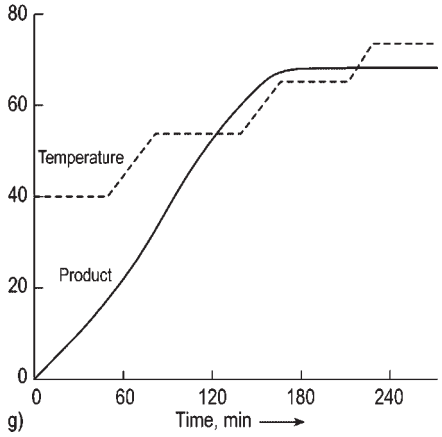

Fig. 43 (continued)

(e.g., temperature) for this enzyme might not completely ideal during mashing, and that a shorter mashing time would optimize the economics of this enzyme.

As a further illustration, Figure 43f and Figure 43g show that for another typical enzyme, a longer mashing period at lower temperature would be better suited.

Since many enzymes act simultaneously, and the results [e.g., high FAN (free alpha amino nitrogen) vs stable foam] often contradict on the molecular scale, the mashing process is a balancing act between obtaining enough activity from one enzyme without destroying another before the goal of its action is achieved [539]. The simulation model in Figure 43e applies to a highly simplified case. In contrast to the simulation, the composition of the solution changes rapidly during real mashing, one enzyme uses the product of another's action as its substrate, proteases also destroy enzymes, and temperature and viscosity change. Even when using identical malt and mashing conditions, relatively small differences between brewing water qualities of two regions can already give rise to substantial differences in mashing results.

Although the mashing process may be too complicated to translate into a kinetic model, brew masters achieve excellent mashing results by skillfully balancing and adapting the many process parameters [540]. In mashing, a premeasured amount of grain is infused with a specific volume of strike water at a predetermined temperature, and the mash is stepped through a series of so-called temperature rests. It is the method used to reach the temperature of these rests which distinguishes the infusion from the decoction mashing methods, and for simplification each rest is given a name according to its major designated effect (Table 12).

The acid rest (see Section 5.1.3.4.2) is rarely employed and has been replaced by the use of brewing salts. The protein rest is used primarily with larger amounts of incompletely modified malts to produce amino acids (as nutrients for the yeast) and to break down other proteins (e.g., those that cause chill haze) without destroying the proteins that provide the beer with a good frothy head.

Table 12. Rest periods in mashing

Temperature	Enzyme	Rest period	Duration
30–40 °C	phytase	acid rest	10–30 min
40–50 °C	protease	protein rest	20–40 min
60–66 °C	β-amylase	conversion rest	40–70 min
68–70 °C	α-amylase	conversion rest	20–40 min

In some cases it can be economically or technically feasible to use exogenous enzyme activities to assist the endogenous malt enzymes, e.g., for the production of a special beer, or to cope with the effects of poor malt modification. A new balance has to be established between parameters such as time and temperature on the one hand, and the combined effects of both endogenous and exogenous enzymes on the other. The combination of different enzymes can result in a more robust, faster, or more economical over-all production process. If malt has been replaced to a large extent by brewing adjuncts such as unmalted barley, corn grits, rice, or sugar syrups, the level of endogenous enzymes will be proportionally reduced, and the additional exogenous enzyme activities become an intrinsic part of the production process.

Sources of exogenous enzymes may be vegetal (papain), fungal (glucanase), or bacterial (protease). Industrial producers of enzymes have been using many sources from as early as the 1960s.

The wide variety of exogenous enzymes range from close-to-malt enzymes to enzymes originating from extromophile bacteria. With the use of bacterial enzymes, the latest achievements of modern biotechnology become available to the brew master. Enzymes hitherto unavailable are now economically feasible for use on an industrial scale, with only personal or commercial sentiments standing between the brew master and the deployment of enzymes from GM organisms.

A traditional all-malt lager brewing process is used as starting point where possible, and the next sections address three areas for using exogeneous enzymes:

1. Solving or preventing problems occurring during the brewing process (Section 5.1.3.3),
2. Improving the brewing process (Section 5.1.3.4),
3. Enabling a nontraditional brewing process (Section 5.1.3.5).

5.1.3.3 Enzymes for Problem Prevention or Solving

5.1.3.3.1 Bacterial α-Amylase in Mashing

The most important change brought about in mashing is the conversion of insoluble starch molecules into soluble, fermentable sugars and unfermentable dextrins. The principal enzymes responsible for starch conversion are α- and β-amylase. α-Amylase (E.C. 3.2.1.1) rapidly splits insoluble and soluble starch into many shorter chains that can be attacked by β-amylase (E.C. 3.2.1.2). In all-malt brewing, a maximum final mash temperature of 78 °C results in the best separation of wort from spent grain. By adding a

bacterial α-amylase, the temperature can be increased to 85 °C in the final mashing phase. The exogenous enzyme extends the degradation of remaining starch into soluble oligosaccharides, continues to do so in the lauter tun, and thus prevents an increase in viscosity due to gelatinization of starch during wort separation. Adding thermostable bacterial α-amylase just before the mash is pumped into the lauter tun degrades any remaining starch. As the mash in the lauter tun is at a higher temperature, it is thinner and passes through the filter medium more quickly and smoothly.

Adding bacterial α-amylases becomes essential when a high fraction of adjuncts has diluted the concentration of endogenous enzymes in the mash tun. Since this dilution factor applies to all endogenous enzymes, additional enzyme dosage or enzyme cocktails containing, e.g., β-glucanase and protease in addition to amylase, are used.

If a thermostable bacterial α-amylase is used, brew masters often prefer separate addition of the β-glucanase and protease to add flexibility (compared to an enzyme cocktail with fixed composition) to adapt to their particular malt/adjunct situation.

5.1.3.3.2 Fungal α-Amylase in Fermentation

Slow fermentation can result from incomplete saccharification in the mashing process. If the problem is diagnosed in an early phase, direct addition of fungal α-amylase (E.C. 3.2.1.1) in the fermenter can correct the problem. The enzyme action at relative low temperatures lowers the attenuation limit (degree of attenuation is the percentage of fermentable carbohydrates in the wort extract), increases fermentability, and more alcohol is produced, resulting in a "drier" more attenuated beer.

5.1.3.3.3 β-Glucanase in Mashing

The β-glucanase (E.C. 3.2.1.4) from barley is very heat labile and survives for only a very short time at mashing temperatures. If not enough β-glucan is broken down, the remaining glucans will partly dissolve, bind water, and cause problems later in the process by increasing viscosity (and thus extending wort runoff times) and by causing haze.

Optimizing the mashing process by varying temperature/time regimes often improves the performance of amylases and increases FAN values. If the new regime decreases β-glucanase performance, this may cause several related problems:

- Poor runoff,
- Poor extract recovery,
- Poor spent-grains drainage,
- Slow yeast sedimentation, poor centrifuge efficiency,
- Poor beer-filter performance, problems with haze.

β-Glucanases from, e.g., fungal origin, selected for improved thermostability under mashing conditions, result in a more robust and stable mashing process. They assist in the mashing of grists containing insufficiently modified or unevenly modified grains, aid runoff from the mash tun by reducing wort viscosity, and improve beer finability and filterability.

5.1.3.3.4 Cysteine Endopeptidases (Postfermentation)

If insufficient protein is removed by hot and cold precipitation (protein is needed throughout as, e.g., yeast nutrient, FAN, and for foam), polypeptides can cross-react with polyphenols during the final stages of beer production (conditioning, lagering) to cause an undesirable, so-called chill haze. Soluble proteolytic enzymes, such as papain (E.C. 3.4.22.2) and, to a lesser extent, bromelain (E.C. 3.4.22.32) and ficin (E.C. 3.4.22.3), are commonly used (mostly in conjunction with other stabilization agents such as poly(vinyl pyrrolidone) (PVP) or silica gels (e.g., Lucilite) before final filtration to improve the colloidal stability of the beer, and thus control chill haze in and increase the shelf-life of packaged beer. Papain, extracted from the fruit of *Carica papaya*, is effective in the temperature range of 30–45 °C and the pH range of 4.0–5.5. Added to conditioning tank, this a-specific endo-peptidase breaks down high molecular weight proteins that react with polyphenols before being destroyed during pasteurization.

The use of immobilized proteases has been studied, but has not been widely adopted.

5.1.3.3.5 Glucoamylase in Mashing

If the amount of dextrins remaining in the beer as nonfermentable sugars is too high, the addition of exogeneous glucoamylase (E.C. 3.2.1.3) to the mash tun results in cleavage of terminal α-1,6 glycosidic bonds of the oligosaccharides, and thus in an additional amount of fermentable glucose in the wort. Since glucoamylase only reacts with oligosaccharides of maximal 10–15 glucose units in length, this enzyme does not prevent starch hazes caused by high molecular weight amylose and amylopectin molecules.

5.1.3.4 Enzymes for Process Improvement

5.1.3.4.1 Adjunct Brewing

In adjunct brewing, malt is partly replaced by another starch source (e.g., corn, rice) for economical reasons or to produce a lighter taste. With malt as the sole enzyme source, the malt enzymes have a more difficult job since their relative concentration during mashing is lower. When higher percentages of adjunct are used a separate pre-mashing step with exogenous enzymes is introduced to enable a more predictable and simpler production process. As heat-stable amylases are much more stable than malt amylases, simpler liquefaction, shorter process times, and an overall increase in productivity can be achieved. Eliminating the malt from the adjunct cooker means less adjunct mash and thus more freedom in balancing volumes and temperatures in the mashing program.

Traditionally, the use of barley has been limited to 10–20 % of the grist when using high-quality malts. At higher contents of barley (> 30 %, or with low-quality malts) processing becomes more difficult, and the mash must be supplemented with extra enzyme activity (apart from α-amylase, some extra β-glucanase and endo-peptidase are needed) if the brewer uses unmalted barley while maintaining brewing performance. Other raw starch adjuncts are used to partially replace malt as the carbohydrate source. Adding thermostable bacterial α-amylase to the mash allows extension to the higher temperatures needed for liquefying starches from, e.g., rice or maize. Brewing systems

that use raw starch or grits from rice, maize, or sorghum will have a separate cooking stage for these materials, preferably at elevated temperatures of up to 108 °C (jet cooking). Malt amylases are not suitable for this, and thermostable bacterial α-amylase, or a protein-engineered more robust version thereof, is needed. Pre-gelatinized adjuncts, such as micronized cereals added to the mash need (nonthermostable) bacterial α-amylase to ensure that no residual starch is present in the worts. The enzyme hydrolyzes both malt and adjunct starches, liberating soluble dextrins. This action is supplemental to the action of the natural malt α- and β-amylases.

The presence of approximately 200 ppm Ca^{2+} is important when using nonthermostable α-amylase, especially when hydrolysis occurs at higher temperatures. Enzyme inactivation occurs when the temperature is raised to ca. 100 °C for 1–20 min. For all practical purposes the enzyme will be destroyed in the brewing kettle during wort boiling.

Liquid adjuncts as carbohydrate source include cane and beet sugar syrups, and the corn based DE (see Section 5.2.3.3.3) syrups produced by the corn starch processing industry. ''Brewer's syrup'' (a maltose syrup produced from, e.g., corn, with a carbohydrate spectrum close to that of sweet wort) has become popular as adjunct in the UK, South Africa, and some Asian countries. The solubilization and partial hydrolysis of corn starch is performed outside of the brewery by starch processors using modern industrial enzymes such as thermostable (protein-engineered) bacterial α-amylase, pullulanase, and β-amylase extracted from malt or barley. By using different saccharification reaction conditions (time, temperature, enzyme), by blending or by introducing fungal α-amylase and glucoamylase, starch processors can nowadays meet any brewer's syrup specification, both in composition and in economics.

5.1.3.4.2 Improved Mashing Processes

Proteases The endogenous endo- and exo-proteases are highly heat labile and act mainly in the malthouse. Carboxy-peptidases are a little less heat sensitive, and continue to operate for a while in the mash. Proteases (and also β-glucanase) are quickly destroyed in an infusion mash at 63–66° C. When using decoction mashing techniques and a reduced initial mash temperature, significant enzyme activity can occur in the early stages of mashing. Therefore, malts for use in decoction mashing systems need not be so well modified as malts in infusion mashes. During the so-called protein rest (30 min at 40–50 °C) protease reduces the overall length of the high molecular weight proteins — the cause of foam instability and haze — to low molecular weight proteins in the mash. Endo-proteases split high molecular weight proteins into simpler peptides by breaking the peptide bonds between amino acids. Endo-proteases are responsible for degrading insoluble globulins, and albumins already dissolved in the wort, into medium-sized polypeptides. The reduction in content of albumins and globulins is important for reducing the haze caused both by proteins and polyphenols (tannins derived from the malt husks and hops). As a rule, reducing the number of large protein molecules in beer makes it less prone to haze. The medium-sized proteins are not useful yeast nutrients, but are important for foam stability and thus head retention, as well as for body or palate fullness. Some brewers prefer to limit the duration of the protein rest to improve the foam quality of the beer. A wide variety of endopeptidases are

readily available; historically, the proteases originating from *Bacillus amyloliquefaciens* have proved adequate for in assisting the available malt proteases.

Pullulanase Endogenous β-amylase (1,4-α-D-glucan maltohydrolase, E.C. 3.2.1.2) is an exo-enzyme that cleaves external α-1,4-glucosidic links to form maltose molecules and β-limit dextrins. The latter contain α-1,6-glycosidic links and cannot be cleaved by either α- or β-amylase; β-limit dextrins remain in the wort throughout fermentation as nonfermentable sugars. The natural enzyme produced in the wort that can cleave this link is limit dextrinase (dextrin α-1,6-glucanohydrolase, E.C. 3.2.1.142), a heat-labile enzyme that is readily inactivated at mashing temperatures. The exogeneous enzyme pullulanase (pullulan 6-glucanohydrolase, E.C. 3.2.1.41) hydrolyses α-1,6 glycosidic linkages in branched polysaccharides, e.g., amylopectin. The enzyme requires at least two α-1,4 glucose units to be on either side of the α-1,6 link, and thus maltose is the main final reaction product. The activity and stability of exogenous pullulanase must be compatible with mashing conditions, and not all industrial enzymes used in the starch processing industry qualify. The tolerance for fermentation pH values and limited thermostability prevent carry-through risk for pullulanase, and limit its action to achieving a set degree attenuation.

Phytase The traditional acid rest used to be responsible for reducing the initial mash pH for traditional decoction mashing of lager beers starting from under-modified malt. The enzyme phytase from barley malt is active at 30–53 °C and breaks down insoluble phytin (a complex organic phosphate containing both calcium and magnesium) to phytic acid. The phytase reaction releases hydrogen ions in the process and may be accelarated and extended by adding a more thermostable phytase of bacterial origin. Highly modified malts contain too little endegenous phytase because of the high kilning temperatures and depend fully on exogeneous phytase to achieve phytase-induced acidification, whether during the acid rest or during the intitial mashing phase at higher temperature. However, the acidity of highly kilned malts is normally sufficient to lower the mash pH without an acid rest.

Diastase/β-Amylase The most obvious method to enrich the enzymatic activity of malt is to add an extract of malt enzymes. Composition (concentrations of various enzymes) may vary between the various products, dependent on malt type and extraction processes. For economical reasons, addition of malt or barley extracts is only used if no high-quality malt is available and the use of bacterial and fungal enzyme is undesired.

5.1.3.4.3 Shelf-Life Improvement

During fermentation, yeast takes up all dissolved oxygen, and in the following processing steps the atmosphere in tanks and equipment is pure carbon dioxide. Typically the oxygen concentration in beer is below 200 ppm. After packaging, the concentration of oxygen in beer may vary from less than 500 to 1000 ppm. Traces of entrapped oxygen (and polyphenols such as delphinidin acting as pro-oxidants), lead to formation of the volatile aldehydes responsible for stale flavor in beer. Antioxidants,

such as sulfite, ascorbic acid, and catechin, can thus protect beer from becoming stale in the presence of oxygen [543].

Various antioxidants have been added to green beer to either remove oxygen or to negate its effects. Ascorbic acid (vitamin C) at 1.5 g/hL reduces oxidation haze and the effect, similar to reducing dissolved oxygen. The reduction of agents containing sulfur can reduce chill hazes. Sodium hyposulfite has some effect on chill haze when used in amounts of 20 ppm, and sodium metabisulfite and ascorbic acid (10–20 ppm each) have a synergistic action in protecting the activity of papain in beer during and after pasteurization. In contrast to the reducing agents, which are needed in equimolar amounts to the dissolved oxygen, enzymatic oxygen removal uses the small concentration of available glucose as the electron donor to scavenge oxygen. The removal of oxygen by glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) plus catalase (H_2O_2 oxidoreductase, EC 1.11.1.6) is the sum of two reactions: glucose oxidase converts glucose and oxygen to gluconic acid and hydrogen peroxide, which is transformed by catalase into water and oxygen (net reaction: glucose $+1/2\ O_2 \rightarrow$ gluconic acid).

In practice, a system with only enzymic deoxygenation is less efficient than a combination of enzymes and chemical reductants. The concentration of free glucose in beer may be too low for effective removal of oxygen, but it was demonstrated [544] that by adding only glucose oxidase and sulfite successfully suppressed the flavor deterioration of the beers. Another possibility is that the peroxide formed in the first reaction, and/or intermediates leading to oxygen formation in the second reaction, are reactive and responsible for oxidative deterioration of beer flavor [545].

5.1.3.4.4 **Accelerated Maturation**

Lagering involves secondary fermentation of remaining fermentable extract at a reduced rate at low temperatures and low yeast count. The low temperature promotes the settling of remaining yeast and precipitation of haze-forming material (protein/polyphenol complexes). The maturation period or diacetyl rest reinvigorates the yeast culture so that it metabolizes byproducts such as diacetyl and 2,3-pentanedione that were excreted early in the fermentation. During the maturation period 96 % of the diacetyl and 2,3-pentadione is used in biosynthesis (i.e., amino acid valine/leucine synthesis in particular) by active yeast and 4 % of α-acetolactate formed in beer is oxidized to diacetyl. The flavor threshold for diacetyl is 0.10 mg/L, and this small molecule causes an unpleasant buttery or butterscotch taste, considered a major off-flavor for lager-type beer.

Depending upon the yeast type, the physical environment, etc., this process takes 5 to 7 weeks with traditional lagering. Employing a diacetyl rest is particularly important when producing beers with high adjunct fraction due to the high levels of diacetyl produced [546]. It is also important in brewing lagers, as they do not have a heavy flavor impression.

In accelerated lagering the beer is fully attenuated, virtually free of yeast, and stored at higher temperatures to reduce the concentration of vicinal diketones, which are responsible for off-flavors in beer. Accelerated lagering reduces the time needed to achieve a product similar to beer brewed with a cold-lagering process to 7 to 14 d, with

the same clarity and flavor stability [547]. Sometimes freshly fermenting wort is added to diacetyl-laden beer in cold storage (kräusen) so that active yeast absorbs diacetyl.

With the exogenous enzyme α-acetolactate decarboxylase (ALDC, (*S*)-2-hydroxy-2-methyl-3-oxobutanoate carboxylyase, E.C. 4.1.1.5) the excess α-acetolactate can be directly converted into harmless butanediol, bypassing the formation of diacetyl.

By eliminating one of the major reasons for an extended lagering period, ALDC enables brewers to extend their peak lagering capacity.

5.1.3.4.5 **Starch-Haze Removal**

Fermentation problems, such as sticking fermentations or unacceptably low attenuation limits, are only noticed after mashing, when certain notable fermentation parameters are not met. Undegraded starch or high molecular weight carbohydrates that will neither fine nor ferment out, rearrange into insoluble complexes that cause beer with a starch or carbohydrate haze. Under these circumstances it is important to apply corrective action swiftly to avoid atypical flavor profiles. At the lower temperatures common in fermentation, fungal α-amylases are capable of rapidly hydrolyzing the interior α-1,4-glucosidic linkages of barley, malt, and cereal starch and the formation of maltose and a carbohydrate profile similar to that derived from indigenous malt amylases.

5.1.3.5 **Special Brewing Processes**

Low-calory beer (diet/light lagers) is based on the American lager style of brewing. Corn is the major adjunct, amounting to 50–65 % of the total grain bill, and processed with added enzymes, e.g., glucoamylase to break down nonfermentable carbohydrates and attenuation is higher than normal beers. *Dry beer*, *super dry beer*, and *sparkling wine* are highly carbonated fermentation products with almost complete conversion of all carbohydrates into alcohol and CO_2 assisted by bacterial and fungal enzymes. The main starch source in *sorghum beer* (bantu or kaffir beer, pombe, burukutu, pito), is unmalted sorghum, supplemented with maize. After acidification with lactic acid to lower the pH to 4, bacterial α-amylase may be added, and the cooker is heated to boiling for 90–120 min. After cooling to 60–62 °C, malted sorghum and/or bacterial glucoamylase is added for a partial saccharification. After coarse filtration and cooling to 30–35 °C, yeast is added. The fermenting turbid mash is packed in open bottles, jerrycans, or vessels, ready for consumption after 16–24 h. Still experimental is the production of *IMO beer*. The consumption of isomaltooligosaccharides (IMO: glucose oligomers with α-1,6-glucosidic linkages) is claimed to stimulate the health-promoting activity of colonic bacteria of genus *Bifidobacterium*, as well as producing a mild sweet taste and low cariogenic properties. IMO-containing syrups are usually produced from starch by means of cooperative reactions, one of which is transglycosylation catalyzed by (immobilized [548]) enzymes of microbial origin, by conversion of high-maltose syrup into IMO-containing syrup. The transglycosylation product contains about 38 % of panose, 4 % of isomaltose, 28 % of glucose and 23 % of maltose, and the usage of IMO syrup in the brewery to replace maltose imparts functional properties to traditional food products with minimal changes in production technology and product taste.

By introducing transglucosidase (EC 2.4.1.) during an enzyme assisted mashing process, it is also possible to produce IMO in situ.

High-gravity brewing involves worts of up to 18 °P and even higher. After fermentation and maturation, the beer is diluted with cold carbonated water to the designated gravity or to a prescribed alcohol concentration. Advantages associated with high-gravity brewing are that it results in beers that are more consistent (alcohol content, original gravity, etc.) and more physically stable because the compounds responsible for haze are more easily precipitated at the higher concentrations [549]. Handling more concentrated wort results in increased utilization of equipment and lower energy costs. The disadvantages are problematic mashing, longer fermentation times, different flavor characteristics, and poorer hop utilization than normal-gravity fermentations [550]. Exogenous enzymes are used to assist in mashing (neutral protease, bacterial α-amylase) and fermentation (fungal α-amylase).

5.1.4 Enzymes in Dairy Applications

5.1.4.1 Introduction

Dairy products are among the classic examples of fermentation-derived foodstuffs, and their history of development goes back several millennia. Through the ages a huge variety of dairy products has been developed based on empirical experimentation. Profound understanding of biochemical, microbiological, and physicochemical processes has mainly been achieved during the second half of the 1900s. Economical interest and scientific progress have led to the development of dairy food science as a special branch within food science, and it is the basis of control in manufacturing a huge variety of dairy products. Emphasis on understanding and controlling taste and texture development has led to a still growing assortment of cheeses and desserts. Enzymes originating from raw materials, microbial starter cultures, or other sources are prime tools in improving existing and creating novel dairy products.

5.1.4.2 Cheesemaking

5.1.4.2.1 Cheesemaking Process

A basic element in the process of cheesemaking is the clotting of the milk. Milk turns into a gel structure through the action of coagulating enzymes, briefly called coagulants. The casein proteins in milk lose their colloidal stability and aggregate to form a gel structure. After cutting of the gel, the liquid whey fraction, containing whey proteins, minerals, and lactose, separates from the casein chunks. The casein material, the curd, is collected in molds. After pressing and brining, the cheese is kept under conditioned storage for ripening. Storage time may last from weeks to months, even years, depending on the type of cheese.

5.1.4.2.2 Mechanism of Renneting

About 80 % of milk protein consists of casein, which is hydrophobic by nature. The bovine caseins may be subdivided in four species of phosphoproteins that exist, due to

their poor solubility in water, in agglomerates. The four species αs_1-, αs_2, β- and κ-casein occur in relative molar concentrations of about 4:1:4:1.6. Caseins aggregate to form submicelles, which together form the casein micelle [551]. The outer surface of the casein micelle consists of submicelles containing a relatively high content of κ-casein molecules. Hydrophilic, negatively charged parts of the κ-casein molecules protrude from the periphery of the micelle and guarantee micelle stability due to electrostatic and entropic repulsion. The coagulating enzymes specifically split off a distinct part of these κ-casein hairs, hydrolyzing the Phe-Met bond (at amino acid 105–106) and thus provoke destabilization of the whole micelles, which aggregate with each other. The part of κ-casein split off is called the caseino macro peptide. Calcium ions facilitate casein micelle aggregation but do not affect the enzyme reaction. The enzyme reaction is very sensitive to pH but less to temperature. The aggregation rate is strongly affected by temperature between 25 and 35 °C. Renneting is thus described by the enzymatic reaction and by Casein Micelle Aggregation [552], [553].

5.1.4.2.3 Types of Coagulants

Commercially available coagulants used in the cheesemaking industry consist of animal rennet and microbial coagulants from non-GM and GM microorganisms. Animal rennet is obtained from the gastric mucosa of young mammals, usually of bovine origin. Animal rennet is a mixture of chymosin and pepsin (E.C. 3.4.23.1). The ratio of chymosin to pepsin depends on the age of the animal, being highest (about 95 %) for unweaned calves. The high ratio of chymosin to pepsin in the calf stomach is explained by the fact that chymosin does not hydrolyze immunoglobulins, which are present in the colostrum and needed to build up immunity, whereas pepsin does.

Due to the limited availability of calf rennet, substitutes from microbial sources have been sought, and nowadays microbial coagulants cover a considerable part of the demand for of coagulating enzymes worldwide. Microbial coagulants are produced by fermentation. Most widely applied are enzymes from *Rhizomucor miehei*, *Rhizomucor pussilus*, and *Cryphonectria parasitica*.

A third group of coagulants is made by genetically modified microorganisms. The bovine gene encoding for chymosin type B is cloned in the genome of fungi and yeasts such as *Aspergillus oryzae* and *Kluyveromyces lactis*. Chymosin type A cloned and expressed in *E. coli* is no longer commercially available. Several efforts have been made to clone microbial genes from *R. miehei* or *R. pussilus* in other microbial expression hosts, but, although technically feasible, none of these have reached commercial application.

In some minor cheese types plant proteases from cardoon and *Solanum dobium* are used.

Coagulants are commercially available in liquid, powdered, tabletted, and paste formulations of varying strengths. Animal rennets are made in standardized ratios of chymosin to pepsin.

5.1.4.2.4 Properties of Coagulating Enzymes

All coagulating enzymes are aspartic endoproteases. Two aspartic acid residues are located in the active center of the enzyme and play an essential role in the proteolytic

Table 13. Biochemical characteristics coagulating enzymes

Coagulant	Source	E. C. no.	M, kDa	IEP	Glycosylation	$T_{opt.}$ °C (milk clotting)
Chymosin	bovine	3.4.23.4	35.7	4.98	no	40–44
Mucorpepsin	*R. miehei*	3.4.23.23	38	4.58	yes	58–62
Mucorpepsin	*R. pussilus*	3.4.23.23	30–39	4.41	yes	42–45
Endothiapepsin	*C. parasitica*	3.4.23.22	33. 8	4.89	no	42

mechanism. All coagulating enzymes are more or less inhibited by pepstatin, a microbial oligopeptide from strains of *Streptomyces* [554]. Coagulants are of comparable size and three-dimensional bilobal, structure and contain highly conserved regions. The amino acid homology between microbial coagulants is high, but they diverge extensively from mammalian ones [555]. The three-dimensional structures have been elucidated by crystallographic studies [556]. Chymosin and pepsin are secreted as inactive zymogens, whereas the microbial enzymes are not. In low-pH environments, as in the stomach, the propeptides are split off to activate the enzyme. Table 13 lists some biochemical characteristics of coagulating enzymes.

Chymosin in animal rennet consists of types A and B, which are point-mutated at position 290, where chymosin A has Asp and chymosin B has Gly [557]. Chymosin A is said to have a higher specific activity for renneting but is less stable [558]. Due to its high specificity for cleaving the Phe-Met bond between amino acid residues 105 and 106 of κ-casein at the natural pH of cheese milk, chymosin is considered the standard renneting enzyme to which others are compared. At lower pH, aspartic proteases lose their specificity and aspecifically hydrolyze proteins randomly, even themselves. Apart from the coagulant from *C. parasitica*, which cleaves between amino acid residues 104 and 105 in κ-casein [555], the other coagulants also hydrolyze the Phe-Met bond of κ-casein. In general, microbial coagulants are more proteolytic than animal rennet. There are differences between the various coagulants in terms of the ratio of their milk clotting to their overall proteolytic activity, whereby a high ratio is important. The milk clotting to proteinase (also called caseinase) ratio (MC/PA) is used as a quality characteristic of coagulants [559]. These ratios vary with pH. At pH 6.5 chymosin derived through fermentation has the highest ratio, followed in order of decreasing ratio by calf rennet, bovine pepsin, and proteases from *R. pusillus*, *R. miehei*, and *C. parasitica*. The MC/PA ratio is directly related to cheese yield, one of the most important economical characteristics in the cheesemaking process. A high caseinase activity results in a relatively large amount of casein protein fragments that are washed away with the whey fraction and thus do not end up in the cheese curd. There is a huge, but also, conflicting amount of scientific literature describing comparison studies of cheese yield with different coagulants. In an elaborate study [560] the most relevant commercial coagulants were studied in a single comparable process (Table 14).

Although the differences appear to be small, considering the huge amounts of milk processed, the economical relevance is considerable.

Heat lability is another important characteristic of coagulants [561]. For Emmental cheese, the cheesemaking process of which involves a cooking step at 50 °C to inactivate

Table 14. Performance of commercial coagulants

Coagulant	Yield reduction, %
Calf rennet	0.0
Calf rennet/pepsin 1/1	0.09
Bovine rennet	0.14
Mucorpepsin (*R. pusillus*)	0.45
Mucorpepsin (*R. miehei*)	0.59
Endothiapepsin (*C. parasitica*)	1.11

all residual coagulant in the cheese, mostly coagulant from *C. parasitica* is used because of its low heat stability. Any proteolytic activity of coagulants in whey, which is nowadays used in many food applications, should disappear following normal pasteurization (12 s, 72 °C). The following order of heat lability is known: porcine pepsin > bovine pepsin > *C. parasitica* > chymosin > *R. pusillus* > *R. miehei*. Commercial types of *R. miehei* coagulants have been chemically modified in order to lose stability and to meet application demands. The heat stability of all coagulants increases with decreasing pH.

5.1.4.2.5 **Cheese Ripening**

Cheese ripening [562] is mainly due to proteolytic breakdown of the casein protein. Endoproteases such as the endogenous milk enzyme plasmin and coagulating enzymes are responsible for the generation of casein polypeptide fragments. Plasmin is a trypsin-like alkaline endoprotease that occurs in milk also in its inactive plasminogen form. It is mainly associated with the casein micelle. Plasmin may become more or less active when plasmin- and plasminogen-activation inhibitors were separated with the whey fraction. Exoproteases, originating from the bacterial starter cultures, further break down part of these peptides to the level of free amino acids. These amino acids function as precursors for flavor compounds to be synthesized by the catabolic enzyme systems of the starter cultures. In this way the various types of starter cultures are responsible for the typical characteristics of cheese varieties. In this simplified description it is clear that coagulants play an important role in the ripening mechanism. At lower pH, as in cheese, coagulants become more and more aspecific. The various coagulants differ considerably in their ability to breakdown casein proteins. For that reason not all coagulants are suitable for all types of cheeses. Microbial coagulants are believed to be less suitable for long-ripening cheeses and are found to develop bitter off-flavors, although chemical modifications and processing optimization result in improved performance.

The separation of whey from curd is a crucial moment in the cheesemaking process in which retention of milk constituents in the curd is determined. Minerals and lactose are not retained in the curd. Particles such as bacteria and fat droplets are entangled in the casein network and end up almost entirely in the curd. The water present in cheese is mainly protein-bound and can not be regarded as a solvent. On the basis of partition

calculations only a few percent of a water-soluble component present in milk would end up in the cheese. Retention of proteins is determined by the extent and nature of interaction of the proteins and casein. Whey proteins are completely expelled, but a lysozyme is fully retained. Retention of coagulants in the curd depends on the pH of the renneted milk at the moment of whey separation. Chymosin is retained to about 15 % at pH 6.5, which is a common pH in semihard cheesemaking (Gouda, Cheddar). In the Camembert cheesemaking process whey separation occurs at pH 6.2, and about 50 % of chymosin is retained. Under the same conditions retention of porcine pepsin is 65 %. Chymosin is electrostatically bound to the para-κ-casein part of κ-casein after having split off the caseinomacropeptide. This interaction, based on differences in isoelectric points of the two proteins, becomes stronger at lower pH [563]. The microbial coagulants are retained to about 18 % in curd, independent of pH [564]. As coagulants participate in the first stage of proteolysis in cheese the extent of retention is of significant importance for the rate of cheese ripening.

5.1.4.2.6 **Cheese Flavors and Ripening Acceleration**

For several semihard and hard cheese types long ripening, and consequently long storage times under strict temperature and humidity conditions, contribute substantially to the cost of cheese. For this reason, ripening acceleration has long been a topic of study. Acceleration of ripening can be achieved by changes in the process, addition of enzymes to the cheese milk, or by manipulation of lactic acid bacteria (LAB) from the so-called starter culture.

The most obvious way to accelerate ripening is by elevating the storage temperature. However, this results in unbalanced flavor and texture characteristics.

As cheese ripening is, for the most part, due to proteolysis of casein, addition of proteases would be an effective way to enhance ripening. The problem of addition of specific proteases, apart from finding the proper proteolytic specificities, is that proteases added to the cheese milk most often do not end up in the curd. Like whey proteins, extra added enzymes usually end up in the whey fraction. For this reason, specific formulation techniques, such as encapsulation in lyposomes, cross-linking, and entrapment of the proteases for targeting into the curd have been sought. In the Cheddar cheesemaking process, in which salt is added to the curd, addition of enzymes along with the dry salt presents an opportunity for addition, in contrast to many other cheese types. An uneven distribution of enzymes, resulting in ''hot spots'' in the cheese matrix may, however, result.

In practice the costly methods of formulation mean that the desired cost reduction is not achieved. Moreover, ripening acceleration results in a mature flavor that often does not match with the textural characteristics. Another approach to accelerate cheese ripening is by modification of starter cultures, both genetically or by composition. Addition of extra LAB is not feasible due to uncontrolled acidification of the cheese milk resulting in unwanted cheese characteristics. An elegant way may be the addition of attenuated starter cultures [565] in which the LAB have been killed by hot/cold temperature shock or by exposure to microwaves, but catabolic enzyme activities are still present. The problems of fast acidification of the cheese milk and low retention in curd have been solved in this way.

In the past the application of lysozyme (to lyse LAB in cheese) and lactase (to convert lactose into glucose and galactose, thereby offering other nutriants to LAB) have been studied, but did not result in commercial application.

Proteases and lipases have found commercial application in the production of spray-dried cheese-flavor protein derived from enzyme-modified cheese (EMC) processing. EMC is made of short-ripened, enzyme-inactivated cheese to which additives such as bile salts, flavors, and enzymes are added. Maturation proceeds at relatively high temperatures and high water content in a short time. Lipases and proteases of microbial origin are preferably used.

5.1.4.2.7 **Lipase**

Milk fat consists mainly of triglycerides. Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.2) hydrolyze triacylglycerols to di- and mono-acylglycerols, free fatty acids, and glycerol.

Lipolysis plays a significant role in the flavor formation of mold-ripened cheeses, blue cheeses, and some Italian types [566]. In other types of cheese the contribution of lypolysis to flavor formation is still obscure. Milk contains the endogenous lipoprotein lipase (LPL, E.C. 3.1.1.34). This LPL is associated with casein, is consequently incorporated in the curd, and may partially survive pasteurization. In milk this lipase is relatively inactive due to its immobilization on the casein micelle and due to the milk fat being present in milk fat globules surrounded by a protective proteinaceous membrane. Severe agitation or homogenization may damage these structures, after which lypolysis may occur, and the milk becomes rancid.

Pregastric esterases are present in rennet paste and are held responsible for the ''piccante'' taste of several Italian hard cheeses. Pregastric lipase powders are now used in Italian types of cheese along with a milk coagulant in view of the poor microbiological quality of rennet pastes. Due to pasteurization of raw milk, endogenous lipases are inactivated. Cheese types that, in the past, were made from raw milk are now made from pasteurized milk and added lipases.

Replacement of mammalian lipases by microbial ones is a topic of interest. Lipases from *R. miehei* and Aspergilli strains are commercially available and useful for the manufacture of Italian cheese types. These enzymes have a preference for hydrolyzing fatty acids located at the 1,3-positions of the glycerol group. The short-chain fatty acids are flavorful and volatile and contribute most to lypolitic flavor development.

Lipases in blue cheeses originate from the *P. roqueforti* surface mold.

5.1.4.2.8 **Lysozyme**

Hen's egg lysozyme (muramidase, E.C. 3.2.1.17) is an enzyme consisting of 129 amino acids, cross-linked by four S—S bridges and able to split β-(1,4) linkages between *N*-acetylmuramic and *N*-acetylglucosamine residues that constitute the cell wall of Gram-positive bacteria. Lysozyme is found in secretory fluids such as human and cow's milk, tears, and cervical mucus. Lysozyme is active in acidic conditions and is able to withstand 100 °C with little loss of activity.

In the cheesemaking industry lysozyme and nitrate are used in the prevention of the defect of ''late blowing'', which is caused by gas (carbon dioxide and hydrogen) produced by outgrowing butyric acid bacteria, in particular *Clostridium tyrobutyricum*.

At the same time organic volatile acids are formed, which negatively affect the taste and flavor.

Lysozyme does not seem to affect lactic acid bacteria when applied in low concentrations, although successful application has been reported in the use of lysozyme to accelerate St. Paulin cheese ripening through enhanced lysis of lactic acid bacteria [567].

In its lytic action against the *Micrococcus lysodeikticus* substrate the enzyme is inhibited by the presence of NaCl. This inhibition is stronger at higher pH [568]. For this reason lysozyme is most effective in stages before brining and before the salt has reached the interior of the cheese by diffusion. Lysozyme associates strongly with αs-casein and β-casein but not with κ-casein. This strong association is responsible for almost full retention of lysozyme in cheese curd.

Lysozyme has found many other applications [569] in the preservation of food (meat, fruits, vegetables, seafood, and wine).

5.1.4.2.9 **Milk Protein Hydrolysates**

A relatively new area of applications has developed for protein hydrolysates. The milk derived caseins and whey proteins are one of the important substrates available in nature. Hydrolysis of proteins has major impact on their functionalities. Fragmentation of the protein due to proteolysis strongly affects its physicochemical characteristics such as molecular shape and size, electrochemical properties, and hydrophobicity, and hence solubility, foaming, and emulsifying and gelation properties. Allergenicity is decreased and taste is severely changed, most often to bitterness. All these characteristics are very much controlled and determined by the degree of hydrolysis of the protein, which in turn is determined by the processing conditions and by the type of proteolytic enzymes used. The degree of hydrolysis can vary from less than 10 to more than 40 %.

Endo- and exoproteases with defined specificities may be employed to control the degree of hydrolysis, the concentration of free amino acids, the peptide size distribution, and even to control the taste, whereby the prevention of bitterness is often a challenge.

Numerous proteases from animal (e.g., pancreatic pepsin, trypsin) or plant (papain, bromealin) origin, but also industrial enzymes from microbial sources (*B. subtilis*, *B. licheniformis*) are used. Aminopeptidases of microbial origin are often used to improve taste, by removing hydrophobic residues from bitter peptides.

Milk protein hydrolysates are used in health and fortifying sports drinks, in infant and low-digestible enteral nutrition, and dietetic foods.

5.1.4.2.10 **Transglutaminase**

An enzyme which is e to play an important role in the texture and rheological properties of dairy food is transglutaminase (E.C. 2.3.2.13). This enzyme catalyzes the acyl transfer reaction between the ψ-carboxyamide group of glutamine residues and ε-amino groups of lysine between proteins. In this way high-molecular proteinaceous polymers are built and influence the hydration, gelation, emulsification, and foaming properties of food substrates such as whey protein, casein, soy, and fish meat proteins. The degree of cross-linking depends on the three-dimensional structure of the protein substrates. The

nonglobular structure of caseins or denatured proteins facilitates this cross-linking [570].

In the dairy, applications in the manufacturing of yogurt, preventing syneresis, and in the renneting ability of skim milk in fresh cheesemaking and in cream whipping have been described [571].

5.1.4.3 Milk Processing

5.1.4.3.1 β-Galactosidase

β-Galactosidase (E.C. 3.2.1.23) or lactase is an enzyme that has found ample application in the hydrolysis of lactose, a disaccharide consisting of a glucose and a galactose moiety, in cow's milk. The consumption of milk is hampered for some people due to a deficiency of lactase in the digestive system. Lactose intolerance in these people, predominantly in Asian countries but also in Africa and South and Middle America, results in symptoms such as bloating, diarrhea, and flatulence [572]. In many cases these symptoms occur after consumption of 200–300 mL of milk.

Hydrolysis of lactose initially results in cleavage next to a glucose molecule, which is released, and an enzyme/galactose complex is formed. Cystein and histidine play an essential role in the active center. The SH group donates a proton to the galactose oxygen atom, and the nucleopholic imidazole group of histidine attacks the C-1 carbon atom of galactose. In the next phase an acceptor ROH (e.g., water) protonates the sulfhydryl anion and liberates free galactose. When the acceptor is another sugar a di-, tri-, or even a polysacharide is synthesized. This activity, also called transferase activity, is made use of in the production of nondigestible oligosacharides. Transferase activity may vary between types of different sources, and oligosaccharide yield is affected by equilibrium concentrations of reactants and reaction conditions.

Ions such as Ca^{2+} and Na^{+} may inhibit the reactions, whereas K^{+}, Mg^{2+}, and Mn^{2+} are activators. The reaction is product-inhibited. Especially galactose strongly inhibits the enzyme-catalyzed reaction.

Table 15 lists microbial sources and characteristics of β-galactosidases [573].

Table 15. Microbial sources and characteristics of β-galactosidases

Origin	$pH_{opt.}$	T_{opt}	K_m (mM) lactose*	*M*, kDa	Activator	Inhibitor
Fungal						
Aspergillus niger	3.5	58	85	124		
Aspergillus oryzae	5.0	55	50	90		
Yeast						
Kluyveromyces lactis	6.5	37	35	115	K^{+}, Mg^{2+}	Ca^{2+}, Na^{2+}, Zn, Cu
Bacterial						
E. coli	7.2	40	2	540	Na^{+}, K^{+}	
B. subtilis	6.5	50	700			
B. stearothermophilus	6.2	55	2	220	Mg^{2+}	
L. thermophilus	6.2	55	6	540		

The specific applications of lactases are determined by the optimum pH and pH-dependent stability characteristics of the different lactases. Neutral lactase is predominantly used in the production of low-lactose sterilized milk. Another concept includes lactase tablets to be added to the milk by the consumer a prescribed period of time before consumption. Acid lactase is able to withstand the acid environment of the stomach and can be consumed with the milk and help the consumer hydrolyze lactose in the digestive tract.

Neutral lactase has also found application in lactose conversion in sweet whey (pH 6.1) and in the manufacture of lactose-free pet milk. Fungal lactase is more suitable in acid whey. Minor applications [573] are found in the manufacturing of yogurt (different outgrowth of cultures), in the sweetening of dairy-based beverages (lactose is less sweet than glucose and galactose), and in preventing the perception of sandiness due to crystallized lactose in ice cream.

5.1.4.3.2 Other Enzymes

Other enzymes such as glucose oxidase (E.C. 1.1.3.4), catalase (E.C. 1.11.1.6), superoxide dismutase, lactoperoxidase (E.C. 1.11.1.7), and sulfydryl oxidase have limited applications. Glucose oxidase, catalase, and lactoperoxidase are involved in the preservation of raw milk. Sulfhydryl oxidase is used to eliminate off-flavors generated due to the UHT treatment of sterilized milk.

5.1.5 Other Food Applications

5.1.5.1 Introduction

The use of enzymes for food applications has increased steadily over the past two decades, not only in traditional application areas such as starch processing, brewing, fruit processing, and dairies. In applications such as baking, the use of enzymes has grown even more [574].

The appearance of new enzyme applications is due to the increasing diversity of the enzymes available, the majority based on GM technology. In future new enzymes for food applications are expected to lead to major developments in the use of industrially produced enzymes [575].

5.1.5.2 Meat and Fish

5.1.5.2.1 Meat Processing

Cooked meat is considered tender if it is easy to chew and, at the same time, retains the desired texture. Tenderness is influenced by a number of factors, which are as yet not well understood. This applies not only to the biochemical reactions involved in rigor mortis, but also to their termination. Indeed, numerous endogenous enzymes take part in this process, including endogenous proteinases, particularly the cathepsins. These enzymes change muscle protein during maturation or aging of meat.

The mechanism of the maturation process has been studied in detail [576]. Natural maturing of carcasses or pieces of meat takes approximately 10 d at 1–2 °C. Slow

maturation has the advantage of producing very tender meat, but also the disadvantage of moisture loss and shrinkage. Optimal meat maturation in special cold-storage depots (1–2°C, 83–86 % humidity) leads to a water loss of up to 7 % over three to four weeks. Since 1940, attempts have been made to use exogenous enzyme preparations as meat tenderizers. Proteinases capable of digesting connective tissue and muscle protein have been chosen for this purpose.

Papain (E.C. 3.4.22.2) [9001-73-4], bromelain (E.C. 3.4.22.33) [9001-00-7], and fiacin (E.C. 3.4.22.3) [9001-33-6] are the enzymes used on a commercial scale. The main problem associated with the use of enzymes is their even distribution in the tissue. Factors influencing this distribution are diffusion, time, salt content, and enzyme concentration.

If enzyme preparations are only sprinkled onto the surface of meat (as recommended for kitchen use) or if pieces of meat are dipped into an enzyme solution, only the surface is tenderized, and the interior remains tough. After enzyme application in the kitchen, e.g., using 2 % NaCl with 0.002 % bromelain, the meat is repeatedly poked to make it easier for the enzyme to penetrate. The main effect of the proteinase, though, is exerted during cooking.

Commercial methods can be divided into premortem and postmortem procedures. In postmortem treatment, a proteinase solution is spread in the carcass by repeated injections, possibly under pressure. In the premortem method (the Swift technique developed in 1960) a very pure, sterilized papain solution is injected intravenously 2–10 min. before the animal is slaughtered.

Yet another method involves injecting a papain solution that has been reversibly inactivated by oxidation. In the last stages of cooking the enzyme is reactivated by the liberation of mercapto groups.

Pancreatic proteinases are used in the maturation of fish. Bacterial proteinase is employed to dissolve bone meat or segments of meat.

5.1.5.2.2 **Fish Processing**

Until recently, industrial enzymes have only been used in limited amounts in the fish industry [577]. Today interest in controlling or aiding traditional fish processes has increased, and uses have emerged for enzymes in deskinning, membrane removal, and roe purification.

Fish sauce is a traditional product in some Asian countries, with an annual production of about 250 000 t. The production procedure consists of mixing small, uneviscerated fish with high concentrations of salt and storing the mixture in sealed vessels at ambient temperature for 6–18 months. During this process hydrolytic enzymes slowly degrade the fish tissue by proteolytic action with the formation of peptides and amino acids. There is some interest in accelerating fish sauce processing. Plant enzymes such as bromalein, fiacin, and papain digest fish tissue over a shorter period [578], and fungal proteinase increases the initial rate of protein hydrolysis.

Enzyme processes are also important in the traditional *salt curing* of herring, proteolytic enzymes having a favorable effect on both the flavor and texture of salted herring.

Another application of proteolytic enzymes is the *deskinning* of fish such as tuna, which are difficult to skin by manual or mechanical means. This process is based on placing the fish in a warm water bath containing a proteolytic enzyme for 10–90 min. After immersion, water jets can remove most of the skin. Enzymes are also used for deskinning and tenderizing squid.

Another use of proteolytic enzymes is in *fish roe purification*. Here the enzyme hydrolyzes the supportive and connective tissue that covers salmon or trout roe eggs and the roe sack, leading to a yield of 85 % compared to 50–65 % when using conventional processing. In Scandinavia and Canada 40–50 t of caviar (1986) are produced by this method.

5.1.5.3 **Protein Cross-linking**

The quality of meat protein is an important factor for meat texture and water binding. The addition of ingredients such as vegetable proteins to meat products is well known to improve water binding and other characteristics. It is, however, also possible to improve the meat product by modifying the meat protein by cross-linking. One such modification uses the protein-cross-linking enzyme transglutaminase, which in nature is known to cross-link proteins. The physiological function of transglutaminase cross-linking is to enhance the strength of the molecular structure of the protein network [579]. Substrate specificity is a key aspect in developing protein–protein cross-linking.

Transglutaminase (E.C. 2.3.2.13) catalyzes acyl-transfer reactions, introducing covalent cross-links between proteins, as well as peptides and primary amines. In protein cross-linking, ε-amino groups of lysine residues and γ-carboxyamide groups of glutamine residues act as acyl acceptors and acyl donors, respectively (Fig. 44).

Transglutaminase of microbial origin is now commercially available [584] and has found several applications in the meat industry, both as a gelling agent and in connection with improving water binding.

Transglutaminase can also produce restructured meat by binding meat pieces [585], [586]. The meat binding system is based on the use of transglutaminase and caseinate simultaneously. Caseinate treated with transglutaminase acts as a glue to hold food

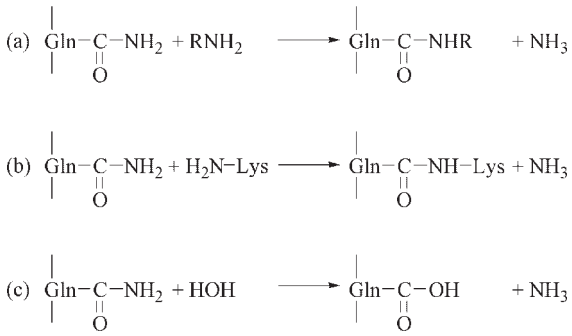

Fig. 44 Reaction catalyzed by transglutaminase (a) acyl-transfer reaction, (b) cross-linking reaction, (c) L. deamidation Source: Ajinomoto information http://www.transglutaminase.com/english/frame/ajinomoto1.htm

components together. Using this system, a larger piece of restructured meat or fish fillet can be prepared from smaller pieces. Minced meat can also be bound together without salt and phosphates to give more consumer acceptable products.

5.1.5.4 Flavor Development

5.1.5.4.1 Protein Hydrolysis

Hydrolyzed protein has been used for centuries to improve the taste of food products. The use of soy protein is well known in the production of the soy sauce by enzymatic hydrolysis during microbial fermentation. Soy and other vegetable proteins can also be used to produce a flavoring called HVP (hydrolyzed vegetable protein) by chemical hydrolysis. This chemical hydrolysis, however, has a number of disadvantages, including very high salt concentration and formation of unacceptable components. More recent developments have shown that better results can be obtained by using enzymatic reactions to produce HVP and other flavor-enhancing proteins.

There are many applications for enzyme-enhanced flavors, including soy and wheat hydrolysates for flavoring soups and sauces, and meat hydrolysates for flavor improvement of meat products, soups, and sauces. Milk protein hydrolysis and lipid hydrolysis contribute to flavor improvement in cheese products.

Naturally occurring food proteins do not contribute chemically to flavor formation in foods. It is, however, well known that peptides and amino acids, which are hydrolyzed protein products, do have a flavor. Amino acids taste either bitter or sweet, with the exception of aspartic and glutamic acid which have a sour taste [580]. Traditionally, these hydrolyzed products, available as flavor enhancers for soups and stock cubes etc., have been produced by acid hydrolysis of proteins during heat treatment. Flavors produced in this way are widely used in the food industry. Increasing concern about the less healthy compounds that develop during acid hydrolysis has led to alternative flavor production processes based on enzymatic hydrolysis of protein.

Protein hydrolysates produced by enzymes often have a bitter taste. This bitterness may be more or less pronounced depending on the type of amino acid and the length of the peptides. The development of bitterness depends on the degree of protein hydrolysis. As the degree of hydrolysis (DH) increases, the level of bitterness rises to a maximum before falling again (Fig. 45) [581].

Bitter peptides are characterised by their high content of hydrophobic amino acids, peptides with a high content of hydrophilic amino acids generally having a bland taste. From present knowledge of the amino acids in common food proteins, it can be predicted that casein-, maize-, and hemoglobin-derived hydrolysates have a tendency to be more bitter, while protein hydrolysates from meat, fish, and gelatin are less bitter.

Flavors produced by enzymatic hydrolyses of protein depend on the type of protein used (animal or vegetable), the type of enzyme, and the combination of enzymes used. By selecting special enzymes, the formation of bitter peptides can to a certain extent be avoided [582].

The ability of protease to develop flavor is used to accelerate the ripening of fermented sausages [583]. The ripening time can be reduced by 30–50 % using a combination of endo- and exoproteases (Table 16). The use of proteases in fermented sausages also has an impact on the microorganism used in the fermentation process.

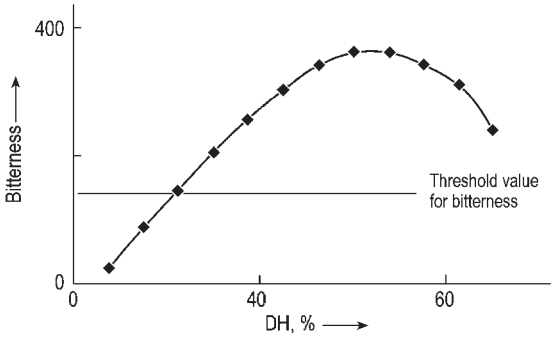

Fig. 45 Quality relation between bitterness and DH of proteins.
Source: Novo, Enzyme Information. IB-number 282-IB. Date 1982-12-23.

Table 16. Proteases used to accelerate ripening of dry fermented sausages [583]

Enzyme	Type	Source
NCCDO151 proteinase	Serine-proteinase	*Lactobacillus paracasei* subsp. *paracasei*
Pronase E	Mixture of proteinases, amino-and carboxypeptidase	*Streptomyces griseus*
Aspartyl-proteinase	Acid proteinase	*Aspergillus oryzae*
Papain	Thiol-proteinase	*Carica papaya*
Alcalase	Serine-proteinase	*Bacillus lichiniformis*
Bromelain	Endoprotease	*Ananas comosus*
Neutrase	Metalloproteinase	*Bacillus subtilis*
Fungal Protease (Protease P)	Mixture of acid, neutral and alkaline proteinase	*Aspergillus oryzae*
Flavozyme	Mixture of proteases with both exo- and endopeptidase	*Aspergillus oryzae*

5.1.5.4.2 **Lipid Hydrolysis**

Traditionally, lipase has been used to enhance the flavor of cheese, especially in strongly-flavored products such as Parmesan [583]. Lipase degrades triglycerides and liberates fatty acids, which are generally not flavors themselves (with the exception of short chain fatty acids) but precursors for flavor substances.

Lipases from different sources (Table 17) have been used to enhance flavor development in cheese and dry-fermented sausages.

Lipases show specificity for fatty acid chain length and esterification position, but it is not completely clear which fatty acid profile is ideal for the flavor profile.

Animal lipases release mainly short-chain fatty acids, whereas microbial lipases release long chain fatty acids. The unsaturated fatty acids released by lipase act as substrates for oxidation reactions and are thus precursors for flavor compounds such as aldehydes and ketones.

Table 17. Lipase for flavour development [583]

Enzyme	Type	Source
Lipase	–	Throat glands of kid goat
Lipid esterase	–	*Rhizopus arrhizus*
Lipase	1,3-specific	*Lactobacillus plantarum* MF 32
Pancreas lipase	1,3-specific	Porcine pancreas
Lipase	–	*Mucor miehei*
Lipase	–	*Candida cylindracea*
Palatase M®	1,3-specific	*Rhizomucor meihei*
Lipozyme®	1,3-specific	*Rhizomucor meihei*
Lipase AP6®	1,3-specific	*Aspergillus* spp.
Novozym® 667BG	1,3-specific	*Thermomyces lanuginosus*

5.1.5.5 Egg Powder

Egg powder has been produced since the early 1900s. One of the problems faced in connection with egg powder production is the Maillard reaction of protein and glucose in eggs. As early as the 1950s, an enzymatic process was developed to remove glucose from liquid whole egg or egg white (albumin) based on the use of glucose oxidase (E.C. 1.1.3.4) and catalase (E.C.1.11.1.6) [587]. The desugaring of egg albumin with catalase and glucose oxidase is performed at approx. 10 °C, and the pH is adjusted to 6.8–7.0 to optimize the performance of the enzymes. This pH adjustment is carried out by slowly adding citric acid solution (to prevent local protein precipitation). Acidification is followed by the addition of hydrogen peroxide. The addition of catalase converts H_2O_2 to oxygen and water (Fig. 46). Glucose is oxidized to glucone δ-lactone by glucose oxidase with oxygen consumption.

If oxygen becomes the limiting factor, more hydrogen peroxide should be added until the desired glucose concentration (0.1 %) is reached. The desugaring procedure for whole egg or egg yolk is similar to the procedure for albumin, but no acid is added to adjust the pH.

Lipase and phospholipase have recently found applications in egg processing. Egg yolk contains phosphatidylcholine (PC), an emulsifier commonly known as egg lecithin. The enzyme-modified product is derived from the catalytic hydrolysis of a free fatty acid from the PC molecule, which yields lysophosphatidylcholine (Fig. 47).

GLUCOSE OXIDASE:

$$\beta\text{-D-Glucose} + \text{Enzyme-FAD} \longrightarrow \text{Enzyme-FADH}_2 + \delta\text{-D-Gluconolactone}$$

$$\text{Enzyme-FADH}_2 + O_2 \longrightarrow \text{Enzyme-FAD} + H_2O_2$$

CATALASE

$$2\,H_2O_2 \longrightarrow 2\,H_2O + O_2$$

Fig. 46 Oxidation of glucose by glucose oxidase. Reduction of hydrogen peroxide by catalase.

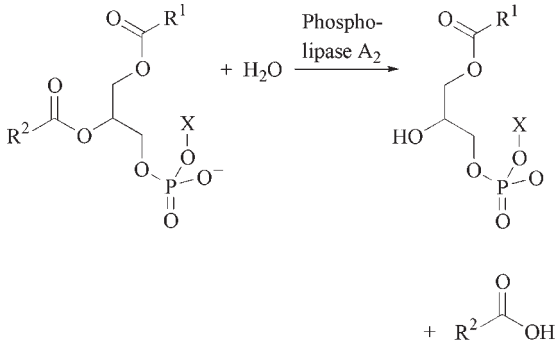

Fig. 47 Hydrolysis of phospholipid by a phospholipase A2

This structural modification improves the emulsification properties of the yolk without altering appearance or nutritional profile. Compared to traditional egg yolk, the structure of emulsions prepared with the enzyme-modified product is more stable and heat-resistant. Generally, the emulsions have greater viscosity and body and are able to emulsify more oil, often at reduced usage levels.

5.1.5.6 Oils and Fats

The commercial application of enzymes in oil and fat processing is still rather limited. This situation is expected to change over the next few years due to growing interest in milder processes and the availability of new enzymes.

Lipases are by far the most widely used enzymes in oil processing. Their natural ability to hydrolyze oils and fats can be used to enrich oil and fat products with a specific fatty acid. Lipases can also be used to rearrange fatty acids and produce fats and oils with other physical characteristics.

Phospholipase (E.C. 3.1.1.4) is commercially used for removing phospholipids during oil refining (degumming).

Lipoxygenase (E.C. 1.13.11.12) can catalyze the oxidation of polyunsaturated fatty acid.

5.1.5.6.1 Fat Splitting

The hydrolysis of fats and oils is of major importance to the oleochemical industry in the production of fatty acids and their derivatives. These products are used in soaps, detergents, and pharmaceuticals as well as in food applications. Hydrolysis or fat splitting is normally carried out under pressure at a high temperature (typically 3–6 MPa and 250 °C). However, this process is not suitable for oils with a high content of polyunsaturated fatty acids. For this type of oil, hydrolysis by an enzymatic process is of great advantage. The enzymatic process is easily carried out at 40 °C in a 50–70 % oil-in-water emulsion. To enable reuse of the enzyme, a number of processes based on immobilized lipase have been developed, making the process more economically feasible.

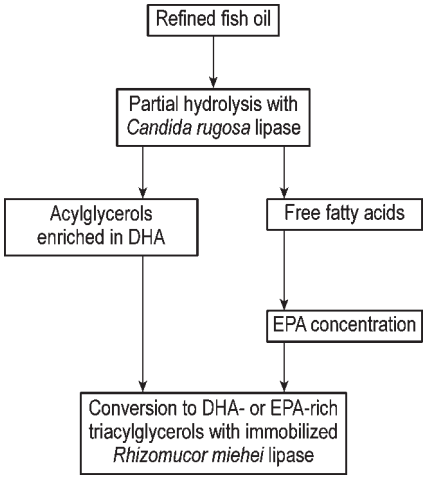

Fig. 48 Reaction scheme for the enrichment of fish oil.
Source: R. Diks, J. Bosleve in T. Uwe, T. Bornscheuer (eds.): *Enzymes in Lipid Modification,* Wiley-VCH, Weinheim 2000.

This principle is of special interest in the enrichment of long-chain polyunsaturated fatty acids from fish oil. Enzymatic hydrolysis of fish oil leads to partial glycerides high in eicosapentaoenic acid (EPA) and docosahexaenoic acid (DHA). *Candida rugosa* lipase is of special interest in this process, because this enzyme shows relatively low activity on DHA compared to EPA [588], [589]. If required, the partial glyceride high in DHA fatty acids can be re-esterified with DHA or EPA to generate triglycerides enriched with these fatty acids (Fig. 48) [590].

5.1.5.6.2 **Interesterification**

It is well-known that polyunsaturated fatty acids (PUFA) play an essential role in human nutrition and have important biomedical properties. Therefore, many physical methods have been developed for extracting and enriching these fatty acids, including crystallization, distillation, and the use of supercritical carbon dioxide. Lipase now offers an alternative, having the advantage of mild processing conditions that minimize fatty acid degradation and oxidation. These reactions rely on the principle that some lipases are able to discriminate between certain fatty acids, and that other lipases are selective for the position of the fatty acid in triglyceride moiety.

A special feature of lipases is their ability to work in nonaqueous environments. This property has been used to develop lipase-catalyzed rearrangement processes to generate tailored triglycerides that would be difficult to obtain by conventional physical and chemical methods. Lipases in nonaqueous media utilise the ''reverse'' reactions, whereby lipases catalyze esterification and rearrangement reactions under low-water conditions (Fig. 49).

These systems, however, must contain small amounts of water to keep the enzyme hydrated. Lipases from different sources differ considerably in their ability to work

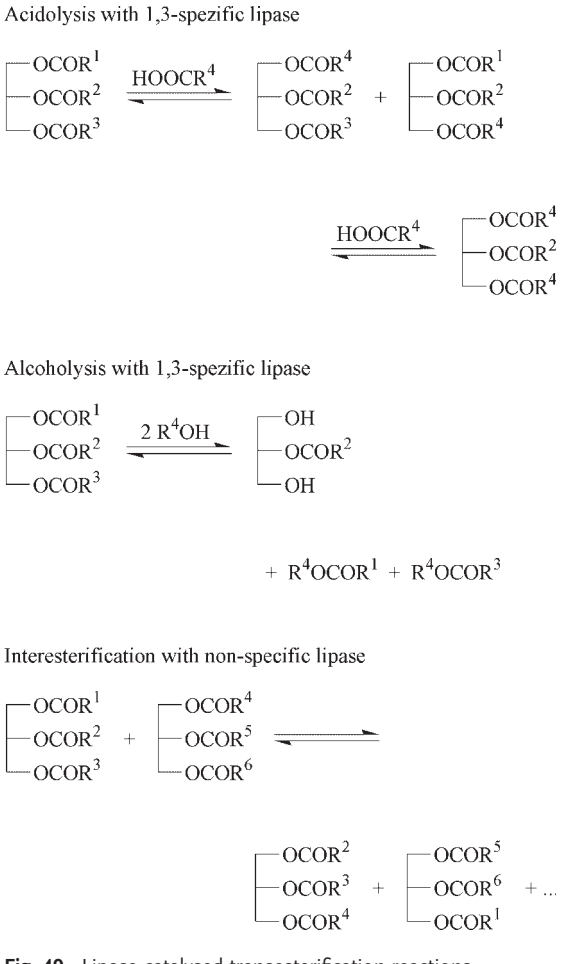

Fig. 49 Lipase catalysed transesterification reactions.
Source: R. Rastall (ed.): Ingredients Handbook, Enzymes, Leatherhead Food International, Surrey 1999.

under low-water conditions. Lipase from *Rhizomucor miehei* displays good activity in low water environments ($a_w < 0.2$) and is often used for these types of applications.

Lipase is normally immobilized for this type of reaction. Immobilization presents opportunities for reusing the enzyme, easy separation of the product from the enzyme, and running a continuous process.

The current main application of immobilized lipase in nonaqueous media is the production of tailored triglycerides for use in confectionery fat and nutritional supplements (Fig. 50).

In the production of cocoa butter substitute (Fig. 51), a mixture of high-oleate sunflower oil (OOO) and stearic acid is passed through a packed bed reactor with immobilised *R. miehei* lipase. The fatty acid exchange occurs in the sn-1 and sn-3

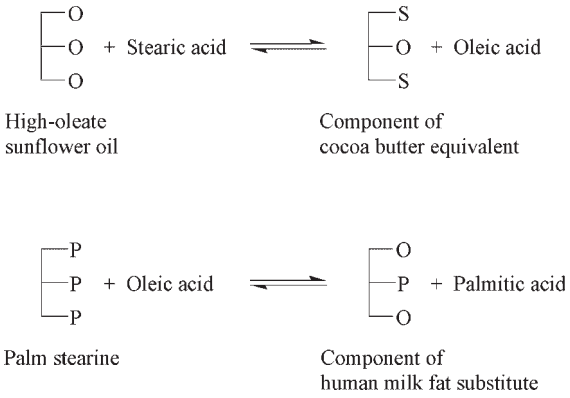

Fig. 50 Reactions catalysed by a 1-,3-specific lipase.
Source: R. Rastall (ed.): Ingredients Handbook, Enzymes, Leatherhead Food International, Survey 1999.

positions of the triglyceride to generate SOS, which is a cocoa butter equivalent. The mixed fatty acid (oleic and stearic acid) is removed and can be hydrogenated and reused. Unwanted triglycerides can be removed by crystallisation and recycled.

Lipase-catalysed interesterification can also be used to improve the nutritional properties of dietary fat. An example of this is the production of tailored triglyceride for use as a nutritional supplement in infant formula [592]. Human milk fat contains a high proportion of unsaturated fatty acids at the sn-1 and sn-3 positions, with palmitic acid in the sn-2 position. Lipase from the human pancreas releases the fatty acid in the

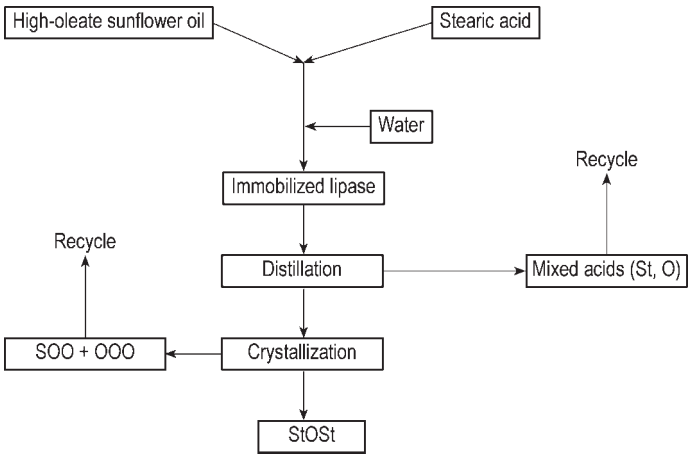

Fig. 51 Schematic production of cocoa butter equivalent (CBE).
Source: R. Rastall (ed.): *Ingredients Handbook, Enzymes*, Leatherhead Food International, Surrey 1999.

sn-1 and sn-3 positions and produces 2-monoacylglycerol. The body readily absorbs these components.

Human milk substitute based on vegetable oils mainly has saturated fatty acid in the sn-1 and sn-3 positions, which, when released by pancreatic lipase, tends to form calcium soaps and is poorly absorbed by the body. Enzymatic interesterification makes it possible to produce a triglyceride with unsaturated fatty acid in the sn-1 and sn-3 positions and palmitic acid in the sn-2 position (OPO). In this process, palm oil high in palmitic acid is treated with oleic acid to form OPO triglyceride.

Similar techniques can be used to incorporate medium-chain fatty acids (C_8–C_{10}) in specific positions in a triglyceride to generate lipids with improved nutritional properties.

Enzymatic interesterification of fats and oils for margarine and shortening has now become an attractive alternative to the traditional interesterification reaction using high temperature and inorganic catalyst. The enzymatic process is more attractive because it is carried out at 40–70 °C, a temperature range in which no *trans* fatty acids are formed.

5.1.5.6.3 **Esterification**

Enzymatic esterification for the production of diglyceride has attracted interest due to the nutritional aspects of 1,3-diglycerides. Commercial products containing high levels of diglyceride are on the market in Japan, where they have increased their share of the cooking oil market. Use of 1,3-diglyceride in cooking oil contributes to a lower serum lipid level, as pancreatic lipase hydrolyzes 1,3-diglyceride into free fatty acids and glycerol, while no 2-monoglyceride (a building block for serum lipid) is formed.

5.1.5.6.4 **Oil Degumming**

Crude vegetable oil such as soy oil contains phospholipids, which are normally removed by chemical and physical methods. During the last decade there has been a tendency to move away from chemical (caustic) refining processes and use only physical processes to reduce the amount of effluent. For reliable physical processing, it is essential that the phospholipid content of vegetable oil is reduced to a very low level (< 15 ppm P) before bleaching and deodorization take place.

Crude vegetable oil has a phospholipid content of up to 3 %, depending on the type of seed and extraction procedure. The first stage of the refining process, water degumming, removes most of these phospholipids, but about 0.6 % nonhydrating phospholipid still remains in the oil. A number of physical methods have been developed to lower the phospholipid content further.

A newer method based on enzymatic hydrolysis of phospholipids by phospholipase A2 has, however, provided an interesting alternative. Phospholipase A2 catalyzes the hydrolysis of fatty acids in the sn-2 position and forms the more water-soluble lysophospholipid, which is more easily washed out of the vegetable oil [593]. This method gives an oil with a residual phosphorus content of less than 10 ppm.

Phospholipase A2 is also used for the production of lysolecithin, which has superior emulsification properties [591]. Lysolecithin is used in the food, cosmetic and pharmaceutical industry.

5.2
Enzymes in Nonfood Applications

5.2.1
Enzymes in Household Detergents

Household detergents need to remove a broad range of complex soil from different fiber surfaces. Soil and stain components with good water solubility are easily removed during the cleaning process. Most other stains are partially removed by the surfactant/builder/bleach system of a detergent, although the result is often unsatisfactory, depending on the washing conditions.

In most cases a suitable detergent enzyme aids the removal of soils and stains. Whereas the detergent components have a purely physicochemical action, enzymes act by degrading the dirt into smaller and more soluble fragments. However, to remove a stain totally still requires the joint action of the enzyme, the surfactant system, and mechanical agitation.

5.2.1.1 Historical Development

The first enzyme-containing detergent was introduced to the household market as early as 1913 when Röhm & Haas in Germany added the protease trypsin, extracted from pig pancreas, to their detergent Burnus, utilizing a patent of Otto Röhm [594].

Because the activity and stability of trypsin are moderate in the presence of typical detergent ingredients, the detergent enzyme concept did not really catch on until 1963, when Novo developed and marketed a much more alkali- and builder-tolerant bacterial protease called Alcalase [595].

Small detergent producers in Switzerland (BIO-40 from Gebrueder Schnyder) and the Netherlands (Biotex from Korman and Schulte) were the pioneers in the commercial utilization of Alcalase, which initially was only considered useful for washing bloodstained laundry from hospitals and slaughterhouses.

Gist-Brocades followed a few years later with the alkaline Maxatase protease (1965).

Within five years of their introduction into detergents, more than 50 % of all heavy-duty laundry detergent for domestic use in Europe contained protease enzymes, compared to only 15 % in the USA.

1970–1980 The rapid growth of the enzyme-containing detergents was temporarily discontinued in the early 1970s when industrial hygiene and safety problems became evident. Significant improvements in detergent manufacturing techniques, the development of low-dust encapsulated enzyme prills, and improvements in industrial hygiene practices and procedures resolved the issue. Since then enzyme sales have again increased steadily.

The first α-amylase for detergents (Termamyl, from Novozymes) was developed in the early 1970s and has increased tolerance to temperature and alkali.

In the mid-1970s the first liquid detergents were introduced in Europe and the USA using specially developed liquid enzyme preparations.

1980–1990 Washing conditions, especially in Europe, gradually shifted further to lower temperatures (from 60 to 40 °C), reduced water quantities, and shorter washing times, and major changes in formulation took place (phosphate-free detergent, activated bleach). New proteases better adapted to these working conditions and new enzyme activities became available.

The first detergent cellulase Celluzyme (Novozymes), a multicomponent cellulase, was developed for improved cleaning and stain removal.

New genetic technology and protein engineering techniques have been introduced for designing further optimized enzymes for detergent conditions or to achieve acceptable production economy. In this way the first bleach-compatible protease (Maxapem, Genencor) and the first detergent lipase (Lipolase, Novozymes) have been developed.

1990–2000 New detergent markets opened up in India, South America, Eastern Europe, the former Soviet Union, and China. Because of the specific washing conditions in these regions, such as cost constraints, low detergent concentrations, very low washing temperatures, and presoaking, new enzyme developments were again needed to cope with these difficult washing conditions.

Second-generation detergent proteases, amylases, and lipases were developed by using protein engineering and genetic techniques for further improved performance under difficult washing conditions (low temperature, single wash effects (cleaning, stain removal, whitening, softening, other, achieved with one single wash treatment.)) and with improved compatibility with new detergent technology (improved activated bleach) and for broader application (e.g., in automatic dish washing, liquid detergents, tablets, gel pads).

Up to the 1980s enzymes were primarily developed and used for aiding stain removal and cleaning of fabrics. Since the mid-1990s also enzymes with specific fabric-care effects (color brightening and fabric softening) appeared on the market. (Carezyme and Endolase monocomponent cellulases from Novozymes) as well as enzymes delivering new benefits for stain removal and cleaning (Mannaway, Novozymes).

Table 18 lists enzyme products for detergents by type and manufacturer.

5.2.1.2 Laundry Soils

Laundry detergents need to remove a broad range of complex soil from different fabrics and fiber types under varying washing conditions. The most frequent soiling on garments and fabrics include all kind of body soils on shirts, underwear, and socks; a variety of food and beverage stains; oily soils; and particulate stains.

The body soils are complex mixtures secreted by sebaceous and sweat glands in the skin or can stem from fecal, urinal, or vaginal secretions. These soils are mixtures of lipids, proteins, glycoproteins, blood and blood degradation components, skin epidermal debris, salts, and particulate materials collected from the environment. The fresh lipid fraction contains polyunsaturated fatty acids (linoleic and linolenic acid) and their triglycerides and unsaturated hydrocarbons (squalene), which can be rapidly autoxidized when exposed to air, heat, and moisture. The lipid autoxidation reactions yield high molecular mass complex compounds with lipids, proteins, and carbohydrates. This results in a gluelike matter that entraps skin debris and particulate soil

Table 18. Enzyme products for detergents by type and manufacturer (2002)

Protease						**Amylase**			**Lipase**	**Cellulases & hemicellulases**
Type	Neutral	Alkaline	Highly alkaline	Bleach-stable	Cold-water	Conventional	Bleach-stable	Cold-water		
Merchant market										
Novozymes	X	X	X	X	–	X	X	X	X	X
Genencor International	–	–	X	X	X	–	X	–	–	X
Captive market										
Kao (Japan)	–	–	X	–	–	X	–	–	–	X
Henkel	–	X	X	X	–	–	–	–	–	–

components. This aged body soil is tough to remove from fabrics and tends to build up on the fabric during the lifetime of the garment.

The food stains encountered in laundry are also complex soils. They contain substantial amounts of animal and vegetable fats and oils; proteins, e.g., from eggs and meat products; carbohydrates such as sugars, starches, and other complex carbohydrates; residues from plants, e.g., from grass or spinach; and mixed oxidation products. Chemical and enzymatic browning of food products occurs during storage and preparation of foods, mostly by formation of dark-colored Maillard reaction products from carbohydrates and proteins at higher temperatures. Natural dyes and pigments present in foodstuffs such as carotenes and chlorophylls add to the deep coloring of some food stains. In particular carotenes are highly soluble in oils and fats, e.g., in tomato-based sauces, and this adds to the difficulty of removing these stains from fabrics. Certain dairy-based foodstuffs can contain substantial amounts of less common carbohydrate-based gums for stabilizing and texturizing the food, such as carrageenan, guar, and locust bean gums, and these are often difficult to remove from fabrics by detergents and even by current amylases.

Colored stains from beverages such as wines, vegetable and fruit juices, and tea contain complex mixtures of natural dyes and pigments such as anthocyanine and colored polyphenolic compounds, sugars, and organic acids. These materials can eventually be polymerized to higher molecular mass substances that are more difficult to remove from fabrics and fibers. Coffee can in addition contain substantial amounts of browning products formed during roasting of the coffee beans.

Particulate soiling can consist of insoluble metal carbonates, oxides, silicates (clay), carbon black, dust, and humus collected from the environment, e.g., airborne soot on shirt collars or garden soil on socks.

Organic soils on fabrics also can stem from the laundry process and fabric care treatments such as surfactant and builder residues, fabric softeners, and from ironing aids, e.g., starches.

However, the frequency and type of soiling on garments is also geographically different, owing to different consumer habits. Figure 52 exemplifies the differences in soiling incidences in a typical north and south European country.

5.2.1.3 Detergent Composition and Washing Process

5.2.1.3.1 Washing Process

Any washing process is an interplay between the washing equipment used; the materials entering the process, i.e., the detergent components and additives; water quality; the wash load composition; and the washing procedures followed, e.g., time, temperature, agitation. The equipment and wash procedures for laundering in three principal geographical areas are summarized in Table 19.

As can be seen the average laundry conditions can vary significantly across the main geographical areas. Within each region also local washing habits exist such as separate addition of hypochlorite-based bleaches in southern Europe; extended boil washing as in Turkey; and multicycle use of the washing water in Japan.

All of this does not facilitate the formulation of detergent effective in the broad range of usage conditions. Often detergent compositions are adapted to these local washing habits.

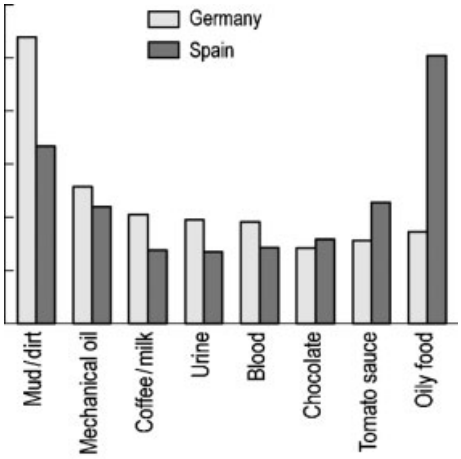

Fig. 52 Soiling in Germany and Spain: incidence ranking [596]

Not all detergent components are effective over the whole range of temperatures from 4 °C up to boil wash and over the range of detergent concentrations. Here enzymes can become important detergency boosters especially for washing in the low temperature range and under conditions of low product usage.

Table 19. Washing equipment and procedures [597]

	USA/Canada	Japan	Western Europe
Machine type	Top-loaded agitator	Top-loaded impeller	Front-loaded rotating drum
Wash time, min	12	10	20–90
Wash load, kg	2–3	1–1.5	3–5
Main wash water volume, L	35–80	30–45	8–15
Main wash temperature, °C	10–50	10–40	30–90
Water hardness, ppm $CaCO_3$	low (100)	very low (50)	high (250)
Recommend product usage, g regular detergent/L	1–5	1–3	5–10

5.2.1.3.2 Detergent Compositions

Detergent compositions also vary by geography and from country to country.

The household laundry detergents available to consumers can be roughly divided into five segments according to the physicochemical properties of the wash solutions prepared from the detergents:

- Near neutral pH, low ionic strength detergents are mostly liquid detergents having solution pH from 7.5 to 9. They contain no bleach and only low levels of salts. Dilute and concentrated liquid detergents are marketed. The latter type of liquid detergent contains only small amounts of water (< 10 %).

- Mildly alkaline pH, medium ionic strength detergents are typically compact powder detergents from Japan and regular powder detergents from the USA. Their solution pH is about 9 and they contain no bleach.
- Alkaline pH, high ionic strength detergents with a solution pH from 9.5 to 11, and with an activated bleaching system, e.g., European regular powder detergents, They also contain sodium sulfate as a filler, and builder systems, e.g., sodium triphosphate or zeolites and sodium carbonate. High dosages are used, which give a wash liquor with a high ionic strength.
- Alkaline pH, medium ionic strength detergents with bleach are represented by European compact powder detergents, which have gained a large market share during the early 1990s They are compact as a result of removal of most or all of the sodium sulfate; solution pH is from 9.5 to 11.

The household detergents are mostly delivered as low-dusting powders or granulates or as liquids with low or high viscosity (gels). Laundry bar soaps are still popular in certain countries (South Europe, Latin America).

More recently (2000) detergents in tablet form or as a single-dose pouch containing liquid detergent have been launched with success in Europe and North America by Unilever and P & G [598–600].

Practically all of these laundry detergents contain enzymes, although some specialty flanker brands are free of any enzymes. Some specialty laundry detergents combine fabric-cleaning and fabric-care technologies delivered during the laundering process without use of additives (two-in-one products).

Examples of laundry detergent compositions are given in Table 20.

Table 20. Examples of laundry detergent composition (in wt %) [601]*

	Liquid	Liquid, concentrated	Granular low bleach, USA/JP	Granular high bleach, Europe	Granular compact, Europe	Tablet
Surfactants	10–50	10–30	7–22	10–20	10–20	15–25
Builders	0–10	5–15	20–70	20–40	20–45	15–30
Bleaches	0	0	0–5	11–27	13–28	10–25
Enzymes	0–6	0–1	0–1	0.3–0.8	0.5–1.5	0–3
pH of 1 % solution	7.5–9	7.5–9	9.5–11	9.5–11	9.5–11	9.5–11

*Surfactants: linear alkyl benzene sulfonates, alcohol sulfates, alcohol ether sulfate, alcohol ethoxylates, cationic surfactants. Builders: zeolites, sodium carbonate, sodium silicate, sodium citrate, sodium tripolyphosphate (STTP), sodium nitrilotriacetate (NTA), polycarboxylates. Bleaches: sodium perborate, sodium percarbonate, tetraacetylethylenediamine (TAED), nonanoyl benzene sulfonate (NOBS) and others. Additives (not listed): foam regulators, stabilizers, processing aids, optical brighteners, antiredeposition agents, corrosion inhibitors, perfumes, colorants, fillers such as sodium sulfate in powders or water in liquid detergents.

5.2.1.4 Enzyme-Aided Detergency and Soil Removal

Soil and stain components with good water solubility are easily removed during the cleaning process. All other stains are partially removed by the surfactant/builder/bleach system of a detergent, although the result is often unsatisfactory, depending on the washing conditions.

In most cases a suitable detergent enzyme may help the removal of soils and stains. Contrary to the purely physical action of the surfactant system enzymes work by degrading the dirt into smaller and more soluble fragments. However, to remove a stain totally still requires the joint effects of the enzyme, surfactant system, and mechanical agitation.

Proteases and amylases can remove protein and starch stains, respectively. Fats and oils are generally difficult to remove at low wash temperatures ($< 40\ ^{\circ}C$) by conventional detergents. By using lipases, it is possible to improve the removal of fats/oils of animal and vegetable origin even at temperatures at which the fatty material is in a solid form.

Particulate soils can be difficult to remove, especially if the particle size is small. Removal of particulate soil from cotton fabric can be improved by use of a cellulase that removes cellulose fibrils from the surface of the yarn.

Various kinds of dirt may adhere to textile surfaces via a glue of proteinaceous, starchy, or fatty material. In such cases of anchored dirt, an enzyme may assist in removing the dirt even though it does not attack the dirt directly. Compounds from several of these classes are intimately mixed in combined soiling, e.g., human sebum on shirt collars, cocoa milk, gravy, or chocolate.

Bleachable stains are the only group of stains for which no enzyme product for detergent applications has been marketed so far. However, the patent literature indicates that efforts are being made by the major enzyme suppliers and detergent producers to develop an enzymatic bleaching system for detergents.

5.2.1.5 Detergent Enzyme Performance Evaluation and Screening

Not all enzymes with a potential for stain degradation and/or removal are suitable for inclusion in detergent products. Enzyme performance is influenced by factors like detergent solution pH, ionic strength, wash temperature, washing time, detergent composition, and mechanical handling. Detergent surfactants, bleach systems, and other enzymes can influence both performance and stability.

The enzymes must have a broad enough substrate specificity because the average load of dirty laundry contains the enzyme substrate in a multitude of forms. For example, in the case of proteases the protein substrate may have been oxidatively modified or complexed with carbohydrates.

A detergent enzyme must be active at the pH of detergent solutions (between 7 and 11) and at the relevant wash temperatures (4 to 60 $^{\circ}C$). Importantly, the enzyme must be compatible with detergent components such as surfactants, builders, bleaches, and other enzymes, not only during the wash process but also upon storage when incorporated in the detergent. In particular, such an enzyme must be resistant toward denaturing surfactants and bleach and towards protease degradation under these conditions.

Enzymes can be screened by assay of their activity on soluble substrates under conditions different from those encountered in a realistic wash. Enzyme screening often uses spectrophotometric assays with chromophoric soluble substrates, and the procedure can be fully automated for high-throughput analysis. The assay results can

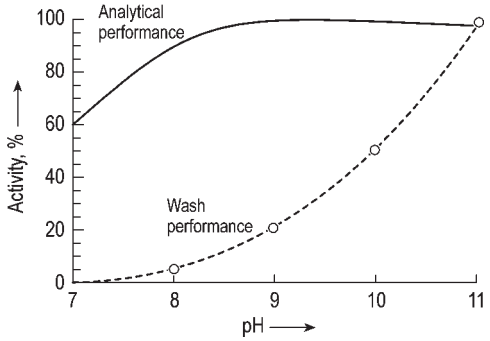

Fig. 53 Example of deviating analytical activity on soluble substrates and laundry scale cleaning performance data [602]. Savinase protease: analytical performance on denatured hemoglobin, 25 °C, 10 min.; wash performance on grass stain, 30 °C, 10 min.

indicate the enzyme's performance on soluble substrates with respect to pH and temperature variations and with respect to enzyme compatibility with detergent components or enzyme-inhibiting conditions, etc. However the analytical data thus obtained with soluble substrates are not necessarily representative of the wash performance of the enzyme, and often low correlation is achieved with performance results obtained with enzymes under realistic laundry conditions (Fig. 53).

It is also important to realize that enzymes can have significant performance effects on substrates that apparently are unrelated to their target substrates. For example, proteases can deliver significant performance benefits on removal of protein-based bloodstains but also on grass stains on fabrics. Therefore real wash trials using a broad range of consumer relevant stains remain necessary to completely evaluate the wash performance of detergent enzymes.

Wash-performance assessments often use pre-soiled test pieces. For example the commonly used stains for protease evaluation are milk, blood, and grass applied to cotton or synthetic fabrics. These stains also may contain particulate matter, e.g., carbon black, as part of the stain matrix. These standardized test materials are available ready-to-use from a number of research and testing institutes in Europe and the USA, e.g., Center for Test Materials, Vlaardingen, Holland; Wascherei Forschungs Institut, Krefeld, Germany [603]; EMPA St. Gallen, Switzerland [604] (Table 21); Instituut voor Reinigingstechnieken TNO, Delft, Holland [605]; Test Fabrics, Middlesex, New Jersey [606] and SDL Textile Innovators [607]. Alternatively, enzyme manufacturers can supply stain preparation procedures, or specific stain material can be made in-house by the enzyme formulator. Finally, naturally soiled and stained fabrics obtained from households can be used for more realistic performance assessment.

Laboratory wash trials are usually conducted in small-scale models of washing machines. The Terg-o-tometer [608] simulates the top-loaded U.S. type of washing machine, and the Launder-o-meter [609] or Linitest [610] simulates the European drum-type machine [611]. These small-scale models use 0.2 to 1 L of wash solution. Both have temperature-controlled heating and a timer. The wash solution is agitated with an

Table 21. Examples of standard performance test materials from EMPA [604]

Soil	Cotton fabrics	Polyester/cotton blend
Carbon black/olive oil	101	104
Blood, milk, carbon black	116	117
Lipstick	141	142
Make-up	143	144
Starch	161	162
Tea	167	168
Grass	164	
Blood	111	
Wine (red)	114	

impeller in the Terg-o-tometer and by rotation of a closed pot in the Launder-o-meter or Linitest equipment. The test stain of defined size is laundered in the wash solution with controlled detergent concentration and solution pH and with or without the enzyme under test. The enzyme concentration in the initial screening tests can be in the range of 1–100 mg/L of enzyme degradable protein.

The evaluation of the enzyme effects on the test pieces can be made visually by grading by an expert panel under controlled illumination conditions and by instrumentally measuring the reflectance of light under specified conditions. Typically, the intensity of light remitted R at 460 nm when illuminating the test pieces with a standardized daylight source is expressed as a percentage, ΔR or ΔE, of the intensity of incident light at the same wavelength. The ΔR or ΔE value is then a measure of the enzyme effect; it is defined as the difference in R between fabrics washed with and without enzyme. The R value is known to correlate well with the visual impression of whiteness of the fabric. Depending on type and degree of soiling, differences in R or E of 2–3 units are detectable by the human eye.

Digital imaging systems can be used for the instrumental evaluation of the performance effects in larger routine performance screening of enzymes using standardized stains and small-scale laboratory performance test methods.

For the complete evaluation of detergency effects of an enzyme two-steps can be considered: (1) ''Primary washing effects'' (cleaning efficacy) refer to the removal of soil and stains after one wash. Testing is carried out as described above using either artificially soiled test fabrics or naturally soiled laundry. (2) ''Secondary washing effects'' (effects after repeated washing) refer to potential multiwash performance (e.g., with Lipase) or to the detection of damage such as loss of tear strength, incrustations (ash residues), and graying. The latter evaluations are usually based on 25 or 50 washes, including a control fabric.

A convenient way to report detergent enzyme performance is the use of dose–response curves. Typically, the enzyme-related performance increases significantly in the lower enzyme concentration range, but reaches a maximum level at higher enzyme doses.

Figure 54 compares protease performance as a function of enzyme dosage. The performances at 60 °C reaches a plateau at an enzyme dosage of approximately 0.04 wt % of the enzyme granulate in the detergent. At this enzyme concentration, the

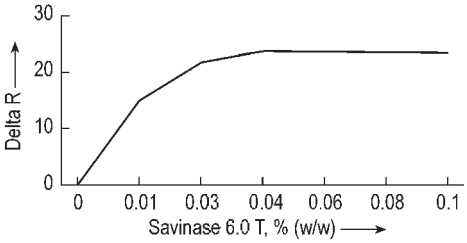

Fig. 54 Example of a dose response curve for Savinase 6.0 T protease (Novozymes) in a powder detergent, 4 g/L in a Launder-o-meter test at 60 °C for 40 min. Source: Novozymes.

fabric is clean and the stain can be totally removed, i.e., the measured *R* value is identical to that of an unsoiled test piece washed under the same conditions.

The extent to which the enzyme is able to remove stains and soils from the fabric depends on the detergent composition, the wash temperature, wash solution pH, washing time, wash load, etc. Enzyme wash performance varies between liquid and powder detergents and with the composition of the soiling. Figure 55 shows that the fabric composition also influences the stain-removal profile for the same soiling (EMPA 117 on polyester–cotton and EMPA 116 on cotton only).

Further scale reduction of the performance evaluation of detergent enzymes has been achieved, and high-throughput performance screens for enzymes are being developed. This type of performance assessment uses multiwell titer plates, each

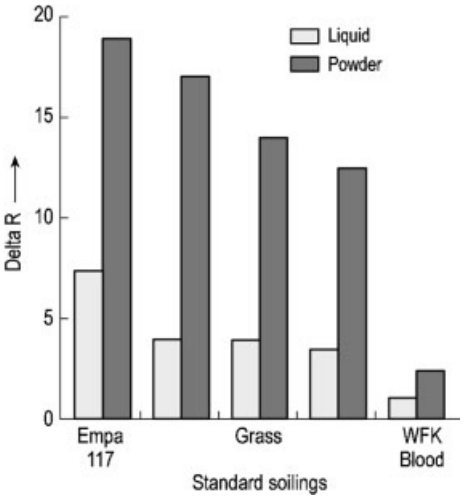

Fig. 55 Stain removal performance of a U.S. liquid detergent (0.4 % Savinase 16L) and a U.S. powder detergent (0.8 % Savinase 8.0T) on different standard soilings in a Terg-o-tometer operating at 6°dH water hardness, 20 °C, 10 min. EMPA 117 (milk, blood, and ink on polyester/cotton); AS 10 (milk, oil, and pigments on 100 % cotton); grass (on 100 % cotton); EMPA 116 (milk, blood, and ink on 100 % cotton); WFK blood soiling (on 100 % cotton). Source: Novozymes.

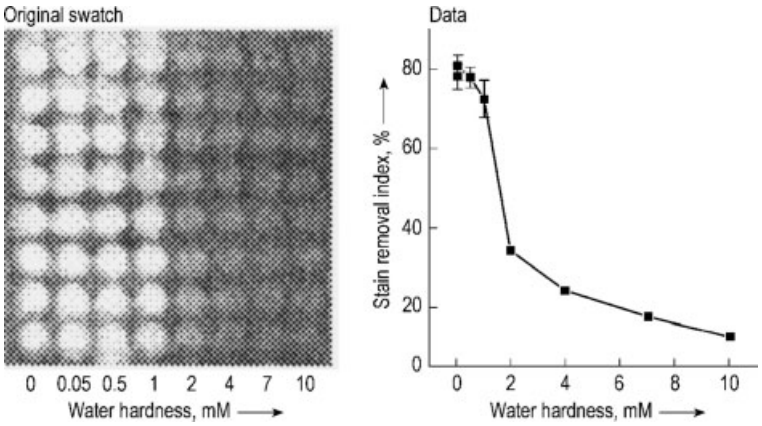

Fig. 56 High-throughput performance screen for detergent enzymes. Enzyme performance on grass stain is evaluated at one enzyme level versus the hardness profile of the wash solution (0–10mmol Ca^{2+}). Left: the multiwell grass stain after treatment; right: instrumental evaluation of the multiwell grass stain. Source: unpublished data courtesy of G. Bechmann, C. Joos, S. Cooremans (Procter and Gamble Eurocor).

holding milliliter volumes of wash solution under controlled temperature conditions (Fig. 56). A realistic level of agitation of the solution is assured with miniaturized stirrers. Using optimized test conditions a good correlation with real laundry conditions can be achieved. The performance can be evaluated visually or preferably instrumentally using titer plate readers and software that are commonly used in protein chemistry. Such miniaturized performance assessment has many advantages such as very low consumption of enzyme, on the order of micrograms versus milligrams in other small-scale tests, and the high-speed evaluation of many performance conditions in a multiwell experiment.

The final conclusion on the performance of a detergent enzyme candidate must be confirmed in a realistic laundry appliance using realistic wash loads and with wash programs at various wash temperatures and detergent usage versus water hardness. Finally these performance tests can be repeated in all important geographical or local washing conditions to confirm the robustness of the enzyme to the wash conditions.

5.2.1.6 **Enzyme Types**

5.2.1.6.1 **Proteases**

Proteases hydrolyze proteins into smaller fragments, i.e., peptides or amino acids. In a detergent context, protein stains on fabric (laundry) or hard surfaces (e.g., in dishwashing) are degraded into fragments which can be removed or dissolved by other components of the washing liquor.

Proteases are well-established ingredients in most household laundry detergents worldwide, except in some developing countries. They are used to remove stains such as blood and grass. The effects of proteases are very clear, and the enzyme cost, considering the effect, is relatively low. On some food stains, e.g., cocoa, a combined effect with amylases or lipases may be observed.

Table 22. Examples of commercially available detergent proteases (Novozymes) [613–617]

Product name	Microorganism	pH application range	Temperature application range
Alcalase	*Bacillus* species	6–10	10–80
Esperase	*Bacillus* species	7–12	10–80
Everlase	GM *Bacillus* spp.	8–11	15–80
Savinase	GM *Bacillus* spp.	8–11	15–75
Durazym	GM *Bacillus* spp.	8–11	15–70

Most of the commercial detergent proteases are subtilisins and are classified as such in the Chemical Abstract Service. The Enzyme Classification number is E.C. 3.4.21.62. The name subtilisin derives from the name of the bacterial species, *Bacillus subtilis*, from which the protease was first isolated. Subtilisins are extracellular serine endo-peptidases, and the term also now covers proteases isolated from related *Bacillus* species.

The first protease of this type was subtilisin Carlsberg from *Bacillus licheniformis*, isolated in 1947 (also known as subtilisin A, Alcalase Novo). Later, in 1954, subtilisin was also isolated from *B. amyloliquefaciens*, also known as subtilisin BPN′, Nagase, Primase, Bacterial Proteinase Novo, Sub Novo.

The commercial detergent proteases are very similar in structure, and there are no fundamental differences between them. They are all characterized by very broad substrate specificity and mainly differ in temperature optimum, pH optimum, bleach sensitivity, and Ca ion demand. Some are highly alkaline, i.e., have a maximum activity in the high pH range, such as Maxacal and Savinase. Others are low-alkaline, e.g., Maxatase, Alcalase, and Subtilisin Novo (BPN′). Table 22 presents examples of commercially available protease and application ranges. They all have a molecular weight between 20 000 and 30 000, and have a serine residue in the active site of the enzyme. Figures 57 and 58 illustrate the pH and temperature profiles of some commercial low- and high-alkaline protease in analytical assay on dimethylcasein (DMC) substrate.

Protease performance can be strongly influenced by detergent pH and ionic strength. Surfactants influence both protease performance and stability in the wash solution. In general, anionic surfactants are more aggressive than amphoteric surfactants, which in turn are more aggressive than nonionic surfactants.

All detergent proteases are destabilized by linear alkylbenzenesulfonate (LAS) and alkyl sulfates (AS), the most common types of anionic surfactants in detergents. The higher the LAS concentration and wash temperature, the greater the inactivation of the enzyme. The presence of nonionic surfactants or ethoxylated alkyl sulfates, however, counteracts to some extent the negative effect of LAS. Since almost all detergents contain some nonionic surfactant or ethoxylated AS, the stability of proteases in a washing context is not problematic.

Phosphate builders such as sodium triphosphate and non-phosphate builders such as zeolite and citrate remove free calcium from the washing solution. Co-builders such as nitrilotriacetic acid and polycarboxylates also may be incorporated into the detergent formulation. Wash performance of detergents decreases with increasing calcium

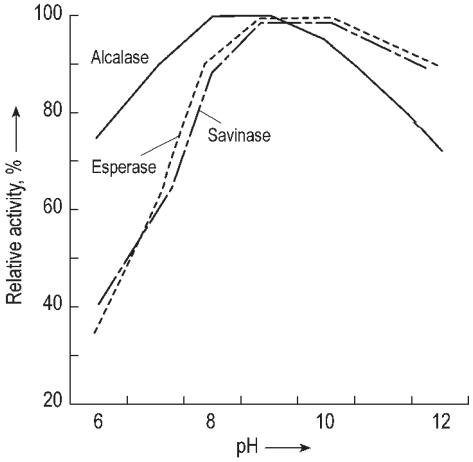

Fig. 57 pH-dependent activity of different detergent proteases at 25 °C, 10 min reaction time, DMC substrate [614–616].

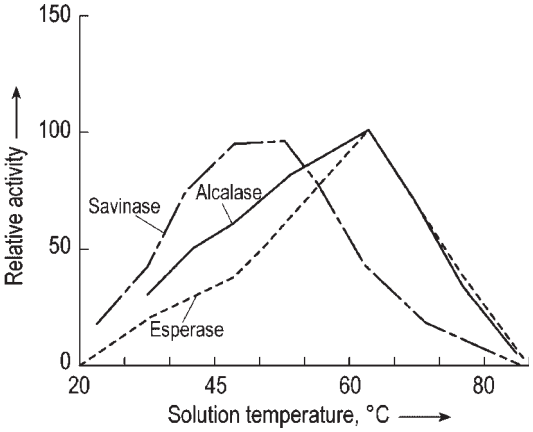

Fig. 58 Temperature-dependent activity of different detergent proteases in solution at pH 8.5 (Alcalase) and 10.1 (Savinase, Esperase), 10 min reaction time, DMC substrate [614–616].

concentration. Protease performance varies, but high calcium concentrations tend to reduce protease performance. Therefore, it is an advantage to add a builder system to the detergent. Proteases need a small amount of calcium for the sake of stability, but even with the most efficient builder systems, stability during washing is generally not a problem.

Bleach systems in laundry products can also oxidize proteinaceous stains on fabric, often making them more difficult to remove. Detergent proteases can partly counteract

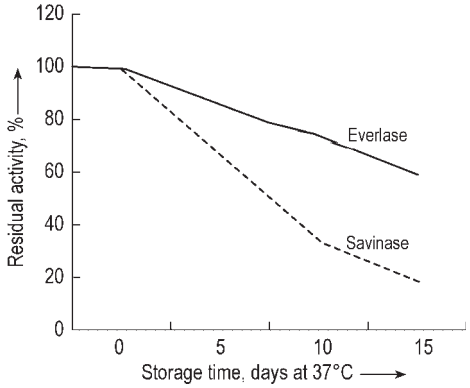

Fig. 59 Storage stability of bleach-stable protease (Everlase, Novozymes) in European powder detergent with activated bleach system. Storage at 37 °C, 70% R.H., open vial [613].

this negative effect of the bleach system. Most detergent proteases are stable during the wash cycle in the presence of such oxygen-based bleach systems.

However, storage stability in detergents containing bleach may be a problem with the established detergent proteases. In particular, the amino acid methionine in the protease molecule can be oxidized during storage by the bleach system in powder detergents, which leads to inactivation of the protease. For example the Savinase detergent protease contains methionine residues in three positions, one of which is next to the active site serine. In new protein-engineered proteases introduced onto the market the most bleach-sensitive amino acid, i.e., methionine close to the active site, is replaced with other amino acids insensitive toward oxidation. This slight change in the molecular structure significantly increases the storage stability in detergents containing bleach. Bleach-stable proteases developed by using protein engineering include Everlase (one amino acid change from Savinase) and Purafect OxP, which are superior to Durazym and Purafect with respect to washing performance in bleach-containing detergents. In addition, the bleach-stabilized proteases also usually have better storage stability when formulated in bleach-containing detergents (Fig. 59).

Chlorine bleach (sodium hypochlorite, NaOCI) is not incorporated into laundry detergents themselves, but can be used as a separate additive to the wash, as is common practice in some parts of the world. Hypochlorite bleach in excess of 200 ppm in the normal wash can quickly oxidize enzymes, resulting in significant loss of enzyme activity. Bleach-stabilized proteases can survive low concentrations of NaOCl, as depicted in Figure 60.

5.2.1.6.2 **Amylases**

Commercial laundry amylases comprise the α-amylase from *Bacillus amyloliquefaciens* and the heat-stable α-amylase from *Bacillus licheniformis* and from *Aspergillus* species.

α-Amylase (E.C. 3.2.2.1) catalyzes the endo-hydrolysis of 1,4-alpha-D-glycosidic linkages in polysaccharides containing three or more 1,4-α-linked glucose units. The enzyme acts on starches, glycogen, and oligosaccharides in a random manner,

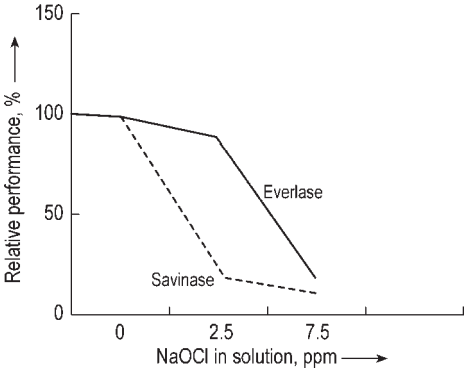

Fig. 60 Performance activity of a bleach-stable protease (Everlase, Novozymes) versus parent protease (Savinase, Novozymes) in the presence of chlorine bleach. Grass stain removal, Terg-o-tometer, U.S. HDP at 1 g/L, pH 9.3, 40 °C, 10 min, 10° dH waterhardness [613].

liberating reducing groups. α-Amylases are characterized by attacking the starch polymer in an endo fashion, randomly cleaving internal α-1,4 bonds to yield shorter, water-soluble dextrins. They are the preferred type of amylase for laundry detergents, and are included in both powder and liquid formulations in many countries. α-Amylases boost overall detergent performance at lower wash temperatures and with milder detergent chemical systems.

Other starch-degrading enzymes of potential interest are β-amylases (E.C. 3.2.1.2) and pullulanase (E.C. 3.2.1.41), but these have not been commercialized on industrial scale for detergent application. β-Amylases differ from α-amylase in that they hydrolyze 1,4-α-D-glycosidic linkages, removing successive maltose units from the nonreducing of the polysaccharide chain. This exo-type activity is not as efficient for stain removal. Pullulanase is a starch-debranching enzyme and hydrolyzes the 1,6-α-D-glycosidic bonds in amylopectin and pullulan, debranching the amylopectin. A mixed amylase and pullulanase enzyme system can accomplish a more complete degradation of starch.

Table 23. lists some application conditions for α-amylases for detergent applications.

α-Amylases catalyze the degradation of starch-containing stains, and improve cleaning by hydrolyzing the starchy glue that binds other dirt and stains to fabric.

Table 23. Commercial amylases (Novozymes) [618], [621], [623], [624]

Product name	Microorganism	pH application range	Temperature application range
Termamyl	GM *Bacillus* species	6–11	25–100
Duramyl	GM *Bacillus* species	6–10	25–100
Natalase	GM *Bacillus* species	5–10	10–60
BAN	*Bacillus* species	5–8	15–90
Fungamyl	*Aspergillus* species	4–7	15–60

However native starch is only slowly degraded by α-amylases. Gelatinization and swelling are needed to make the starch susceptible to enzymatic breakdown. For most food stains, various degrees of gelatinization result from cooking of starch-containing foodstuffs, e.g., pasta, potato, gravy, and baby food.

If the food processes did not exceed the starch gelatinization temperature, the starch may be in the form of partly or nongelatinized granules, or it may be partly retrograded. Such starch may be amorphous and is usually difficult to remove from surfaces without boil washing. The presence of an amylase renders boil wash superfluous in laundry process and enhances laundry-cleaning performance of detergents at lower wash temperature.

Gelatinized starch may form a film on fabric that can result in an increased pick-up of particulate soil after washing [619]. Starch stains combined with particulate soiling are more difficult to remove than starch alone. As a result white laundry items turn increasingly gray after repeated wash cycles, an effect that has been demonstrated by adding about 0.5 g starch per kilogram of cotton fabric [620]. Starches may react differently depending on their amylose content, which is thought to be the film-forming component of the starch. Film formation is favored under European laundry conditions, in which the temperature may be closest to the starch gelatinization temperature.

In laundry detergents, amylases may maintain or even contribute to increased whitening of dingy fabrics [619] and inhibit the graying of white fabrics resulting from a combination of starch and particulate soiling, as illustrated in Figure 61. A noticeable amylase effect is obtained with pre-spotting (at high concentration), and with presoaking (prolonged reaction time).

Examples of artificially soiled test pieces used to test the performance of amylases include cocoa/milk/sugar, cocoa/sugar/potato starch, cocoa/milk/sugar/potato starch, and starch/carbon black, all on cotton or polyester/cotton.

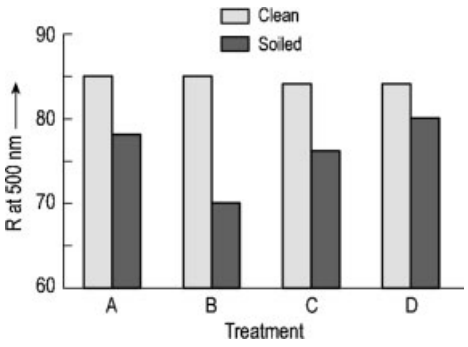

Fig. 61 Effects of added starch in solution on particulate soiling and removal with amylase and European granular detergent. Treatments: A) no starch added and washed with amylase-free detergent; B) starch added and washed with amylase-free detergent; C) starch added, washed with 0.4 KNU/L (Kilo Novo Units per Liter) Termamyl and detergent, D) starch added, washed with 1.2 KNU/L Termamyl and detergent [620]

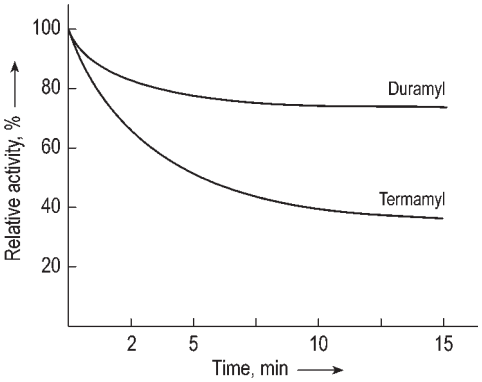

Fig. 62 Residual activity of bleach-stable α-amylase (Duramyl) compared to standard amylase (Termamyl) in 100 mM hydrogen peroxide at pH 9.0, 40 °C, 0.1 mM Ca^{2+} [623]

Bacterial α-amylases used in laundry detergents are fully compatible with detergent proteases, i.e., the two enzymes work together in the wash process. During storage in both powder and liquid detergents, the amylases are stable in the presence of proteases.

The stability of some calcium-dependent α-amylases can be enhanced by addition of small amounts of calcium salts to the detergent composition.

For bleach-containing detergents, bleach-resistant α-amylases have been developed by using protein engineering and genetic techniques, as indicated above for proteases. Figure 62 illustrates the gain in amylase enzyme stability, as indicated by the improved residual enzyme activity over time in a bleach-containing solution.

Protein-engineering and genetic techniques have been used to develop α-amylase with lowered optimum temperature for enzyme activity, specifically for low-temperature wash conditions with heavy-duty liquid detergents. Figure 63 illustrates the reduced optimum working temperature for such a low-temperature amylase developed by Novozymes. The significantly improved performance of such low-temperature α-amylase in a cold-water wash (25 °C) in a Mexican powder detergent is shown in Figure 64.

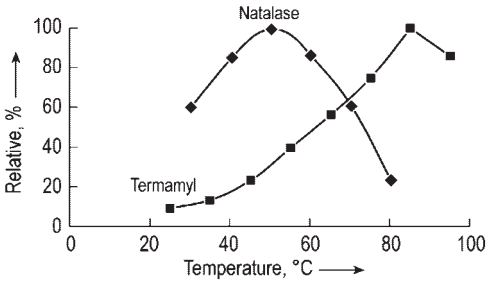

Fig. 63 Optimum temperature of low-temperature amylase (Natalase) versus standard (Termamyl) amylase [621], [624]

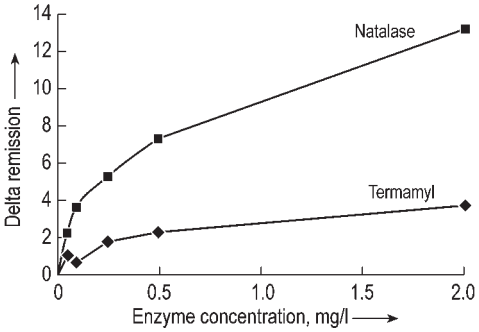

Fig. 64 Stain-removal performance of low-temperature amylase (Natalase) versus standard amylase (Termamyl) at 25 °C in Mexican commercial detergent (P&G). Orange-dyed starch swatch, small-scale wash test [621], [624].

5.2.1.6.3 **Lipases**

Lipases (E.C. 3.1.1.3) hydrolyze triacylglycerol (triglyceride) substrates, as are present in fats and oils. Because of their strong hydrophobicity, fats and oils are difficult to remove from laundry at low temperatures. A triglyceride molecule is composed of three fatty acid moieties linked to a glycerol backbone by ester bonds. Lipases hydrolyze triglycerides to more hydrophilic mono- and diglycerides, free fatty acids, and glycerol. These hydrolysis products are all soluble under alkaline wash conditions.

Many patents have demonstrated that lipases can improve the removal of fatty stains when used in powder and liquid detergents, special pre-soakers, or other cleaning agents. Intense research activity is also reflected in the literature.

Lipolase was the first commercially available lipase for detergents and the first detergent enzyme produced by genetic engineering to achieve an acceptable production economy. Leading brands in the USA and Europe included lipase from 1990/1991 [625] in powder and in liquid detergents.

The slow development of a commercial detergent lipase was due to low fermentation yields and to difficulties in finding lipases with the appropriate characteristics for application in household detergent products. Patent literature indicates the only feasible way to produce lipases at an acceptable cost/performance ratio is by genetic engineering [626–628]. Lipolase was originally isolated from the fungus *Thermomyces lanuginosus* (formerly *Humicola lanuginosa*) with low levels of enzyme expression. By using rDNA techniques the lipase is expressed in acceptable yields in the harmless host microorganism *Aspergillus oryzae* [624, 629] in the current commercial production process.

Protein engineering and genetic techniques delivered improved lipase enzymes for detergent applications. For example Lipolase Ultra by Novozymes is a protein-engineered variant of Lipolase that has improved washing performance at lower temperatures, i.e., below 20 °C. The change made by protein engineering involves replacing a negatively charged amino acid in the lipid-contact zone of the Lipolase molecule. More precisely, aspartic acid (negatively charged) in position 96 has been replaced with

leucine (neutral, hydrophobic). The change reduces the repellent electrostatic forces between the soil and the enzyme, making the active site more hydrophobic so that the affinity to a lipid contact zone on the textile surface was improved.

Another protein-engineered variant of Lipolase is LipoPrime for better first-wash effect, especially under European washing conditions. Further efforts are now being made to develop more ''first-wash lipases'' [630].

Surface-active molecules such as surfactants and fatty acids/soaps can strongly inhibit lipase enzymes [631]. Because of the presence of free fatty acids in the mix of hydrolysis products, pH strongly influences lipase activity and the removal of decomposed stains. The best rate of removal requires a pH above 8 [632]. Above pH 8 the hydrolysis reaction may be favored by small amounts of free Ca ions due to the formation of Ca soap, although lipases are also effective at low concentrations of free calcium [633]. The more the fatty stain is hydrolyzed by the lipase, the easier it is to remove the stain from the fabric due to its increased hydrophilicity.

However the effects of conventional lipases are seen only after several wash cycles (''multi-cycle wash performance''), as illustrated in Figure 65.

Surprisingly, lipases are also active during a certain period of the drying step [630], [636] during line drying, and in tumble dryers, Lipolase displays maximum activity when the moisture content of the fabric is 20–30 wt %. This means that significant decomposition of any residual fatty matter will take place while the laundry is drying. This hydrolytic activity does not result in an immediate advantage in terms of fat removal, but the next time the stained fabric is washed the stain will be removed more effectively. Figure 66 illustrates lipase enzyme activity in the drying step.

Lipases have proven to be effective in pre-spotters and other liquid detergent formulations when used in undiluted form for pretreatment of tough fatty stains.

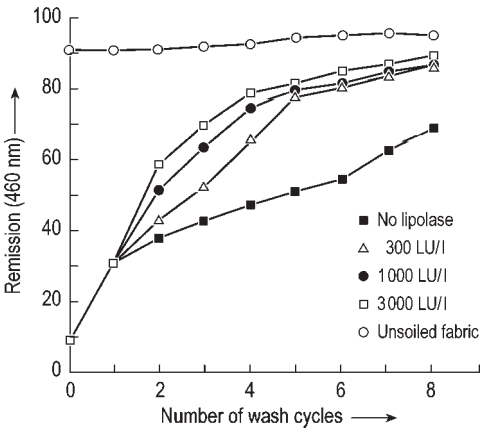

Fig. 65 Multi-cycle wash performance of lipase in detergents. Stain removal as function of number of wash cycles. European wash conditions 5 g/L powder detergent, 30 °C, 20 min, wash at pH 9.7. Polyester swatch soiled with lard fat plus Sudan red [636]. LU/L = Lipase Units per Liter

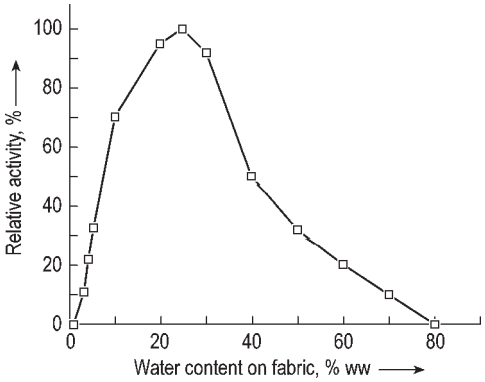

Fig. 66 Lipase performance in the laundry drying step. Relative enzyme activity during drying after a European wash and one rinse with tap water [638].

The low water content on the fabric in this situation is believed to be responsible for the high lipase activity and stain-removal performance in these conditions.

In *performance testing* of lipase detergent enzymes, three consecutive washing/drying cycles are usually employed. Washing temperature, time, and Ca^{2+} concentration depend on the geographical area where the detergent is used. After washing and drying, the reflectance of the colored, lard-containing test swatches is measured, and the remaining lard may be extracted for quantitative determination.

The multiple-wash performance effects of a detergent lipase are shown in Figure 65.

Lipase can cause *malodors* on butter- or fat-stained fabrics by releasing short-chain free fatty acids [631]. Lipases also may indirectly create malodor from laundry items such as sweaty sports clothes. The main reason for malodor (at least in the case of sports clothes) is the survival of microflora. The problem is therefore most pronounced in detergents without bleaching agents. Under normal drying conditions, lipases do not give rise to additional malodor, but if the drying takes several days some lipase-associated malodor may develop. In laundry detergents, the dosage of lipase is often limited by the maximum acceptable malodor.

5.2.1.6.4 **Cellulases**

Cellulases cleave β-1,4-glycosidic bonds in cellulose and operate directly on the natural cotton fibers or cotton/flax blends and on the cellulose portion in blended cotton/synthetic fibers. This enzyme class is divided into endo-cellulases (endo-glucanase, EG; E.C. 3.2.1.4) and exo-cellulases (cellobiohydrolase, CBH; E.C. 3.2.1.91). EG-type cellulases catalyze the endo-hydrolysis of 1,4-β-D-glycosidic linkages in cellulose, lichenin, and cereal β-D-glucans. CBH-type cellulases hydrolyze the 1,4-β-D-glycosidic link in cellulose and cellotetraose, releasing cellobiose from the nonreducing ends of the cellulose chain. For both EG and CBH cellulases, many iso-enzymes with slightly different substrate specificities and optimum working conditions have been identified.

The cellulase enzyme molecule is composed of up to three types of functionally different domains, as illustrated schematically in Figure 67: (1) the catalytically active

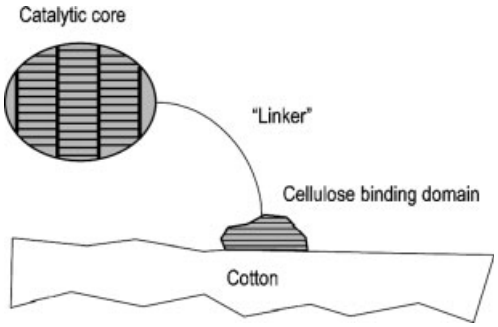

Fig. 67 Schematic presentation of a multi-domain cellulase adsorbed to cellulose substrate. The enzyme having a catalytic core, a linker, and a cellulose-binding domain [618].

core, which is a large, spherical domain; (2) the linker domain, which is an elongated and flexible spacer; and (3) a spherical cellulose-binding domain (CBD).

The nature of the core determines catalytic properties such as endo activity versus exo activity, substrate specificity, and the type of reaction products that are formed. The presence of a CBD is of particular importance for binding of the enzyme on insoluble and crystalline cellulose and for hydrolytic effects. Both EG- and CBH-type cellulases can contain linkers and cellulose-binding domains.

Table 24 lists some application conditions and a few comparative characteristics for some commercially available detergent cellulases from Novozymes. These cellulases can be used in heavy-duty liquid detergents, in bleach-containing powder detergents, and in a number of ''color'' and compact detergent powders.

As an example the pH and temperature profiles of Carezyme are presented in more detail in Figures 68 and 69.

Cleaning by removal of particulate soils, softening, and improved color brightness are the three basic benefits obtained from cellulases. Cellulases are unique in providing these effects.

When a textile is exposed to shear stress, either during wear or washing and tumble drying, the surface becomes slightly damaged. The worn yarns form fibrils and ''pills'' ranging in size from a few micrometers to a few millimeters on the surface. The pills

Table 24. Examples of commercially available cellulases from Novozymes and application conditions. Source: Novozymes cellulase application sheets

Product name	Microorganisms	Cellulase type(s)	pH range	Temperature range
Celluzyme	*Thermomyces lanuginosus*	CBH 1, CBH II, EG I, EG II, EG III, EG V, and EG VI	4–10	25–70
Carezyme	*Thermomyces lanuginosus*	EG V	5–10.5	25–70
Endolase	*Thermomyces lanuginosus*	EG II	5–9	25–70

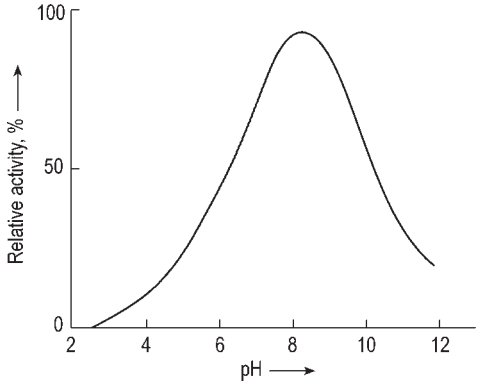

Fig. 68 pH-dependent activity of EG V cellulase (Carezyme, Novozymes) at 40 °C and 100 ECU(CP) enzyme concentration, 20 min. reaction time [642].

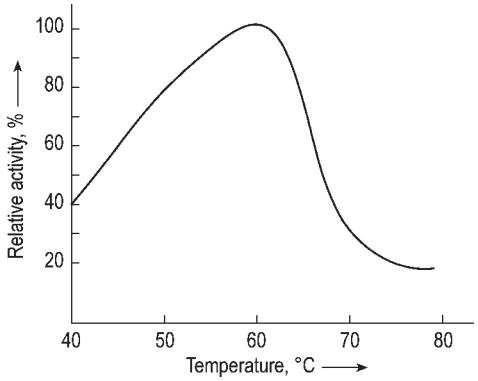

Fig. 69 Temperature-dependent activity of EG V cellulase (Carezyme, Novozymes) at solution pH 8.5 and 100 ECU(CP) [Endo Cellulose Unit (Carezyme Product)] enzyme concentration, 20 min. reaction time [642].

scatter light, giving the fabric a grayish or dull appearance, and affecting color brightness and contrast. Dust particles also tend to stick to the areas of damaged cellulose fibers, and this further adds to the gray appearance. Damaged fibers are also thought to be responsible for making the fabric surface more rigid by entanglement, thereby reducing softness and altering size and shape of a garment.

Cellulases hydrolyze the exposed β-1,4 bonds in the cellulose fibrils and pills, which leads to their removal during the treatment. This is believed to be the mechanism behind the softening and color-brightening effects. Published literature indicates that little is known about the mechanism behind the cleaning action of cellulases. Possible explanations for the cleaning effect are that by removing the fibrils, the soil attached to

them is released, and that the enzymatic action facilitates cleaning by exposing dirt trapped in the fiber matrix to the washing solution [639], [640].

Extremely high dosages of detergent cellulases can cause fabric damage in some cotton products after repeated laundering. Damage may appear as loss of fabric strength and excessive softening of the mechanically exposed parts of laundry items, such as hems and edges. These effects may be eliminated by balancing the cellulase dosage to manage the desired benefits. Application tests include small-scale laundering in Terg-o-tometers and Launder-o-meters and full-scale multicycle laundering in commercial washing machines.

5.2.1.6.5 **Mannanase**

The mannanase of interest is a mannan endo-mannosidase, which degrades the β-1,4-mannose linkage of galactomannans, e.g., in neutral pectins such as guar gum. The hydrolysis products are mannose oligosaccharides. Galactomannans such as guar gums are widely used as thickeners and stabilizers in foods, household agents, cosmetics, and toiletries. Because of high affinity to the cotton surface due to hydrogen bonding effects, stains containing guar gum can be difficult to remove. Guar gum also has a glueing effect on particulate soil and can flocculate dispersed clay particles in the wash. Hence, invisible residual spots of guar gum can bind particulate soil released during the wash cycle, so that the stain re-appears.

Mannaway [37288-54-3] (E.C. 3.2.1.78) is a mannanase for use in detergents commercialized by Novozymes and developed in close cooperation with Procter and Gamble [643]. It was first introduced in Procter and Gamble's HDL Ariel Liquid in 2000. The mannanase in this enzyme product is a 33 kDa hemicellulase (pI 4.7) that was cloned into *Bacillus licheniformis* from alkalophilic *Bacillus* strain I633. The optimal temperature for the enzyme at pH 10 in an analytical system is 50 °C (Fig. 70). In the same system the optimal pH is determined to be 6–8 (Fig. 71).

The enzyme activity of Mannaway is expressed in mannanase immuno units (Mannaway), MIU(M). One unit is equal to one milligram of pure enzyme protein

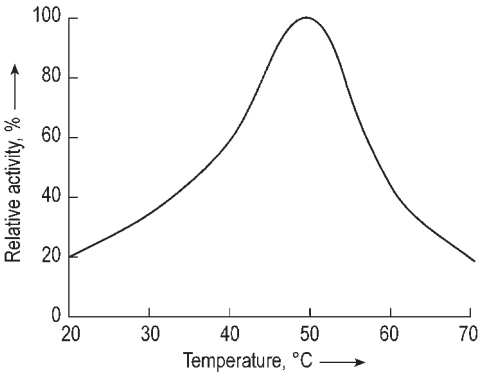

Fig. 70 Temperature profile of Mannaway at pH 10. Determined by spectrophotometer at 600 nm, AZCL galactomannan in 0.1 M glycine [644].

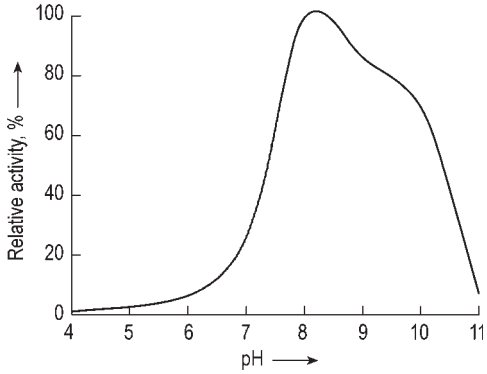

Fig. 71 pH profile of Mannoway at 40 °C. Determined by spectrophotometry at 600 nm, AZCL (Azurine Cross Linked) galactomannan in 0.1 M glycine [644].

and is defined relative to an enzyme standard under specific conditions. A specific enzyme activity of 1 MIU/M) mg is achieved in the commercial liquid preparation with a total of 25 MIU(M)/g.

Using Mannaway in a detergent formulation thus improves its overall cleaning efficacy as well as its stain-removal effect on stains like BBQ sauce, ice cream, gravy, and fudge.

5.2.1.7 **Future Trends**

With water becoming an increasingly precious commodity, increased environmental awareness and care for continued energy conservation will impact consumer washing habits. Detergents will have to become even more efficient and effective for maintaining high performance standards at further reduced dosage using less wash liquor at lower wash temperatures in short wash cycles. In addition altered consumer demands and new fiber developments will require further specialized detergents. All of the above dictate development of smart detergents and wash processes, which certainly will include new enzymes [596].

The contribution of enzymes will continuously increase as more cost-effective enzyme production processes become available as well as new enzyme types that can deliver unique performance benefits in detergents. Increased effort to develop low-allergenicity enzymes, redox enzymes; targeting enzymes, smart enzyme delivery and new improved enzymes from psychrophilic and extremophilic organisms are underway, as indicated by many patent applications. Further progress in protein engineering, genomics, and proteomics will allow the discovery of new and better adapted enzymes for detergent applications.

New hydrolases for broadening the range of difficult to remove stains and for cleaning and whitening benefits are being investigated by the major detergent manufacturers and enzyme suppliers across the world. Many hemicellulases such as xylan-degrading enzymes [645], pectin-degrading enzymes [646], and dextran-degrading enzymes [647] are of interest for specific stain-removal benefits.

Redox enzymes, mainly peroxidases, haloperoxidases, and laccases, and other oxidases are being intensively researched as potential components for novel bleaching systems that can provide bleaching effects at lower washing temperatures than current bleaching systems. This would provide energy savings apart from utilizing less harsh chemicals and also would ensure enhanced fabric care of garments being washed [648].

Until now a peroxidase (Guardzyme, Novozymes) and a laccase have been commercially developed by Novozymes for use in dye-transfer inhibition (DTI) in textile processing in combination, respectively, with a low concentration of hydrogen peroxide and oxygen and with a polyphenolic mediator [649]. Also the potential for removing bleach-sensitive stains in the wash using these peroxidase- and laccase-based enzymatic bleach systems and sanitization effects have been reported in patents from Novozymes, Genencor, Unilever, and Procter and Gamble [650–652]. These enzymatic bleach systems have not been applied in detergents so far.

More recently mono- and dioxygenase enzymes have been investigated for bleaching performance according to patents by Unilever, Procter and Gamble [653], and Novozymes. These enzymes incorporate oxygen directly into the substrate to be oxidized or bleached. The dioxygenases such as lipoxygenases do not require added cofactors or mediators for achieving bleachlike performance effects. However, many, if not all, oxygenase enzymes are intracellularly active and are difficult to express as extracellular enzymes, as are preferred for economical and industrial production.

Also enzymes will start to appear in other household detergents (apart from automatic dishwashing detergents) as developments proceed. Protease enzymes have already been introduced in hand dishwashing detergent by Procter and Gamble for providing skin-care benefits to consumers. Household cleaners also can benefit from enzymes. Many enzyme-susceptible soils can be found on hard surfaces of floors and workbenches in the kitchen (dried-in fats, sticky sugars, dairy products, dust, and so on), and in bathrooms on tiles and bath tubs (calcium soaps, sebum, hair, and others).

5.2.2 Enzymes in Automatic Dishwashing

5.2.2.1 Introduction

Proteases and α-amylases have become state-of-the-art worldwide for automatic dishwashing detergents, one of the last remaining growing markets in the field of detergents. Because modern automatic dishwashing detergents are required to be safe for consumers and to take account of environmental aspects, proteases and α-amylases are now indispensable ingredients. Their incorporation made possible the development of automatic dishwashing detergents with reduced alkalinity, lower dosage, and active-oxygen-based bleach systems, as well as lower application temperatures. The enzymes offered on the market for this application are the same as for laundry detergents. But due to differences in the performance profile some proteases and α-amylases are preferred. Therefore the search for better performing enzymes by microbiological screening or protein engineering, such as site-directed mutagenesis or DNA shuffling, is still going on. Although an official, well-established method for

testing the performance of enzymes does not yet exist, there are some methods that are commonly used.

The concept of using enzymes in detergents first appeared in 1913 in German Patent DRP 283 923, assigned to Otto Röhm and describing the introduction of a pancreatic protease into a laundry detergent [654]. The first enzymatic product, a soaking detergent launched in Germany under the trade name Burnus, was essentially made of soda ash and pancreatin, a protease mixture, mainly of trypsin and chymotrypsin, obtained from crude extracts of animal pancreatic glands. Since then protease, like surfactants and builders, has become a standard ingredient of detergents. Later, protease was joined by other enzymes such as amylase and lipase. Whereas enzymes had been used successfully in laundry detergents for many years as an aid to remove tough stains, the incorporation in automatic dishwashing detergents (ADD) only began in the early 1990s [655].

Though washing clothes and cleaning dishes are two different tasks, both laundry detergents and dishwashing detergents share similar concerns, such as removal of stains derived mostly from foods such as egg and milk, as well as starch-based soils. The main difference between the two areas and thus the reason for the late use of enzymes in ADD is the formulation and application conditions, which have a strong influence on the performance of enzymes. The development of ADD with reduced alkalinity (pH < 11.5) and lower dosage, the use of active-oxygen-based bleach systems instead of hypochlorite, and the trend to lower temperatures led to the incorporation of first protease and, shortly afterwards, amylase to maintain the high performance level [656].

5.2.2.2 Characteristics of Enzymes for ADDs

Proteases and α-amylases have become state-of-the-art worldwide for automatic dishwashing detergents. Both belong to the group of hydrolases because they degrade their substrates by hydrolysis, using water as co-substrate. The enzymes offered on the market for this application are the same as for laundry detergents [657], but due to differences in the performance profile some proteases and amylases are preferred. Sometimes the use of lipase has been discussed [658], as ADDs contain only a low content of surfactants compared with laundry detergents. Furthermore, enzymes belonging to the group of hemicellulases [659] are also described in patent literature for use in ADDs. However, so far no product containing lipase and/or hemicellulase has been offered on the market.

Natural enzymes which can be found in the stomach, saliva, or pancreas of animals, as well as in some fruits, are not really suitable for detergents because they only work under acidic or neutral conditions. Moreover, they are extremely specific, usually able to degrade only one particular type of bond, e.g., in protein molecules. However, enzymes used in ADDs must fulfill several requirements set by the environment (detergent and cleaning liquor) and by the soil substrate, which consists of a huge variety of foodstuffs and is therefore not very specific.

The currently used enzymes are usually produced by high-performance bacterial strains, conditioned to resist alkaline pH and therefore to produce alkaline-resistant enzymes. The enzymes are extracellular enzymes, secreted by the bacteria into the surrounding medium. Thus, they can be isolated without breaking the cells. This

makes the purification process easier and more economical. In case of proteases, these bacterial enzymes are less specific and will degrade almost all kinds of proteins.

Enzymes are sensitive to some ingredients of automatic dishwashing detergents, both during the shelf life of the product and in the cleaning liquor. Anionic surfactants, especially alkylbenzene sulfonates, can degrade enzymes, whereas nonionic surfactants do not destabilize them [660]. Enzymes are also deactivated by oxidizing agents such as hypochlorite, which was the commonly used bleaching agent in ADDs for a long time, and to a lesser extent by hydrogen peroxide, peroxides, and peroxyacids. Application conditions are also important. For instance, any temperature increase favors the enzymatic reaction, as for most chemical reactions, but at the same time the degradation of the enzymes will increase. Heat degradation of proteases occurs above 55 °C in pH 9 buffers and sometimes at lower temperatures when surfactants and builders are present.

To be well suited for the use in ADDs, enzymes must exhibit the following properties:

- Activity optimum at alkaline pH,
- Efficacy at temperatures of 20–70 °C,
- Stability at temperatures up to 60 °C,
- Stability in the presence of other detergent ingredients, such as surfactants, builders (e.g., phosphates, silicates, etc.), and activated bleach, both during storage and use,
- Low specificity to soils, i.e., a specificity broad enough to enable the degradation of a large variety of proteins and starches.

Enzymes are commercially available as dust-free granulates for solid products and as yellowish to brown liquids for liquid products. Because they are biocatalysts, they act at very low concentrations. Their efficacy does not increase linearly with concentration, but levels off above a certain concentration.

5.2.2.3 **Proteases**

5.2.2.3.1 **Proteins: The Substrate of Proteases**

A fresh proteinaceous stain on textile or hard surfaces is generally not stubborn and can often be removed simply by cold water. It is only when the proteinaceous soil is dried, aged, or heated, for instance, after cooking, that it becomes difficult to remove, even with surfactants, as it coagulates and hinders the penetration of the cleaning liquor. Moreover, protein residues may be oxidized and thus denatured upon aging due to presence of oxygen or a bleach system as part of an ADD, so that the soil/stain becomes permanent.

Furthermore, there are some stains which contain only small amounts of protein and are not completely removed by surfactants and bleaching systems. Proteins are present in small amounts in many natural oils as well as starchy stains from different foodstuffs, such as gravy or cocoa [661]. In these types of stains, the coagulated proteins act as a glue fixing the soil to the surface.

5.2.2.3.2 Proteases for ADDs

Proteases catalyze the breakdown of large and complex protein molecules into peptides and amino acids (Fig. 72), which are more easily removed by the detergent. According to the site of catalytic hydrolysis of the protein, proteases can be classified in two groups: endopeptidases, which hydrolyze peptide bonds within the protein chain, yielding water-soluble peptides, and exopeptidases, which split terminal peptide bonds only, releasing free amino acids.

Proteases for detergents are endopeptidases belonging to the class of serine proteases, and within this class to the subtilases (subtilopeptidases [9014-01-1], E.C. 3.4.21.62) [662–666]. Similar to another large class of proteases, the trypsin family, these enzymes all have the same active-site residues, i.e., a serine, a histidine, and an aspartate residue. For example, in subtilisin BPN′ the catalytic triad is formed by Asp-32, His-64, and Ser-221 [667]. Structurally, the positions of the active-site serine and histidine residues are very well conserved, whereas the location of the carboxylate function of the aspartate residue shows more scatter.

Subtilisins have several characteristics that make them well suited for detergents: stability in the presence of detergents, broad specificity for the amino acid sequence of the peptides to be hydrolyzed, high specific activity at alkaline pH, and the possibility for production in industrial amounts. Due to a pH profile ranging from 9 to 11, the subtilisin from *Bacillus lentus* shows superior performance to subtilisin BPN′ or Carlsberg and is thus the preferred protease for solid detergents, both laundry and dishwashing (Table 25). The *Bacillus lentus* subtilisin (same as *Bacillus alcalophilus* and *Bacillus clausii*) consists of 269 amino acid residues, slightly shorter than subtilisin BPN′ and Carlsberg, consisting of 275 and 274 amino acid residues, respectively.

Modification of enzymatic properties of the subtilisins by protein engineering started as soon as the technique became available in 1984. One objective is to stabilize the enzyme against harsh application conditions such as bleach and detergents. Surface-exposed methionine residues belong to the group of amino acids that are likely to be oxidized by peroxides and peracids. The subtilisins contain a methionine residue next to the active site serine-221. Also other methionine or aromatic residues may be oxidized, leading to destabilization and thus to increased autocatalytic breakdown. Oxidation of the exposed methionine-222 drastically lowers catalytic activity. This methionine residue was replaced by several other residues to give increased oxidative stability, but also relatively poor catalytic power on small peptides as well as protein substrates [668], [669].

The other objective is to improve the performance of the enzyme in general by increasing the turnover on given protein substrates or by improved or modified targeting of the enzyme. Besides site-directed mutagenesis, fusion techniques, e.g., by means of DNA shuffling, have also been described for this purpose [670], [671]. The

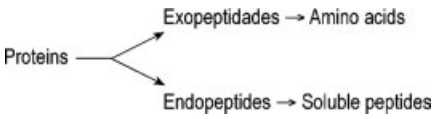

Fig. 72 Schematic of enzymatic protein hydrolysis

Table 25. Proteases available on the market

Supplier: Trade name	Origin	Optimum pH*	Optimum temperature, °C*	Characteristics*
Genencor:				
Purafect®	*B. lentus*	10	60	highly alkaline
Purafect® OxP	GM Purafect	10	50	highly alkaline, oxidatively stable
Properase®	GM *B. alcalophilus*	11	50	highly alkaline, low temperature
Novozymes:				
Alcalase®	*B. licheniformis*	8–9	60	moderate alkalinity
Savinase®	*B. clausii*	9–11	55	highly alkaline
Everlase®	GM Savinase	10	50	highly alkaline, oxidatively stable
Esperase®	*B. lentus*	9–12	60	highly alkaline

*All data according to supplier.

use of these techniques led to the development of proteases with modified substrate specificity, e.g., higher performance on proteins derived from egg or reduced inhibition by substances present in egg, such as trypsin inhibitor. This was achieved by changing the charge ratio within the catalytic domain [672], [673] or by insertion of at least one amino acid in the active loop site region [674–677]. Both methods resulted in a protease with a modified substrate specificity. The protease according to refs. [672], [673] can be found in European ADD, but is not a commercial product. The other protease has been introduced to the market under the trade name Ovozyme®.

5.2.2.4 Amylases

5.2.2.4.1 Starch: The Substrate of Amylases

Starch is a carbohydrate food reserve in plants and the major energy source for nonphotosynthetic organisms. It is abundant in nature and is composed of two distinct polysaccharides, amylose and amylopectin. Amylose is essentially a linear, water-soluble polymer consisting of α-1,4-linked D-glucopyranose, and amylopectin is a highly branched, water-insoluble polymer consisting of amylose chains linked at branch points by α-1,6-bonds (Fig. 73). The relative amount of amylose and amylopectin depends on the source of the starch. The major component is usually amylopectin (73–86 %) and the minor component is amylose (14–27 %). Some cultured variants, such as waxy corn or rice, can deviate from this general ratio of amylopectin and amylose.

As a major component of foodstuffs, starch is frequently found in food residues on dishes. Such starch-containing residues are derived from pasta, potatoes, oatmeal, and sauces, etc. [678].

Household cooking as the final step of starch modification leads to the formation of gels with different viscosity and solubility in water. When starch is heated in water, it swells when the specific gelatinization temperature is reached. The concomitant water

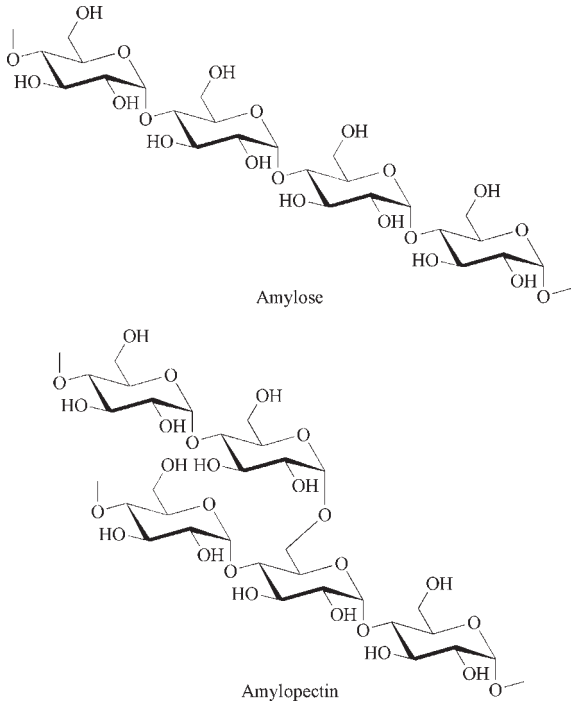

Fig. 73 Structure of amylose and amylopectin

adsorption leads to a viscosity increase and to a change in the chemical and enzymatic degradability of starch. During cooling, the amylose fraction recrystallizes. This process is referred to as retrogradation. Foodstuffs may contain starch both in retrograded and in nongelatinized granular forms.

Before cooking, however, starches for food application are modified to facilitate handling during food manufacturing or to improve the taste and texture of the final product. The simplest starch modification is the separation of amylose and amylopectin. Enriched amylose is used for flexible films, production of capsules for food use, and in sweets. Enriched amylopectin can be used as a shape stabilizer in prepared food such as skinless sausages. Starches can also be modified by chemical and enzymatic methods. Oxidation by hydrogen peroxide or sodium hypochlorite gives starches that are good aids for the production of sweets and suspensions. Phosphorylated starches and enzymatically degraded starches like dextrins are used as emulsifiers, binders, or thickeners for the preparations of mayonnaise, dressings, mustard, gravy, and similar food products.

5.2.2.4.2 **Amylases for ADDs**

Amylases are specific starch-cleaving enzymes [679], [680]. Amylases degrade starch and related compounds (Fig. 74) by hydrolyzing the α-1,4 and/or α-1,6 glucosidic linkages in an endo- or an exo-acting fashion [681–683]. The variety of enzymes

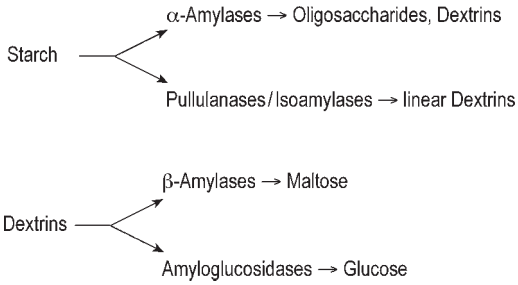

Fig. 74 Schematic of enzymatic starch hydrolysis

catalyzing such reactions include α-amylases (α-1,4-D-glucan glucanohydrolases [9000-90-2], E.C. 3.2.1.1), β-amylases (α-1,4-D-glucan maltohydrolases, E.C. 3.2.1.2), amyloglucosidases (α-1,4-D-glucan glucohydrolases [9032-08-0], E.C. 3.2.1.3), α-glucosidases (α-D-glucoside glucohydrolases, E.C. 3.2.1.20), pullulanases (α-dextrin 6-glucanohydrolases [9075-68-7], E.C. 3.2.1.41), and isoamylases (glycogen 6-glucanohydrolases, E.C. 3.2.1.68). Each of these enzymes can be distinguished from the others on the basis of reaction specificity.

α-Amylases catalyze the hydrolysis of the amylose fractions of starch with cleavage of the α-1,4-glycosidic bonds in the interior of the starch chain. In the first step, this endo reaction leads to oligosaccharides and further to short-chain, water-soluble dextrins. The dextrins can be further degraded by β-amylases by splitting maltose from the reducing end of the chain. Additionally, there are amyloglucosidases that release glucose and pullulanases, or isoamylases that degrade starch directly into linear dextrins, for they also attack α-1,6-glycosidic bonds.

Of these different amylases, only α-amylases are used in automatic dishwashing detergents, although recently other carbohydrate-cleaving enzymes (e.g., pullulanases or isoamylases) have also been described for this application [684–687]. Usually, the primary hydrolysis of starch by α-amylases into oligosaccharides and dextrins is sufficient for easier removal of starch from hard surfaces.

α-Amylases for detergents are obtained from bacteria by fermentation. Submerged cultures of *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are the common production method. The α-amylase that is predominantly used today for automatic dishwashing detergents is a genetically modified α-amylase produced from *B. licheniformis*, available under different trade names (Table 26). The primary target of the modification of the traditionally used α-amylase was improved resistance against oxidants, especially active-oxygen-based systems. This was achieved by replacement of methionine-197 in the α-amylase [688], [689]. Besides this improvement, performance advantages on particular starch-containing stains led to the preferred use of this modified amylase in ADD.

The α-amylases of *B. licheniformis* are more suited for the use in ADDs than the formerly used types from *B. amyloliquefaciens*. The activity optimum of these thermally stable bacterial α-amylases strongly depends on the pH, the temperature, and the presence of calcium ions as stabilizer [690], [691]. The molecular weight is about 58 000.

Table 26. Amylases available on the market

Supplier: Trade name	Origin	Optimum pH*	Optimum temperature,* °C	Characteristics*
Genencor:				
Purastar®	*B. licheniformis*	6–9	75–90	thermostable
Purastar® OxAm	GM Purastar	6–9	75–90	thermostable, oxidatively stable
Novozymes:				
BAN®	*B. amyloliquefaci-ens*	6–6,5	70–90	
Termamyl®	*B. licheniformis*	6–9	70–90	thermostable
Duramyl®	GM Termamyl	6–9	65–85	thermostable, oxidatively stable
Termamyl® ultra	GM Termamyl			reduced Ca dependancy
Stainzyme®	GM α-amylase			altered substrate specificity

*All data according to supplier.

At neutral pH the optimum temperature is near 90°C. A certain concentration of calcium ions is required to maintain the activity of α-amylases. Calcium(II) stabilizes the enzyme against denaturation and the attack of proteases. In the range of 40–60 °C the optimum activity lies in a relatively broad pH range from 5 to 9. In the pH range of 9–11, which is relevant for ADD, the activity decreases; however, it is still sufficient to give a good cleaning effect.

The builder environment of detergent applications sometimes causes a problem for calcium-dependent enzymes like amylases [692, 693]. These builders, such as phosphates, citrate, silicates, or chelating agents, form stable complexes with ions of the alkaline earth group such as calcium(II). As a result, the concentration of free calcium in the liquor decreases. If the calcium binders have a higher complex-forming constant than the enzyme they can even deplete the structural calcium ion of the α-amylases to give a destabilized enzyme. The destabilized enzyme is inactivated early in the cleaning cycle and, as a result, the performance decreases. Therefore, α-amylases with less calcium dependency have been developed and products have been offered on the market since 2002.

Optimization of α-amylases by using modern techniques of protein engineering is also under progress [694]. With regard to new dishwashers with increased energy efficiency, i.e., lower cleaning temperatures and shorter cleaning cycles, the development of a-amylases with a combination of different characteristics (improved resistance against oxidants, reduced calcium dependency, altered substrate specificity, and/or specific activity) has started [695–697]. A first product of this development has been available on the market since 2004. Furthermore, new α-amylases derived from other bacilli than *B. amyloliquefaciens* or *B. licheniformis* are described in the patent literature [698], [699]. Some of these amylases show additional activities, such as cyclodextrin glycosyltransferase (CGTase) activity, besides their amylolytic properties.

5.2.2.5 Other Enzymes

Usually, all enzymes claimed for detergency are also described for the use in ADDs. Consequently, besides protease and amylase, which are state-of-the-art in ADD, other enzymes belonging to the group of hydrolases can be found in the literature for this purpose.

There is a variety of patent applications dealing with the use of esterases, especially lipases, in ADDs [658], [700–703]. Due to a low content of surfactants compared with laundry detergents the improved cleaning of fatty stains by using lipase was the target, especially with regard to lower application temperatures and reduced alkalinity. On the other hand, the use of lipase requires the reformulation of the ADD to avoid the formation of calcium soap (limesoap) on dishware.

The use of hemicellulases for ADD is also mentioned in the patent literature. Hemicellulases such as β-glucanases [659] or mannanases [704] deal with specific parts of starch-containing stains, such as β-glucan or mannan. For instance, β-glucan can be found in food residues with a high content of oatmeal, and mannan is used as binder/thickener in some milk products and special diet food. Therefore, compared to broad-application α-amylase, both hemicellulases can be regarded as enzymes with specific effects.

5.2.2.6 Automatic Dishwashing Detergents

During the 20th century electrical domestic appliances reduced the effort of housework in a dramatic way. The washing machine is considered the most important household appliance, because, in the past, laundry washing was the toughest housework job. However, dishwashing is also very time-consuming as it is done several times a day. In the USA the automatic dishwasher had already become fairly common in the household by the early 1960s, and the penetration of dishwashers exceeded the 50 % mark by about 1990. In Europe, dishwasher penetration differs dramatically from country to country [705]. For example, in northern Europe the penetration is quite high at approximately 60 % in Norway and Sweden. In countries like Switzerland, Germany, and Austria more than 40 % of households own an automatic dishwasher. However, in southern and eastern Europe the penetration is still quite low. Thus, substantial growth has been observed, especially in the last-mentioned regions. Therefore, automatic dishwashing is considered one of the last remaining growing markets for producers of electrical household appliances and ADDs.

5.2.2.6.1 Composition of Automatic Dishwashing Detergents

With a dishwasher cleaning can be done easier and even more economically than by hand [706], [707]. The amount of water necessary for a cleaning program in an automatic dishwasher has been drastically reduced in the last decade from 45 to about 20 L. In the cleaning cycle, water consumption has been reduced from about 10 to 4–5 L. On the other hand, the amount of crockery, and therefore the amount of soil, remained unchanged. Today modern European dishwashers consume, for the cleaning of 12 table settings, 18 L of water, 1.6 kWh of electrical energy, 20–30 mL of detergent, 2–3 mL of rinse aid, and 20–30 g of salt. These values for the standard 65°C program can be lowered by using the 55 or even 45°C program.

The first automatic dishwashing detergents contained almost 100 % sodium tripolyphosphate. Until the end of the 1980s, sodium tripolyphosphate remained the main ingredient, but in combination with sodium metasilicate. In addition, a chlorine-based bleaching agent such as sodium trichloroisocyanurate was incorporated to achieve an visible removal of bleachable stains like tea.

Modern detergents are required to be safer for consumers and to take concern of environmental aspects (e.g., chlorine bleach, phosphates, energy consumption). In some countries, household products containing ''corrosive'' metasilicate are no longer allowed or have strong restrictions for sale to the public. Consequently, automatic dishwashing detergents have been undergoing an evolutionary process similar to that of laundry detergents. Pancreas extracts have been described for dishwashing powders, but the use of enzymes was taken into consideration for this product category only after alkali-stable and thermostable enzymes became commercially available in granulated form. Dishwashing detergents based on metasilicates and thus of high alkalinity can very efficiently remove most types of soils that can swell under alkaline conditions. This effect decreases dramatically if the pH of the cleaning solution falls below 11.5, leading to an unacceptable loss in performance. By incorporating enzymes such as α-amylases and proteases into products with reduced alkalinity, the performance loss can be partially or completely compensated. It can even lead to an improved performance on specific soils, e.g., dried-on starch.

The availability of relatively alkali-stable, granulated, and dust-free enzymes was the basis for the development of a new generation of enzymatic automatic dishwashing detergents which are called ''compacts'' in contrast to the ''conventionals'' and whose composition is totally different from that of the conventionals (Table 27).

5.2.2.6.2 **Application of Enzymes in ADD**

The main task of protease and α-amylase in automatic dishwashing detergents is the cleaning of dishes by removal of tough stains, mainly consisting of proteins and starches, without damaging the surface and/or décor of china, glass, and metal. To evaluate this performance and to differentiate between various enzymes of a class like subtilisins, the producers of enzymes and manufacturers of ADDs apply different test methods for protease and amylase performance, but only a few have been published [655], [656], [708], [709]. Official methods exist in different countries for testing the performance of dishwashers, for example, IEC 436 and DIN 44990, the American AHAM, and the Australian Standard.

Some commonly used methods for testing protease and amylase are based on dried-on protein or starch, respectively, and are classified in terms of their soil (Table 28):

By using these soils and their corresponding methods differentiation between different proteases and amylases is possible. The influence of an increasing enzyme content can also be detected. For soils like minced meat on porcelain plates and porridge a minor contribution of the alkalinity must be considered.

The following figures illustrate the efficiency of different proteases and amylases dependant on concentration, given as protein content per liter and temperature. The performance of the different enzymes were tested according to the aforementioned methods (Figs. 75–77) in a compact phosphate-based European ADD with a dosage of

Table 27. Types of automatic dishwashing formulations (in wt %)

Ingredient	Conventional detergents (powder/tablet)		Compact detergents (powder / tablet)	Phosphate	Non-phosphate
Alkaline carriers	metasilicate/ disilicate	30–70	sodium carbonate	0–40	0–40
	sodium carbonate	0–10	sodium bicarbonate		0–40
			disilicates	0–40	0–40
Complexing and dispersing agents	phosphate	15–40	phosphate	> 30	
	polymers	0–10	citrate		> 30
			phosphonate	0–2	0–2
			polycarboxylate	0–5	0–15
Bleach system	chlorine carrier	0–2	oxygen carrier	3–20	3–20
			TAED	0–6	0–6
			Mn accelerator		< 1
Wetting agents	surfactants	0–2	surfactants	0–4	0–4
Enzymes				< 6	< 6
Auxiliaries	paraffin oil	ca. 1	perfume	< 0.5	< 0.5
			paraffin oil	< 1	< 1
			silver-protection agent	< 1	< 1
pH of 1 % solution		12–13		< 11	11–12

Table 28. Soils for performance tests of protease and amylase

Enzyme	Soil	Evaluation	Method
Protease	egg yolk	gravimetric	[710]
	egg/milk	gravimetric	[710]
	minced meat on porcelain plates	visual (color reaction) /photo catalogue	[710] by analogy with EN 50242
Amylase	starch mix consisting of potato, maize, rice, and wheat starch	gravimetric	[710] by analogy with IEC SC 59A/WG II
	porridge	visual (color reaction) /photo catalogue	[710] in accordance with EN 50242

4 g/L in a 55 °C program at 16° German water hardness (corresponds to 286 ppm $CaCO_3$ with a ratio of Ca:Mg of 5:1). Within the group of proteases and amylases the enzymes were compared on equal protein concentration.

In Figures 77, 78, and 79 the preference for some proteases is evident. Protease A — a protease in accordance with WO 92/21760 and WO 95/23221 [672], [673] — achieves

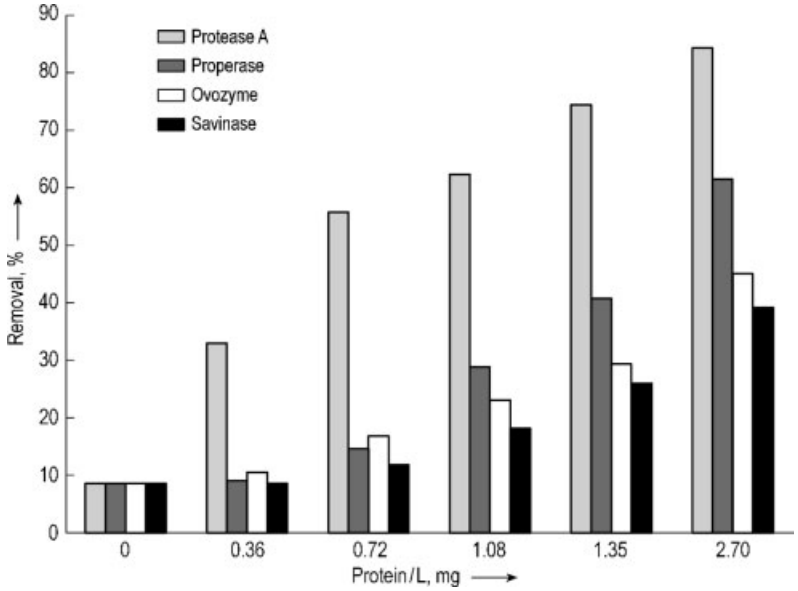

Fig. 75 Efficiency of different proteases on egg yolk at 55 °C

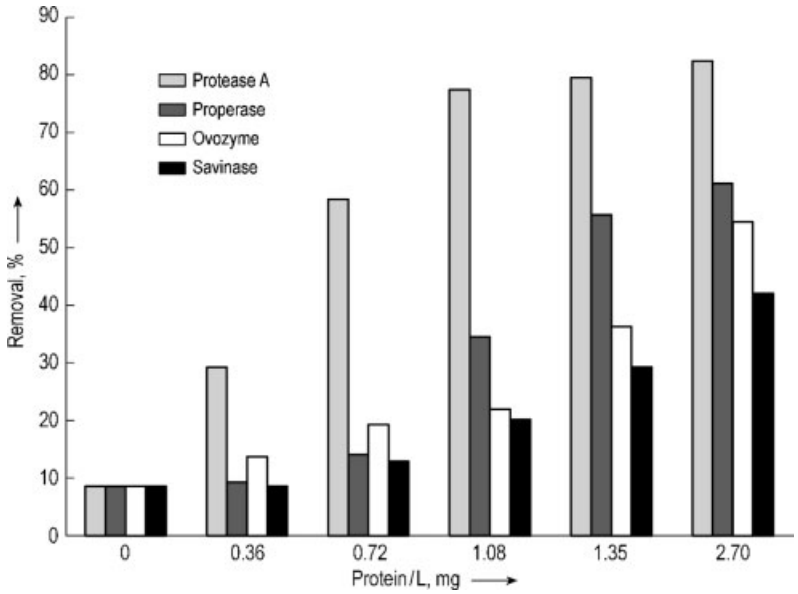

Fig. 76 Efficiency of different proteases on egg/milk at 55 °C

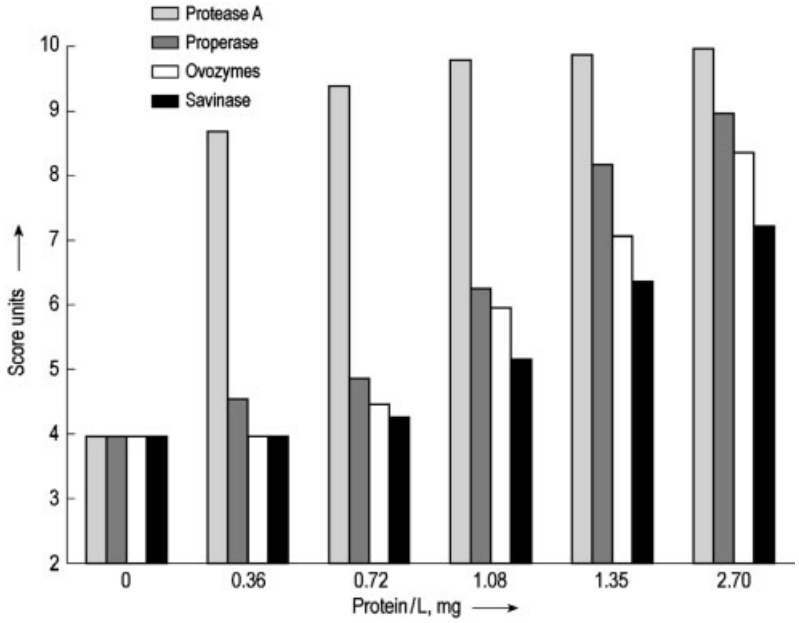

Fig. 77 Efficiency of different proteases on minced meat at 55 °C

the best performance in all three criteria compared with the other proteases. Furthermore, the fact that this high performance level is already reached at low protein contents indicates a high turnover. This effect grows even stronger when the temperature is reduced from 55 to 45 °C (Fig. 78).

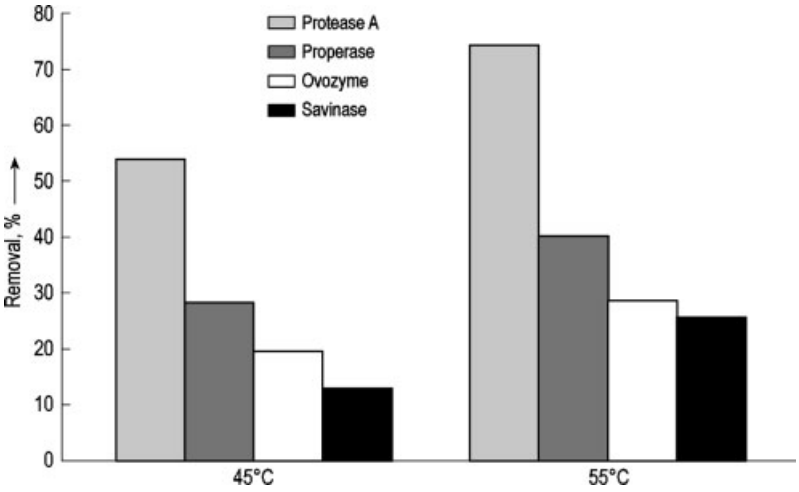

Fig. 78 Performance of different proteases (1,35 mg protein / L) on egg yolk at 45 and 55 °C

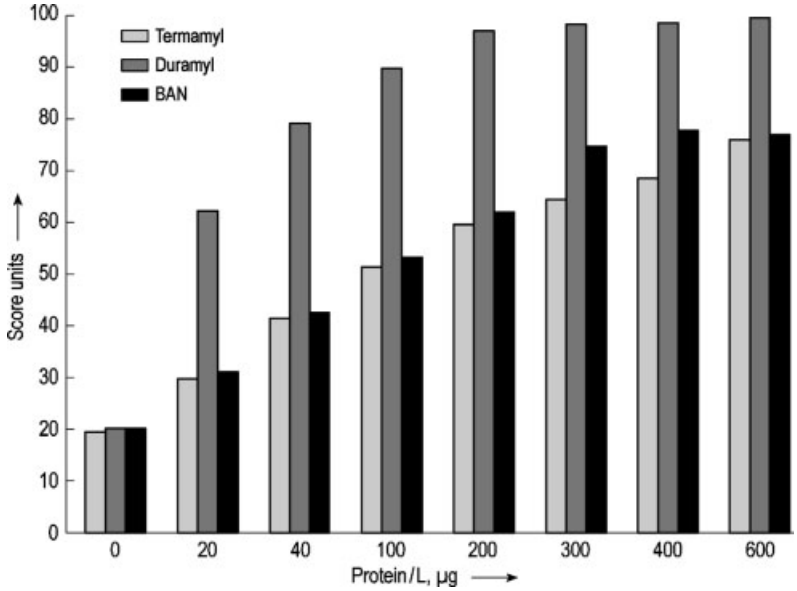

Fig. 79 Efficiency of different amylases on starch mix at 55 °C

Moreover, the efforts to improve protease performance in ADD by using protein engineering can also be recognized, as three of the four proteases are genetically modified (see Section 5.2.2.3.2), and the wild-type protease gives the poorest results.

In the field of amylases a similar trend as for proteases can be observed. By using protein engineering the performance profile of a-amylases has been improved step by step over the years (Figs. 79, 80 and 81) and thereby a better fit to the requirements of

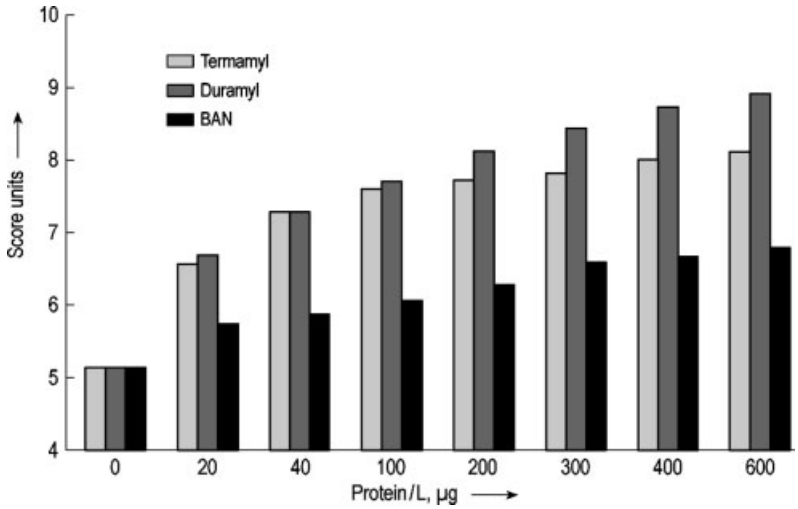

Fig. 80 Efficiency of different amylases on porridge at 55 °C

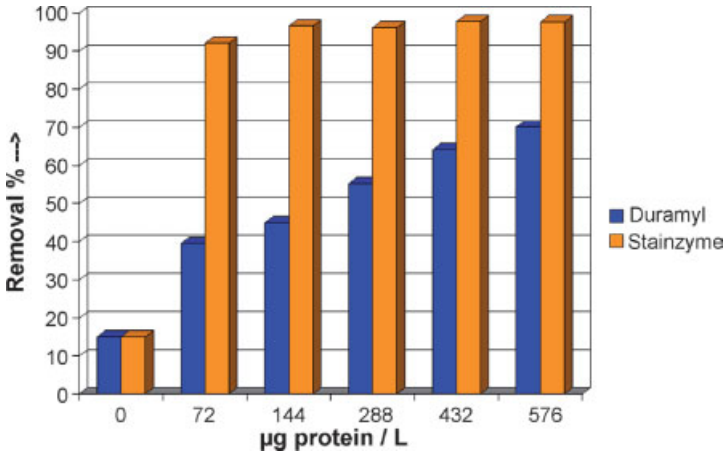

Fig. 81 Efficiency of different amylases on starch mix at 45 °C

ADDs is evident. In a first step the modification of a-amylases according to WO 94/02597 [688] and WO 94/18314 [689] increased the performance, especially on starch mix at 55 °C (Fig. 81), compared with wild-type a-amylases, whereas the superiority of this amylase on porridge only becomes evident with increasing protein content. These amylases are sold under the trade names Duramyl and Purastar OxAm, respectively. Further modification of a-amylase with regard to calcium dependency, specific activity, and/or substrate specificity led to significant improvement at lower temperatures (e.g., 45°C, Fig. 81).

Summarizing all these results a clear statement can be given:

- A significant increase of the performance of modern automatic dishwashing detergent is detectable when using protease and α-amylase. This advantage is especially apparent when comparing ADDs with and without enzymes.
- A major contribution to this effect is based on the modification of protease and amylase by protein engineering and thereby improvement of their performance profile with regard to application and requirements in ADDs.

5.2.2.6.3 **Stability and Compatibility**

In addition to the aforementioned performance aspects, stability during storage is a further important aspect in the application of enzymes in automatic dishwashing detergents. For enzyme granulates, it is influenced by the formulating process of the granulate and of detergent in powder or tablet form. Here, especially the presence of bleaching systems plays an important role. The developments in ADDs, starting with a conventional powder, the one-phase tablet as the next step, and finally the two-phase ADD tablets, which are nowadays the leading ADD product in Europe, makes the separation of enzymes and active oxygen carrier possible and thereby improves

the storage stability of enzymes. However, this requires enzyme granulates which are resistant to pressure. A further possibility of this two-phase concept [705] is the time-shifted release of enzymes and bleach, which is also in favor of the enzymes and their performance.

5.2.3 Enzymes in Grain Wet-Milling

5.2.3.1 Introduction

Wet milling of grain, in which the kernels are soaked and then separated into their individual components by grinding and centrifugation, is one of the highest volume processes for converting agricultural raw materials to finished products. The vast bulk of the grain that is wet-milled is maize (corn), and most wet milling occurs in the American Midwest, primarily in the states of Iowa, Illinois, and Indiana, where most of the corn is produced. The main products made in the USA and sold to consumers outside the corn-milling industry, including exports, in 10^6 t/a on a dry or pure basis in 2002 are fuel ethanol (10.4), high-fructose corn syrup (HFCS, 8.3), starch (3.8), and glucose syrup (2.4) [711].

Corn wet milling requires a complex series of processes, as described in Section 5.2.3.2. Important among them are three enzymatic steps: the hydrolysis of starch to shorter maltooligosaccharide chains (dextrin) with α-amylase, the further hydrolysis of dextrin to glucose with glucoamylase, often with some pullulanase added, and the isomerization of glucose to a near-equilibrium mixture of glucose and fructose with glucose isomerase. Because of the large scale of these processes, the enzymes used in them, along with the proteases and amylases used in synthetic detergents, are produced in tonnages higher than any other enzymes.

Several other enzymes, such as β-amylase and maltogenic α-amylase, are used in various peripheral processes but are not described here.

5.2.3.2 Overview of the Conversion of Corn to HFCS

There are six main steps in the conversion of corn to starch by wet milling and a further five to convert starch to HFCS (Fig. 82):

1. Steeping of corn kernels in a sulfur dioxide solution to soften them,
2. Coarse grinding of the kernels to liberate the germ, which contains oil, from starch, fiber, and protein,
3. Cyclone separation to separate the germ,
4. Fine grinding to liberate starch from fiber and protein,
5. Screening to separate fiber from protein and starch,
6. Centrifugation to separate protein from starch,
7. Hydrolysis of starch with α-amylase to form dextrin,

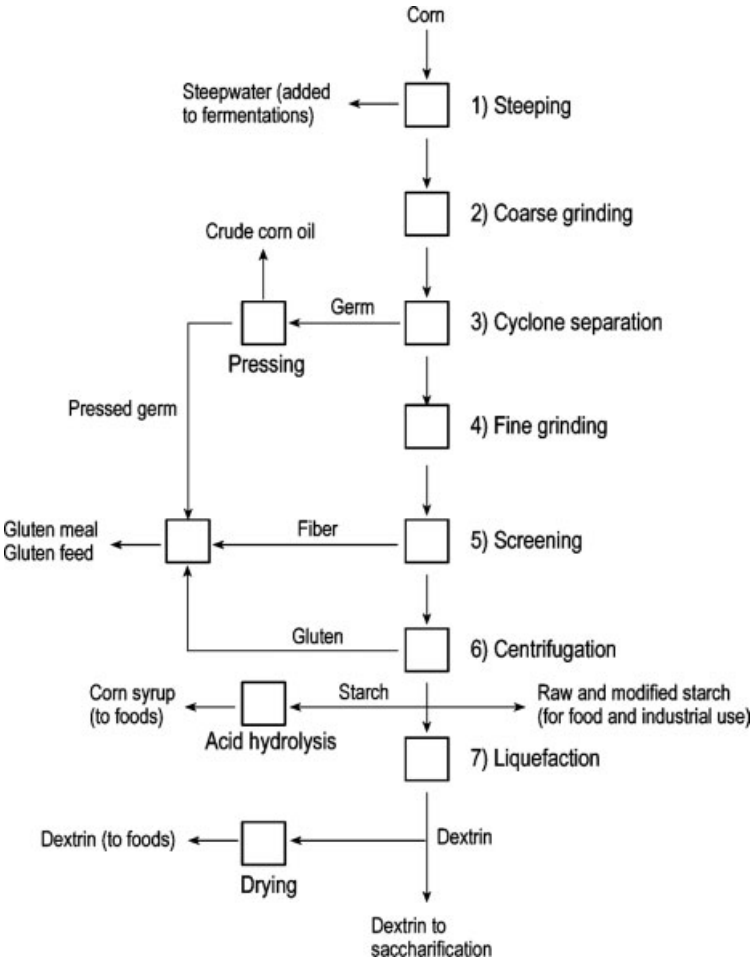

Fig. 82 Schematic of the main steps of the corn wet milling process.

8. Hydrolysis of dextrin with glucoamylase and often with a little pullulanase to form glucose,
9. Isomerization of glucose to a glucose–fructose mixture with glucose isomerase,
10. Separation of fructose from glucose and other products by liquid chromatography,
11. Blending of the fructose with some of the glucose and byproducts to give HFCS 55 (55 % fructose).

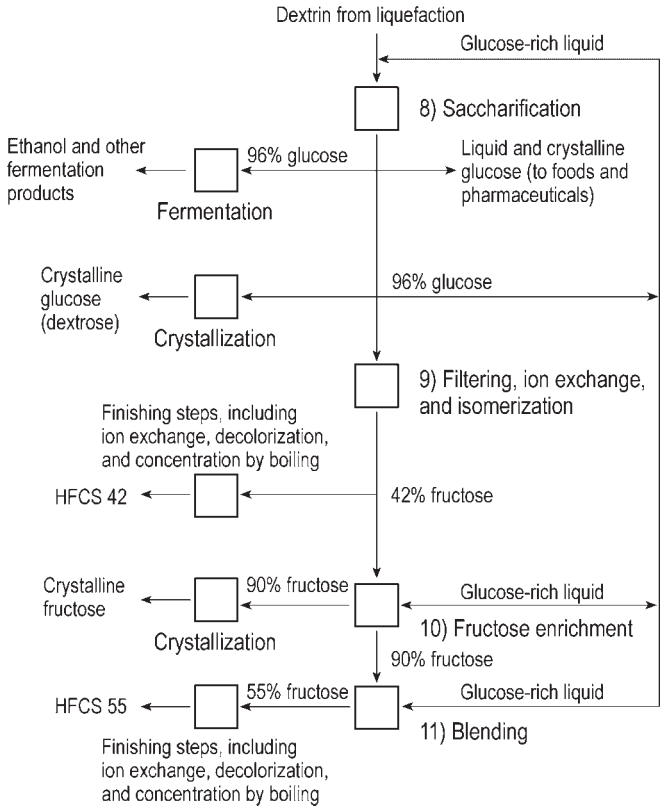

Fig. 82 (continued)

5.2.3.2.1 **Corn Steeping**

Yellow No. 2 corn, the chief grade grown in the U.S. Midwest, is soaked for 24–36 h in large tanks filled with a weak aqueous sulfur dioxide solution (Step 1). A fermentation that produces lactic acid occurs in the tanks. In general several tanks are used, with those containing the corn steeped the shortest length of time having the oldest solution, and those having the corn that has been steeped the longest having fresh solution. This is arranged by having each tank hold corn soaked for different lengths of time and by passing the steeping solution from the tank holding corn for the longest time to the tank holding it for the shortest time.

The goal of steeping is to soften the kernels, which originally contain between 10 and 15 % water. Steeping also loosens the components of the kernel, which is composed of the germ, a small oil- and protein-rich structure; the endosperm, the largest part of the kernel, which contains mainly microscopic granules of starch; and the pericarp, or hull, which is composed mainly of fiber and protein. The steeping solution, known as steepwater or corn steep liquor, is a valuable addition to many fermentation processes, since it is rich in vitamins and growth factors.

5.2.3.2.2 Coarse Grinding and Germ Removal by Cyclone Separation

Coarse grinding (Step 2) of the softened corn is designed to break the kernel into many pieces without destroying the germ. The germ is separated in cyclones (Step 3), since it is lighter than the other kernel components. The oil it contains is pressed out and refined, and the remainder of the germ, which is protein-rich, usually is combined with the gluten feed and meal produced in Step.

5.2.3.2.3 Fine Grinding and Fiber Removal by Screening

Fine grinding of the remaining contents of the kernel (Step 4) is designed to separate the starch and protein as much as possible from the fiber. Filtering over screens (Step 5) removes fiber, largely the remains of the corn hulls, from starch and protein, since it is in larger pieces. The fiber always contains some starch and a little protein that cannot easily be separated from it. This fraction usually is combined with the gluten feed and meal produced in the next step.

5.2.3.2.4 Centrifugation and Washing to Separate Starch from Protein

The starch and protein fractions, neither of which dissolve in water, can be separated from each other by centrifugation (Step 6). The starch is washed many times by successive water addition followed each time by dewatering in cyclone separators. This brings its purity to above 98 %, the remaining impurities mainly being contained within the starch granules themselves.

The protein, or gluten, fraction is used along with the fiber and that part of the germ from which the oil has been pressed for animal feed, mainly in two different products, gluten meal and gluten feed, of different protein content.

The starch can be dried and sold at this point, to be used as it is or after being modified by chemical processes, or it can be further processed as described below to give dextrin, glucose, ethanol, and HFCS. Some of the starch may be acid-hydrolyzed to make a series of corn syrups. How much of each product is made is at the discretion of the producer.

5.2.3.2.5 Hydrolysis with α-Amylase (Liquefaction)

Starch is contained in microscopic granules and is composed of long chains of glucosyl residues linked by α-(1→4) glycosidic bonds, with branches in its amylopectin fraction being initiated by α-(1→6) glycosidic bonds. Although α-amylases are capable in nature of attacking starch granules, in industrial practice steam under pressure is used to dissolve (liquefy) them as, simultaneously, highly stable bacterial forms of α-amylase hydrolyze α-(1→4)-glycosidic bonds in the now greatly increased number of accessible chains (Step 7). Since α-amylases in general attack interior bonds of these chains faster than those near chain termini, a mixture of products with intermediate numbers of glucosyl residues (dextrin) is formed in preference to glucose or maltose.

The two reasons for using α-amylase are (1) to make the starch chains shorter so that they will not form a gel if the temperature is decreased, and (2) to make more chain ends, since glucoamylase, the enzyme used in the next step, cleaves glucose molecules only from the nonreducing ends of the chains. A more detailed description of this process can be found in Section 5.2.3.3.

At this point the producer can sell the dextrin as a commercial product after drying or further process it, as described in the following sections 5.2.3.3.

5.2.3.2.6 **Hydrolysis with Glucoamylase (Saccharification)**

Glucoamylase, often called amyloglucosidase or AMG in the industry, and often pullulanase are added to dextrin, which is composed of chains of moderate length, mainly between one and twenty glucose molecules, linked by α-(1→4) glycosidic bonds (Step 8). There are occasional branch points initiated by α-(1→6) glycosidic bonds. The glucoamylase breaks the main bonds in the chain, and both glucoamylase and pullulanase break the branch points. This hydrolysis leads to about 96 % yield of glucose, with about 4 % of the original dextrin being found as other products, some coming from the liquefaction step and the majority from the saccharification step.

A more detailed description of the process is given in Sections 5.2.3.4 and 5.2.3.5.

The glucose solution is subjected to ultrafiltration or rotary precoat filtration to remove solids, while soluble impurities and salts added during the α-amylase step and formed during the change of pH in the glucoamylase step are removed by carbon and ion exchange, respectively. The producer has a choice at this point to divert the glucose solution to fermentation processes to make ethanol or other products. The glucose solution may also be evaporated and crystallized, giving a solid often sold under the name dextrose. Alternatively it can be converted to HFCS, as described for Step 9 (Section 5.2.3.2.7).

The glucose solution diverted for ethanol production is fermented with yeast. The liquid fermentation mixture is distilled successively in three columns, each increasing the ethanol concentration by separating water, yeast cells, and dissolved matter, so that the ethanol finally reaches almost complete purity. This can be mixed with gasoline to make automotive fuel, serve as raw material for a number of industrial products, or used in alcoholic beverages. Corn millers making both HFCS and ethanol have more process flexibility than those making only one or the other.

5.2.3.2.7 **Isomerization with Glucose Isomerase**

Glucose solution is passed through a column containing glucose isomerase immobilized on porous solid particles (Step 9). Since the enzyme is much more expensive than α-amylase or glucoamylase, it must be immobilized so that it remains in the column to be used for long periods rather than being dissolved in the sugar solution. However, glucose isomerase does lose activity over time, so often several columns are used side-by-side, with the column holding the freshest glucose isomerase, that with the highest activity, having the highest rate of flow through it, and that with the oldest glucose isomerase having the lowest flow rate. This is done so that each column gives about the same fructose yield.

Glucose isomerase converts glucose to fructose by isomerization. The reaction does not yield only fructose, since glucose and fructose have a roughly equimolar equilibrium. Instead the product is a mixture of about 50–53 % glucose, 42–45 % fructose, and 5 % other products.

A more detailed description of process kinetics and conditions can be found in Section 5.2.3.6.

The producer can either subject this mixture to finishing steps including ion exchange, decolorization, and evaporation to give HFCS 42, or can enrich it to increase its fructose content.

5.2.3.2.8 Fructose Enrichment and Blending

The sugar mixture produced in the previous step can be passed through a column containing strongly acidic ion-exchange particles with Ca^{2+} counterions (Step 10). These separate the fructose from the glucose and other products, with the fructose-rich stream, whose sugars are 90 % or more fructose, exiting the column last. This mixture can be evaporated and crystallized to give pure fructose crystals. That part of the fructose-rich stream that is not subjected to crystallization is sent to the next step. Streams rich in glucose and other products can either be recycled to earlier steps or sent to Step 11.

The high-fructose stream that is not crystallized is blended with streams rich in glucose and other products from Steps 8, 9, and 10 to bring its sugar content to about 55 % fructose (Step 11). Various finishing steps then make HFCS 55. They include ion exchange to remove salts, decolorization with charcoal, and evaporation of water to bring the sugar concentration from about 45 % to over 70 %, so that the finished product is microbially stable and can be shipped with less water.

HFCS 55 is virtually identical in sweetness, taste, and mouth feel to an equivalent concentration of sucrose, and since under present U.S. trade policy it is nearly always cheaper than sucrose, it has captured over half the U.S. natural sweetener market.

5.2.3.3 α-Amylase

5.2.3.3.1 Origin and Enzymatic Properties

α-Amylases (1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1) are found throughout the living world. All forms cleave α-(1→4) glycosidic bonds in starch, glycogen, and maltooligosaccharides to give shorter maltooligosaccharides and little glucose, so α-amylases are classified as endo-hydrolases. Hydrolysis rates are faster when these bonds are located away from chain termini. This is because α-amylase active sites are clefts without barriers at either end, so that substrate chains can be bound with different α-(1→4) glycosidic bonds adjacent to the enzyme cleavage point. Productive binding near chain termini is less likely since fewer glucosyl residues are bound, resulting in lower binding energies and hence lower binding equilibrium constants. Longer maltooligosaccharide chains are hydrolyzed faster than shorter ones, with higher values of ν_{max} and lower values of K_M [712–715], for the same reasons.

In general α-amylases can cleave α-(1→4) glycosidic bonds sufficiently distant from an α-(1→6)-initiated branch on a maltooligosaccharide chain and on either side of it, but they cannot attack α-(1→6) glycosidic bonds. With *Bacillus amyloliquefacians* α-amylase, a close relative of the *Bacillus licheniformis* α-amylase presently used in industry, the smallest molecule remaining after prolonged incubation is 6^2-α-maltosylmaltotriose (a maltosyl residue linked through an α-glycosidic bond to the 6-OH group of the middle glucosyl residue of maltotriose) [716]. This indicates that the three α-(1→4) glycosidic bonds immediately adjacent to the branch point are totally resistant to attack by this α-amylase. Shorter, but still lengthy, incubation leaves the hexaoses 6^2-α-maltosylmaltotetraose (linkage through the second glucosyl residue from the reducing end), 6^3-α-maltosylmaltotetraose, and 6^2-α-maltotriosylmaltotriose [717]. Attack on any chain is processive (multiple attack); a single chain is cleaved several times before being released [718]. All α-amylases yield products by the retaining mechanism [719], [720], which means that the anomeric hydroxyl group that is formed upon hydrolysis has the same α configuration as the bond that was hydrolyzed. Ca^{2+} is required for activity and stability.

5.2.3.3.2 **Structure**

α-Amylases are the most numerous members of glycoside hydrolase Family 13 [719]. There are references to enzymes with α-amylase activity in other families, but they are not of commercial interest, and therefore they are not discussed here. Although most enzymes in Family 13 are hydrolases that cleave α-(1→4) glycosidic bonds or in a few cases α-(1→6) bonds between glucosyl residues (E.C. 3.2.1.–), some are glucosyltransferases (E.C. 2.4.1.–), and one is a trehalose synthase (E.C. 5.4.99.16) [719]. Considering that glycoside hydrolase families are classified on the basis of similarity in amino acid sequences (primary structures), it follows that these different enzymes have arisen through divergent evolution.

Tertiary structures have been determined for α-amylases from the bacteria *B. licheniformis* (Protein Data Bank structures 1BPL [721] and 1VJS (Fig. 83) [722]), *Bacillus* (recently reclassified as *Geobacillus*) *stearothermophilus* (1HVX) [723], *Bacillus subtilis* (1BAG) [724], *Pseudoalteromonas haloplanctis* (1AQM) [725], and *Thermoactinomyces vulgaris* (1JI1) [726]; the fungi *Aspergillus niger* (2AAA) [727] and *Aspergillus oryzae*

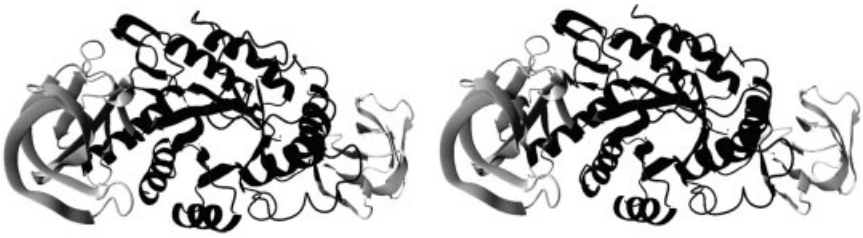

Fig. 83 Stereo view of the tertiary structure of unligated *B. licheniformis* α-amylase. Domains B and C are gray, with B to the left and C to the right (1VJS) [722].

(7TAA) [728]; the plant *Hordeum vulgare* (1AMY) [729]; the insect *Tenebrio molitor* (1JAE) [730]; and the mammals *Homo sapiens* [both pancreatic (1JXJ) [731] and salivary forms (1HNY) [732]] and *Sus scrofa* (1PPI) [733] (other structures and articles can be accessed elsewhere [719]). Tertiary structures for maltogenic α-amylases (EC 3.2.1.133) from *G. stearothermophilus* and *Thermus* sp. and the maltotetraose-forming exo-amylase (E.C. 3.2.1.60) from *Pseudomonas stutzeri*, as well as from enzymes with less closely related specificities, are also available.

All Family 13 α-amylases so far known have three domains: A, the catalytic domain, containing a $(\beta\alpha)_8$ barrel structure, as their cores are composed of eight β-strands surrounded by eight α-helices; B, a long loop with a β-strand between β-strand 3 and α-helix 3 of Domain A that binds Ca^{2+}; and C, a β-sheet. Family 13 members all have aspartate residues as their catalytic bases/nucleophiles and glutamate residues as their catalytic acids/proton donors [719]. The two catalytic residues in hydrolases with retaining mechanisms, such as α-amylases, are approximately 6.5 apart, except in very unusual cases.

The *B. licheniformis* α-amylase, the one most used in industry, contains 483 amino acid residues and has a protein molecular mass of 55 268 Da. Its Domain A encompasses residues 1–103 and 207–396, while Domain B comprises residues 104–206. Residues 397–483 are in Domain C [722], which in this case is composed of eight β-strands that appear to bind granular starch [722]. A comprehensive account of the alignment of Domains A and B across different species and their evolution can be found elsewhere [734].

Enzyme domains that bind carbohydrates rather than having catalytic activity have been classified by their amino acid sequences into different families of carbohydrate-binding modules, of which four encompass starch-binding domains (SBDs) [719]. α-Amylases from different sources have SBDs in each of the four families, with many, including that from *B. licheniformis*, still unclassified. Those from the bacterial genera *Bacillus*, *Streptomyces*, *Thermoanaerobacter*, and *Thermomonospora* and the fungal genera *Aspergillus* and *Cryptococcus* are members of Family 20, along with SBDs from glucoamylases, cyclomaltodextrin glucanotransferases, β-amylases, maltotetraose-forming exo-amylases, maltogenic α-amylases, and other hydrolases. These domains are found at the C-termini of the catalytic domains. Although no tertiary structure of an α-amylase SBD is available, there are sufficient structures available from other Family 20 members to strongly imply that those from α-amylases are β-sandwich folds composed only of β-strands and associated loops.

Tertiary structures have not yet been determined for any members of the other three SBD families. Family 21 contains SBDs from an α-amylase from the fungus *Lipomyces kononenkoae*, along with those from two glucoamylases and several different protein phosphatases [719]. Family 25 has SBDS from *Bacillus* and *Clostridium* α-amylases and from various β-amylases and maltopentaose- and maltohexaose-forming amylases, while Family 26 has SBDs from α-amylases from the bacterial genera *Bacillus*, *Butyrivibrio*, *Lactobacillus*, and *Streptococcus*, and from maltopentaose- and maltohexaose-forming amylases.

The fact that closely related strains produce α-amylases with SBDs from different families (for instance, different *Bacillus* α-amylases have SBDs from three different

families) suggests that the acquisition of SBDs is a relatively recent occurrence, more recent than the evolution of α-amylases themselves.

5.2.3.3.3 Industrial Use

A slurry of starch granules is mixed with *B. licheniformis* or *G. stearothermophilus* α-amylase, and then steam is added through an orifice at high velocity into a tube through which the slurry is flowing so that mixing of the two streams is complete. The mixture is held at about 105 °C and pH 5.6–6.5 for several minutes as it passes through the tube. This dissolves the granules and exposes the starch chains to α-amylase, which breaks them into shorter maltooligosaccharide chains. This dextrin mixture is often held in a tank for another hour or more at about 90°C, sometimes with more α-amylase added.

If glucose or HFCS is the desired final product, then the reaction mixture is hydrolyzed to a DE (dextrose equivalent) value of 10 to 15, where DE is defined as a percentage of reducing power to that of glucose, glucose and starch therefore having DE values of 100 and close to zero, respectively. Hydrolysis to DE < 10 leaves the mixture susceptible to retrogradation, in which the resulting long maltooligosaccharide chains form a pseudocrystalline mass. On the other hand, if the mixture is hydrolyzed for a sufficiently long time at high temperature so that its DE exceeds 15, various other products, especially maltulose [α-D-glucopyranosyl α-(1→4)-D-fructose], accumulate through base-catalyzed rearrangement of the reducing-end glucosyl residue to a fructosyl residue. The combined rate of acid-, base-, and water-catalyzed glucose rearrangement is lowest at approximately pH 4.5, but the α-amylases used industrially have optimal pHs for activity and thermostability between 6 and 7, so a balance between these two optimal pHs is chosen to best minimize rearrangement and enzyme inactivation and to maximize enzyme activity, so that the reaction time is minimized to reduce later losses of glucose and fructose yield.

If the desired final product is a solid dextrin mixture or a moderate-DE syrup, up to DE 42, then various mixtures of α-amylase and acid are used to achieve this. Total reaction times increase with increasing DE.

5.2.3.4 Glucoamylase

5.2.3.4.1 Origin and Enzymatic Properties

Glucoamylases (1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.3) are found only in bacteria, archaea, and fungi. They attack the nonreducing ends of maltooligosaccharide chains to produce glucose. Glucoamylases can cleave both the α-(1→4) and α-(1→6) glycosidic bonds in these chains, as well as every other α-glycosidic bond between two glucosyl residues except that in α, α-trehalose (α-D-glucopyranosyl α-D-glucopyranoside) [735]. Hydrolysis rates increase with increasing substrate chain lengths [736], [737], but given sufficient time and sufficiently low concentrations of dissolved solids, glucoamylases will completely convert maltooligosaccharides to glucose. Glucoamylases are very slow enzymes; this is compensated by the large amounts produced by many fungi. Their initial product is β-glucose, so they act by an inverting mechanism.

At the high concentrations of dissolved solids that are present in industrial saccharifications, glucoamylases will produce substantial amounts of di-, tri-, and

tetrasaccharides by condensation of glucose to the nonreducing ends of glucose and di- and trisaccharides [738]. Although glucoamylases hydrolyze α-(1→4) glycosidic bonds and therefore form them at much higher rates than the cleavage or formation of any other glycosidic bonds, thermodynamics favor formation of α-(1→6) glycosidic bonds because of their one extra degree of freedom. For that reason and because during most of the saccharification glucose is the major component of the reaction mixture, isomaltose [α-D-glucopyranosyl-(1→6)-D-glucose] is the chief condensation product, followed by isomaltotriose [738].

5.2.3.4.2 **Structure**

Glucoamylases are essentially the sole members of glycoside hydrolase Family 15 [719]. Only three tertiary structures of the catalytic domain have been described, one each from the filamentous fungus *A. niger* (1GAH) [739], [740], the yeast *Saccharomycopsis fibuligera* (1AYX) [741], and the bacterium *Thermoanaerobacterium thermosaccharolyticum* (1LF9) [742]. All glucoamylase catalytic domains have $(\alpha,\alpha)_6$ barrel structures, with six inner α-helices being surrounded by six outer ones [739], [743] (Fig. 84). In eukaryotic forms a peripheral thirteenth α-helix is present. The active site is a conical well, and this explains why glucoamylases act as exo-hydrolases and why they are more active on longer rather than on shorter substrates, since several glucosyl residues at the nonreducing end of the substrate are strongly bound. This also explains why glucoamylases act by a single-attack mechanism and why they are so slow, with each chain being released from the active site after a single glucosyl residue is cleaved from it. All glucoamylases have glutamate residues as both their catalytic nucleophiles/bases and as their catalytic proton donors [719].

Most glucoamylases produced by filamentous fungi have SBDs connected to the C-termini of their catalytic domains by O-glycosylated linkers of variable lengths, with O-glycosylation extending into a thirty-residue belt at the C-terminus of the catalytic

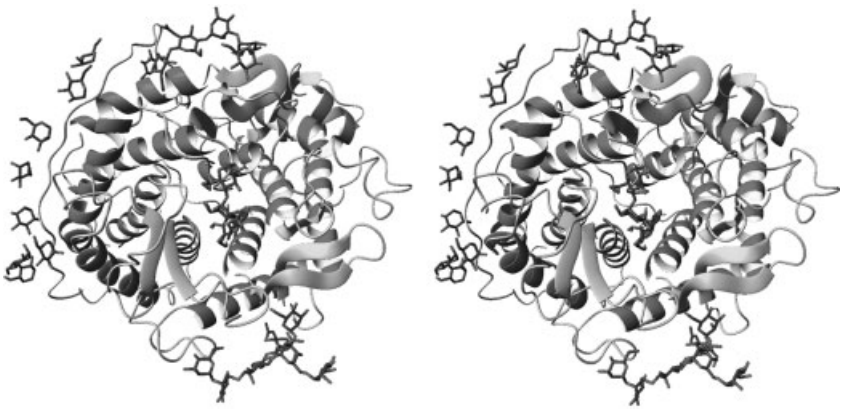

Fig. 84 Stereo view of the tertiary structure of the *A. awamori* (now classified as *A. niger*) var. X-100 glucoamylase catalytic domain with acarbose ligand (1GAH) [740]

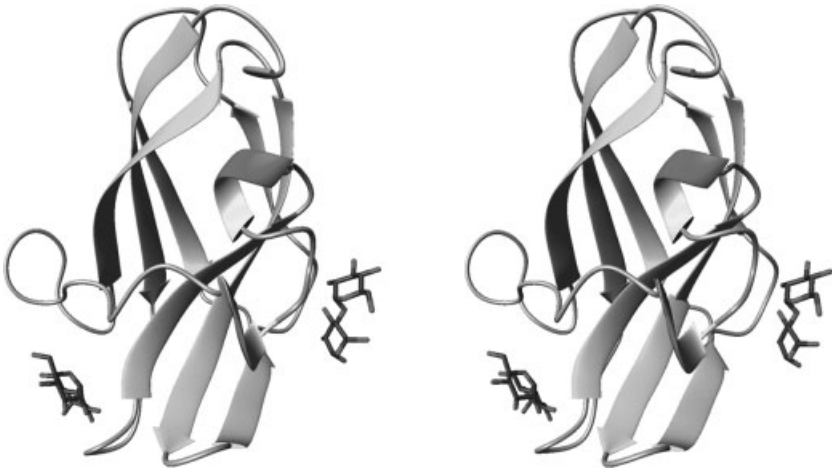

Fig. 85 Stereo view of the tertiary structure of the *A. niger* glucoamylase starch-binding domain with two maltose ligands (1KUM) [744]

domain [743]. These SBDs are members of carbohydrate-binding modulus Family 20 [719], as are those of many α-amylases, with a β-sandwich fold composed of β-strands and loops but no helices [744] (Fig. 85). The protein molecular mass of the *A. niger* glucoamylase used industrially is 65 790 Da, with that of the catalytic domain, residues 1–480, being 50 456 Da, that of the linker, residues 481–508, 3 454 Da, and that of the SBD, residues 509–616, 11 880 Da. N- and O-glycosylation of the enzyme varies among strains, and can add a further 12–15 % to the total molecular mass. The catalytic acid/proton donor of *A. niger* glucoamylase is Glu179 [745] and its catalytic base/nucleophile is Glu400 [746].

A. niger glucoamylase used industrially is a mixture of GAI and GAII species, the first containing all 616 amino acid residues, and the second only the first 512 residues. GAI can bind starch granules much better [747], since it contains the SBD, but in industrial practice this confers no advantage, since the two forms encounter soluble dextrin molecules, which they hydrolyze at the same rate [735], [736].

SBDs of glucoamylases produced by the filamentous fungus *Rhizopus oryzae* and the yeast *Arxula adeninivorans* are connected by O-glycosylated linkers to the N-termini of catalytic domains and are members of carbohydrate-binding modulus Family 21 [719], for which no tertiary structures are available. A recently determined tertiary structure of *T. thermosaccharolyticum* glucoamylase has an N-terminal domain of unknown function with eighteen antiparallel β-strands in two β-sheets arranged in a super-β-sandwich [742].

5.2.3.4.3 Industrial Use

Glucoamylase is used industrially to hydrolyze maltooligosaccharide chains of moderate length produced by α-amylase hydrolysis of starch. The enzyme is most stable and active around pH 4.0–4.5, and since this is also the pH of highest glucose stability, no

compromises need be made to operate there. Glucoamylase is appreciably less stable than α-amylase, rapidly unfolding above 60 °C, so it is used industrially at 55–60°C in soluble form, since it is quite inexpensive. This temperature is also sufficiently high to prevent microbial contamination of the reaction mixture.

The saccharification step is conducted over roughly 48 h at 27–30 % dry solids and is stopped when glucose concentration reaches its highest value at about 96 % of the total sugar content. Further operation leads to lower glucose yield, since condensation reactions continue until equilibrium is attained. Since these reactions are essentially second-order (first-order each in glucose and the acceptor), while hydrolysis reactions are essentially pseudo-first-order in maltooligosaccharide (the molar concentration of water is very high and is almost invariable), increased concentrations of dissolved solids lead to higher concentrations of condensation products. However, the lower concentrations of dissolved solids that would be necessary to appreciably increase the glucose yield would result in much more water having to be removed from the reaction mixture before shipping, an uneconomical task.

5.2.3.5 **Pullulanase**

5.2.3.5.1 **Origin and Enzymatic Properties**

Pullulanase (α-dextrin endo-1,6-α-glucosidase or pullulan 6-glucanohydrolase, E.C. 3.2.1.41) is named for its ability to hydrolyze the α-(1→6) glycosidic bonds in pullulan, a polysaccharide composed of repeating α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl units. However, it also attacks the α-(1→6) glycosidic bonds in β-limit dextrin, the α-(1→6)-bond-rich residue left upon extended hydrolysis of maltooligosaccharides, starch, or amylopectin by β-amylase. The enzyme also attacks glucoamylase-trimmed dextrins. Pullulanase is not effective at even moderately high dosages in hydrolyzing α-(1→6) glycosidic bonds in starch, amylopectin, or in starch hydrolyzed by α-amylase (liquefied starch). The exact chain length of glucoamylase-trimmed dextrins necessary to provide an effective substrate for pullulanase is not known, but based upon known substrates for pullulanase (e.g., β-limit dextrins), it is safe to assume that it is no more than two to four glucosyl residues. The smallest molecule pullulanase can produce when it cleaves α-(1→6) glycosidic bonds is maltose [748]. Pullulanases are found in many bacteria, especially those in the *Bacillus*/*Clostridium* group, in occasional archaea and yeasts, and in some plants, especially cereals [719].

5.2.3.5.2 **Structure**

Pullulanases are members of glycoside hydrolase Family 13, which is dominated by α-amylases. This classification is determined by primary sequence homology, and in fact no tertiary structure is available for any pullulanase. However, one would expect pullulanases to resemble α-amylases, with $(\beta,\alpha)_8$ barrel folds, but with the active site modified by divergent evolution so that α-(1→6) rather than α-(1→4) glycosidic bonds in molecules with a number of glucosyl residues would be hydrolyzed. There are reports of Family 20 SBDs being found in various bacterial amylase-pullulanases, but not yet in enzymes with strictly pullulanase function.

5.2.3.5.3 Industrial Use

Glucose yield from the saccharification step is limited by several factors:

1. The liquefaction step produces some materials, such as maltulose, that glucoamylase cannot further cleave;
2. Glucoamylase catalyzes condensation reactions that lead to formation of a number of di-, tri-, and tetrasaccharides, most containing α-(1→6) glycosidic bonds;
3. Glucoamylase rather slowly cleaves the α-(1→6) glycosidic bonds arising from the amylopectin fraction of starch and remaining in the maltooligosaccharide chains arriving from the liquefaction step. The maximal glucose yield occurs when condensation reactions have not yet substantially progressed but when incoming α-(1→6) glycosidic bonds have been largely hydrolyzed.

Pullulanase, since it hydrolyzes α-(1→6) glycosidic bonds in certain molecules containing a number of α-(1→4) bonds faster than does glucoamylase, allows a higher glucose yield than would be achieved by glucoamylase alone. It has no effect on the extent of condensation reactions, since it cannot attack isomaltose or isomaltotriose, the chief condensation products.

The addition of pullulanase to saccharification mixtures does not require a change in pH or temperature. However, its addition may allow higher contents of starch dry solids and shorter saccharification times.

Although the increase in glucose yield using pullulanase in saccharifications is only 0.3 to 0.5 %, pullulanase is widely used due to the competitive nature of commercial dextrose and HFCS production. Pullulanase is usually added to the glucoamylase preparation by the enzyme manufacturer.

5.2.3.6 Glucose Isomerase

5.2.3.6.1 Origin and Enzymatic Properties

A number of enzymes isolated from microbial sources over the past forty years have been called glucose isomerases. Technically however, they are D-xylose isomerases (E.C. 5.3.1.5, D-xylose ketol-isomerase) due to their substantially higher affinity for xylose than for glucose. All known xylose isomerases, in addition to isomerizing D-xylose to D-xylulose, also isomerize D-glucose to D-fructose. Since their only industrial application is the latter, they are referred to as glucose isomerases throughout this section.

Most if not all glucose isomerases require metal ions such as Co^{2+}, Mn^{2+}, Mg^{2+}, or Cr^{2+} for their catalytic activity [749]. A number of inhibitors of the enzyme are known, such as Ca^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Ag^{+}, sugar alcohols, and various sugar breakdown products. Both Ca^{2+} and the sugar breakdown products are present from previous steps, and it is commercially important to minimize their concentrations by ion exchange and adsorption processes.

The reaction catalyzed by soluble glucose isomerase follows simple reversible Michaelis–Menten kinetics, since the equilibrium state is approximately equal in glucose and fructose concentrations (at an initial glucose concentration of 2 M, equilibrium fructose concentrations expressed as a percentage of glucose plus fructose

range from about 48 % at 45 °C to 55 % at 85 °C), and because the Michaelis constants for glucose and fructose are also almost equal (0.25 M and 0.26 M for glucose and fructose, respectively, at 60 °C) [750]. Rate equations have been derived relating rate of fructose formation to rate coefficients, substrate and enzyme concentrations, and Michaelis constants for fructose and glucose [750]. The soluble *Streptomyces rubiginosus* enzyme is relatively fast, having a turnover number of approximately 7300 min^{-1}.

Kinetic equations have also been derived for use of an immobilized form of glucose isomerase in packed-bed reactors [750]. The equations relate enzyme performance to a reaction rate constant, and express enzyme stability as a function of operational variables such as substrate and product composition, flow rate through the reactor, residence time of the substrate in the reactor, and amount of enzyme in the reactor. A basic working equation can be derived relating glucose isomerase performance (productivity) to measurable operating parameters. Enzyme efficiency can be defined as the weight of fructose syrup solids produced per unit of enzyme when the degree of isomerization (fructose concentration) is held constant and the enzyme is used for a given number of half-lives. Efficiency can therefore be calculated as

$$\text{Eff} = \frac{1.44 k_f \tau (1 - e^{-0.693H})}{d \ln \frac{X_{eq} - X_0}{X_{eq} - X}}$$

where k_f is the rate coefficient expressed in terms of g (glucose + fructose) $IU^{-1} h^{-1}$, τ is the enzyme half-life in hours, H is the number of half-lives the reactor is on stream, d is the fraction of glucose and fructose in the total sugar solids in the substrate, and X is the fractional conversion of glucose to fructose:

$$X = \frac{F}{[F] + [G]}$$

X_{eq} is the fractional conversion at equilibrium, and X_0 is the fractional conversion at $t = 0$ [750].

5.2.3.6.2 **Structure**

Tertiary structures of many glucose isomerases are now known, including those from *Actinoplanes missouriensis* (1BHW, 1XIM–9XIM, 1XIN–3XIN, 5XIN) [751–755], *Arthrobacter* sp. (1XIA, 4XIA, 5XIA, 1XLA–1XLL) [756–759], *G. stearothermophilus* (1A0D) [760], *Streptomyces albus* (6XIA) [761], *Steptomyces diastaticus* (1CLK, 1QT1) [762], *Streptomyces murinus* (1DXI) [763], *Streptomyces olivochromogenes* (1XYA–1XYC, 1XYL, 1XYM, 2GYI) [764–766], *S. rubiginosus* (1XIB–1XIJ, 8XIA, 9XIA, 1XIS–4XIS; Fig. 86) [767–769], *Thermoanaerobacterium thermosulfurigenes* (1A0C) [760], *Thermotoga neapolitana* (1A0E) [760], *Thermus caldophilus* (1BXC) [770], and *Thermus thermophilus* (1BXB) [770]. So far all have been from bacteria, and it does not appear that glucose isomerases are found in any other part of nature.

Glucose isomerases are members of two different families. Those in Family I have about 390 residues, while members of Family II have about 440 residues. For instance,

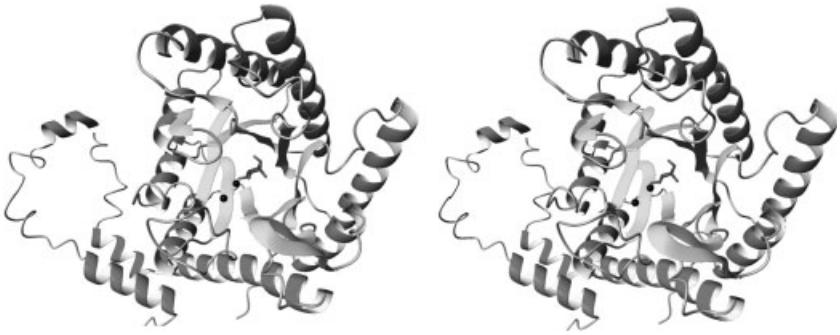

Fig. 86 Stereo view of the tertiary structure of *S. rubiginosus* glucose isomerase with glucose, Mg^{2+}, and Mn^{2+}, ligands (1XIF) [768]

the industrially important glucose isomerase from *S. rubiginosus* has a monomeric form with 388 residues and a molecular mass of 43 199 Da. All glucose isomerases for which tertiary structures are available, except those from *G. stearothermophilus*, *T. thermosulfurogenes*, and *T. neapolitana*, are members of Family I. This is not an indication that the extra residues confer thermostability, since glucose isomerases of several *Thermus* species belong to Family I.

Most if not all forms of glucose isomerase are homotetramers composed of two tightly bound dimers. Monomers have $(\beta,\alpha)_8$ barrels in which eight β-strands are surrounded by eight α-helices. The active site is in a deep pocket near the C-terminal ends of the β-strands. A C-terminal loop of about 65 residues with strong α-helical character binds to an adjacent subunit. Two divalent cations are required for activity; depending on the form, Co^{2+}, Mg^{2+}, or Mn^{2+} have different effects on activity, as well as on specificity between xylose isomerase and glucose isomerase activities.

The glucose isomerase mechanism consists of ring opening, with a cation hydrogen-bonded to O2 and O4 of the substrate, while the other cation binds to O1 after a substantial change of position. Isomerization occurs through a cation-mediated hydride shift from C1 to C2 and then ring closure to give the α-ketol [753], [754], [759], [766], [769].

5.2.3.6.3 Industrial Use of Glucose Isomerase

As mentioned in Section 5.2.3.2.7, enzymatic conversion of glucose syrup to an equilibrium mixture of glucose and fructose is carried out by passing glucose syrup through immobilized glucose isomerase in controlled-temperature reactors.

In the 1970s six immobilized glucose isomerases derived from *Streptomyces*, *Bacillus*, *Actinoplanes*, and *Arthrobacter* were being used commercially in the USA. Immobilization techniques included electrostatic adsorption, cross-linked lysed cells, whole cells entrapped in glutaraldehyde-cross-linked gelatin, flocculated whole cells, and glutaraldehyde-cross-linked whole cells. Today (ca. 2002) there are only four immobilized glucose isomerases, all derived from *Streptomyces*, in use in the USA. Two of the

enzymes are immobilized electrostatically on charged carriers (granular DEAE–cellulose and a silica-based material) and two are covalently cross-linked with whole or macerated *Streptomyces* cells by glutaraldehyde. This is, by a large margin, the largest use of immobilized enzyme in the world.

A reactor, usually a large column, is filled with the immobilized enzyme, kept in place by a perforated floor. Glucose solution containing a minimum of 93 % glucose based on total dry solids, which has been exhaustively refined by ion-exchange and carbon adsorption, is adjusted to 40–50 % dry solids, pH 7.5–7.8, 1.5 mM Mg^{2+}, and about 2 mM HSO_3^-. The solution is passed downwards through the reactor at a predetermined flow rate to produce 42–45 % fructose. Commercial glucose isomerases are relatively thermostable, being used at temperatures ranging from 50 to 65 °C, and more preferably about 58 °C, to provide optimal enzyme performance and stability and to prevent microbial growth. Typical reactor lifetime is on the order of twelve months, allowing it to produce many tonnes of HFCS.

Use of immobilized glucose isomerase in large reactors today (2003) is relatively problem-free. In the early days of HFCS production there were occasional problems with blinding, channeling, and high pressure drops in the deep-bed reactors due to breakdown of the enzyme particles or to insoluble impurities in the feed syrup. Reactor life could be shortened by improperly refined syrup that allowed inactivators to contact the enzyme, or by temperature or pH excursions. Today, consistent quality of the commercial enzymes and over 30 years of user experience in handling them have greatly reduced the cost of producing HFCS.

After exiting the reactor, the fructose syrup is immediately adjusted to a lower pH in the range of 4–5 to prevent unwanted formation of base-catalyzed byproducts and color. The syrup is refined by ion exchange and carbon adsorption before it is fractionated into glucose, oligosaccharides, and fructose.

5.2.3.7 **Use of Wheat Starch**

Due to various governmental policies, wheat starch rather than corn starch was in the 1990s, and still is, enzymatically converted to syrups in Europe. The amount of syrup made there is small relative to that produced in the USA.

Although wheat is intrinsically more expensive than corn, the production of vital wheat gluten destined for human consumption rather than corn gluten used for animal consumption at least partly compensates for the extra cost. However, to obtain this material, wheat must be dry milled rather than wet milled, as wet milling accompanied by steeping renders the gluten unusable by humans [771].

Starch from dry-milled wheat is obtained by washing it out of a thick flour water dough, leading to a quite pure A fraction and a much less pure B fraction contaminated with a nonstarch polysaccharide, together with a buoyant mixture containing gluten, bran, and fiber [771]. The polysaccharide contributes to high viscosity during starch hydrolysis, so commercial hemicellulases or xylanases may be used to reduce the viscosity. Furthermore, an accompanying lipid, lysophospholecithin, acts as an emulsifier in saccharified starch and prevents a clean break of the insoluble protein/lipid fraction from the soluble glucose fraction. The enzyme lysophospholipase is used, at least in some plants, to break the complex.

5.2.3.8 New Technology for Fuel Ethanol Production

As of 2005 the use of fermentation-derived ethanol as automotive fuel (fuel ethanol) has dramatically increased to a production level in the USA alone of about 15×10^9 L. Global production has reached about 45×10^9 L, of which about 60 % is produced from sucrose. In Europe and the USA most fuel ethanol is produced from grain such as corn and wheat.

Until recently, grain-derived fuel ethanol was produced by fermentation of glucose which was produced from either wet-milled starch or dry-milled, ground whole corn. The starch or dry, ground corn slurry was liquefied at high temperatures (e.g., 105°C in the presence of α-amylases) and then the starch dextrins were saccharified to glucose at a lower temperature (60°C, pH 4.5). Fermentation of the glucose to 12–18 % ethanol was carried out by addition of *Saccharomyces cerevisiae* yeast either before or after completion of saccharification.

Recently, trade journals and patent applications have disclosed technologies which purport to provide significant advantages over conventional fuel ethanol production. The primary advantages seem to be reduced energy usage and lower capital investment, made possible mostly by elimination of the high-temperature liquefaction process [772–778]. Although actual details of any new commercial processes are not revealed in the patent applications, the published commercial processes appear to be based on the use of finely ground corn or granular starch, and replacement of the conventional, separate high-temperature liquefaction and saccharification operations with a single, lower temperature treatment of granular starch. A blend of proprietary glucoamylases and α-amylases is used to convert the granular starch to glucose. This granular-starch conversion appears to be made possible by either development or discovery of new enzymes that have greater catalytic activity toward granular starch than those previously known. The combined liquefaction–saccharification operation appears to be carried out at temperatures of 50–65°C and a pH range of about 3–6. Although it is not entirely clear from the literature whether the novelty lies in new activity patterns for α-amylase, glucoamylase, or both, suffice it to say, a new combination of enzymes and modified process conditions appear to provide a low-temperature fuel ethanol production process which is gaining popularity. Reportedly, the amount of fuel ethanol produced by such new processes is in the range of $2.3–3.4 \times 10^9$ L.

5.2.4 Enzymes in Animal Feeds

5.2.4.1 Introduction

The use of enzymes in animal feed is now well accepted throughout the industry. In simple terms it has long been recognized that the addition of enzymes to animal feed will improve the efficiency with which animals digest the raw material.

The largest component of animal feed is in the form of cereal, and depending on the local availability this can be either a viscous cereal (wheat, barley, rye, or triticale) or nonviscous cereal (maize or sorghum). These cereals, with the addition of soybean meal and other protein sources, contain many antinutritional factors (ANFs), which restrict their value to the animal. Although animals produce their own endogenous enzymes, these are not sufficient to digest all the feed; for example, swine are unable to digest

approximately one-fifth of their daily ration. Feed supplemented by the addition of commercial enzyme products is used to overcome this problem, and as the understanding of the levels of ANFs present in the feedstuff and the effect they have on the animal performance increases, enzymes can be targeted more effectively.

In general terms there are a wide variety of reasons for using commercial enzyme products in animal feed:

1. To break down the ANFs inherent in the feedstuff, and therefore to improve the performance of the animal, either through a higher weight gain, or better feed utilization.
2. To break down the chemical structure of the cereal base of the diet, and thus release extra nutrients, which will increase the feeding value of the cereal.
3. To increase the availability to the animal of proteins, starches, and minerals from sources that are not usually accessible.
4. To supplement the endogenous enzymes of the young animal.
5. To reduce the variability inherent in feedstuffs, and increase the uniformity of the animals, which in turn increases profitability.
6. To reduce the environmental pollution caused by the excretion of undigested feed, especially phytase, which reduces phosphorus excretion.

Enzymes were first used commercially in animal feed in Finland in the 1980s when a β-glucanase was added to a barley-based feed for poultry, and it has been the poultry industry that has seen the biggest use of feed enzymes. The swine industry is following this trend, and to a lesser extent the ruminant sector. The size of the animal feed production industry means that there is huge potential for enzymes, and the current penetration rate of approximately 10 % of all monogastric feeds [779] shows that there is room for growth. However, in the EU the level of use is already very high, with the majority of poultry feed already containing enzyme supplements.

Enzymes used in animal feed must be able to attack the target substrate relatively quickly, as the passage through the animal's digestive system is rapid, particularly for broiler chickens. The enzymes must also be able to work under the conditions of pH associated with the gastrointestinal tract, as well as being able to withstand the processing regime of the feed before it is fed to the animals. Most feed is pelleted, a process that involves conditioning of the feed with steam, followed by pressing through a die. The temperature at which this process occurs varies between 65 and 100 °C. Enzymes can be added directly to the feed as a dry product, in a premix containing vitamins and minerals, or applied after pelleting as a liquid if the regime of the feed mill is too harsh and may cause a loss of enzyme activity.

This section concentrates on the enzymes currently available (ca. 2003) for use in animal feed, their mode of action, and methods of analysis. There is still some debate as to the exact method by which the enzymes bring about the benefit to the animals, but that is outside the scope of this article. A more detailed treatment of the use of particular enzymes in animal diets is given in recent comprehensive reviews [780], [781].

5.2.4.2 Enzymes Used in Animal Feed

For the purposes of this section the enzymes currently used are discussed on the basis of their target substrates.

5.2.4.2.1 Fiber-Degrading Enzymes

In viscous diets that contain a large proportion of wheat, barley, rye, or triticale the main antinutritive factor is the high fiber content of the diet. Poultry and swine do not have the endogenous enzymes needed to degrade fiber and thus rely on supplementation of their diet. The fiber present consists mainly of nonstarch polysaccharides — arabinoxylan (pentosan) in wheat and rye, and β-glucan in barley and oats — with the levels of each depending on many factors, including environment, temperature, and species.

The nonstarch polysaccharides are antinutritive because in the soluble form they can increase the viscosity of the digesta in the animals' small intestine and thus reduce the degree and rate of nutrient digestion and decrease the animals' performance.

Enzymes targeted against these antinutritional factors formed the initial basis of commercial products, with xylanases aimed at the arabinoxylan backbone of wheat-based diets, and β-glucanases at the β-glucan backbone of barley.

Xylanases Xylanases or endo-1,4-β-xylanases (E.C. 3.2.1.8) are the most commonly used feed enzymes [782]. Their target substrate is the xylan structure found in plant cell walls (Fig. 87). This consists of a β-1,4-linked D-xylose backbone that has side chains of α-arabinose, and varying degrees of substitution, for example, with ferulic acid, galactose, or coumaric acid [783]. The breakdown products of the xylanase are short-chain sugars.

The mode of action by which xylanases increase the digestibility of cereals has been the subject of debate. One theory is that xylanases release starch and protein molecules that are held within the xylan structure, and these are then utilized by the animal. The other theory is that the cell wall components are dissolved under conditions present in the digestive tract of poultry and swine, and then form viscous aggregates. This increase in viscosity in the intestine is a problem particularly in poultry [782], and leads to a reduction in digestive efficiency. In this theory xylanase works by reducing the viscosity in the gastrointestinal tract, as the oligomers that are produced by the action of the enzyme are less viscous, and therefore allow an increased rate of digestion.

Xylanases are produced by many bacteria and fungi. The source of xylanases for use in animal feed is mainly fungal, with species of *Trichoderma* and *Aspergillus* being among the most commonly used. *Trichoderma* xylanases have been shown to have

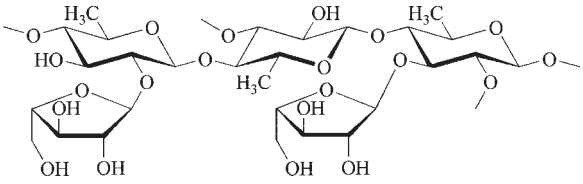

Fig. 87 Schematic xylan structure

optimum temperature ranges between 45 and 65 °C and pH ranges between 3.5 and 6.5 [784]. Bacterial xylanases, mainly from *Bacillus* species, have been investigated and some are available commercially. The catalytic activity of the xylanases differs from source to source, and in vitro data will depend on the substrate used for characterization.

The benefits of adding a xylanase to a wheat-based diet can be seen in Table 29, which demonstrates the addition of a *Trichoderma* xylanase to a wheat-based piglet diet at a rate of 5000 units per kilogram of feed. The results show an increase in the final weight of the piglet, and a reduction in the feed:gain ratio, both of which have large economic implications for the farmer. In broilers, increased weight gain, an improvement in feed conversion, and a reduction in gut viscosity are routinely observed.

Table 29. Piglet trial to show the effects of a xylanase product in wheat-based diets (Finnfeeds International)*

	Wheat control	**Wheat** + *Trichoderma xylanase*
Initial weight, kg	10.3	10.3
Final weight, kg	22.6	26.6
Daily gain, g	352	466
Daily feed intake, g	724	794
Feed:gain ratio	2.05	1.71

*Pietran x DL pigs were used in the growth trial, with 9 replicates per treatment and 2 pigs/pen. Castrates and gilts were evenly represented. All diets (Table 30) were pelleted (ca. 80 °C) and offered *ad libitum*.

Table 30. Diet composition for trial shown in Table 29

Diet	**kg/t**
Wheat	400
Rye	200
Barley	200
Soybean meal	100
Fishmeal	70
L-lysine HCl	2
DL-Methionine	1
L-Threonine	1
Tryptophan	0.5
Vitamins and minerals	25
Protein, %	19.9
Total lysine, %	1.12
Digestible lysine %	0.94
Total NSP* (soluble) %	11.3 (6.4)
DE,** MJ/kg	13.6

*NSP = non-starch polysaccharide.
**DE = digestible energy

Assaying feed xylanases is important both in the product and after addition to the feed. The analysis is needed for quality control of the products and to allow traceability through the feed manufacturing process.

As a number of xylanase products are available, there are a number of assays, each with their own definition of a xylanase unit. For product analysis the most common method is the colorimetric dinitrosalicyclic acid (DNS) assay [785] or the dye-linked method of [786]. Within these the pH, temperature, and substrate used all differ. Therefore, it is difficult to compare two xylanases using a standard assay.

Measuring the enzyme after it has been added to the feed and the feed has been processed is more difficult. Any method used will have to overcome the following problems:

1. Low activity in the finished feed. A typical xylanase product (e.g., Avizyme 1302, Finnfeeds International) is added to the feed at a rate of 2.5×10^6 units of xylanase per tonne. When assaying the feed a routine 10 g sample would therefore contain only 25 units.
2. Xylanase inhibitors are known to exist in wheat [787].
3. As the matrix of the feed is complex, binding of the enzyme to the feed components may occur, and specific extraction may be needed to release the xylanase.

One of the most commonly used methods for in-feed analysis is the Xylazyme dyed cross-linked wheat arabinoxylan method [788], which can be measured spectrophotometrically. This method is reported to be more sensitive than assays that use chromogenic substrates, for example, azo-wheat arabinoxylan [786].

β-Glucanases Barley contains approximately 3–4 % of β-glucan, which acts as an antinutritional factor for poultry and swine by increasing intestinal viscosity, and has been shown to increase the incidence of sticky droppings in poultry [789]. It is present in the aleurone layer and the endosperm of barley and oats. The β-glucan consists of linear glucose units linked by β-1,3 and β-1,4 linkages (Fig. 88), which are cleaved by β-glucanases (E.C. 3.2.1.6) to produce free glucose and oligosaccharides.

The use of β-glucanase in animal feeds is long established and the benefits are well recorded. In general terms the use of β-glucanase improves the weight gain and the feed efficiency of both poultry and swine fed diets that contain 30 % or more barley. Broiler chickens are the largest target market, with improvements of 50 % in weight gain reported [790]. A typical trial result using β-glucanase in broiler diets is shown in Table 31, with a significant increase in weight gain, and an improvement in the feed:gain ratio.

As with xylanases most commercial β-glucanases are either fungal, mainly *Trichoderma* or *Aspergillus* species, or bacterial, usually *Bacillus* species, in origin.

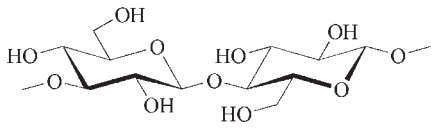

Fig. 88 Schematic β-glucan structure

Table 31. The use of β-glucanase for broiler chickens fed barley-based diets

	Control	+ Avizyme
Weight gain, g	2238	2305
Feed:gain ratio	1.91	1.86
Hock lesions, %	0.72	0.33
Mortality, %	4.7	4.4

Design: A total of 720 one-day-old male Ross chicks were assigned to 12 pens of 60 each, with 6 pen replicates per diet. The pelleted (80 °C) diets (Table 32) were fed *ad libitum*. Mortality and hock lesions were recorded to 42 d.

Table 32. Diet composition for the trial reported in Table 31

Formulation	Starter (0–21 d)	Grower (21–42 d)
Barley	584	608
Soybean meal 48	291	279
Fishmeal 60	25	
Maize oil	59	78
Amino acids	3.8	1.6
Vitamins & minerals	36	32
AME,* MJ/kg	12.5	13.0
Lysine, %	1.31	1.09
Met + cys, %	0.95	0.81

*AME = apparent metabolisable energy

The problems associated with the in-feed analysis of β-glucanase are similar to those of xylanases, with the lower levels of activity used creating further problems with the detection limits. In a commercial β-glucanase product for piglets (Porzyme 8100) the recommended level of inclusion is 600 units of β-glucanase per kilogram, while for broilers it tends to be even lower. The most common assays for β-glucanase in feed utilize dye-labeled substrates, either soluble or insoluble [791], although the problem of sensitivity at lower levels remains. Insoluble dye-labeled substrates are more sensitive and are based on cross-linked polysaccharides.

As with xylanase assays, different companies use different conditions to assay their products, with a range of pH levels, temperatures, and substrates, and different definitions of activity. For example, Finnfeeds International uses pH 5.0 and 30 °C to assay their β-glucanase and defines one unit as the amount that will liberate 1.0 mol of glucose per minute. Novozymes uses the same definition but the conditions used in their assay are pH 5.5 and 37 °C, while Roche uses pH 5.0 and 40 °C and defines their unit as the amount of β-glucanase needed to liberate 0.10 mmol of glucose per minute.

5.2.4.2.2 Phytic Acid Degrading Enzymes (Phytases)

Phytic acid (*myo*-inositol hexakis-dihydrogenphosphate) is present as the storage form of phosphorus in many of the cereals and legumes fed to animals and acts as the primary source of inositol. The contents vary from plant to plant, with phytate forming

60 % of the total phosphorus in soybean meal, 72 % in corn, and 77 % in wheat [792]. Monogastric animals can only partly digest it as they lack the endogenous enzymes required. This leads to the excretion of the undigested phytic acid, which is responsible for phytate phosphorus pollution [792], most notably water pollution after runoff from agricultural land.

Phosphorus is a vital part of the diet for poultry and swine as it is used in the formation and the maintenance of the skeletal structure and is implicated in many metabolic processes. Therefore, due to the unavailability of the phosphorus in the form of phytic acid, and the effects of a deficient level of phosphorus, including a loss of appetite leading to a low weight gain, leg disorders, and reduced fertility, when poultry and swine diets are formulated they have to be supplemented with inorganic phosphorus sources. However, the level of phosphorus in the diet then often exceeds the amount needed by the animal due to the estimate of the availability of the plant phosphorus. Therefore much of the additional phosphorus is also excreted by the animals, again leading to increased environmental pollution.

Another disadvantage of high levels of phytic acid in the diet is its ability to chelate potassium, calcium, copper, zinc, and magnesium [793], thus decreasing their availability to the animal and making phytic acid an ANF.

As poultry and swine have very little endogenous phytase activity, supplementation of the feed with phytase is now widely accepted within the animal feed industry and it is currently the fastest growing product sector. The addition of phytase makes the phosphorus stored as phytic acid available, and thus it helps to reduce the amount of phosphorus excreted and is thought to increase the nutrient availability from the feed. By replacing added inorganic phosphorus it also achieves an economic benefit for the animal feed producer.

Phytase, also known as *myo*-inositol hexaphosphate phosphohydrolase, acts by selectively removing orthophosphate from phytic acid, thus producing inositol phosphate intermediates, and *myo*-inositol. Phytases occur in two forms, either as 3-phytase (E.C. 3.1.3.8), with which the phosphate group at position C3 is the first to be hydrolyzed, or 6-phytase (3.1.3.26), which begins by hydrolyzing the phosphate group at the C6 position. They both belong to the histidine acid phosphatase subclass of phosphatases, and as such have a histidine residue in the highly conserved active center. The histidine residue is involved in nucleophilic attack on the phytate phosphorus atom, while the overall reaction yields inorganic phosphate that can then be utilized by the animal.

In 1984 the Dutch government introduced legislation that restricted the application of phosphorus per hectare of land, either from bought sources or home-produced. Failure to comply with the regulations resulted in a fine. This lead has been followed by various European countries and U.S. states, producing a large market for phytase products.

The improvement in phytate phosphorus utilization has been estimated to be between 20 and 45 % [792] in a review of the available literature. The absorption of zinc, copper, iron, and magnesium has also been shown to increase in pigs fed phytase-supplemented feed [794].

In nature phytases are found in plants, animals, and microorganisms. The main source of commercially available phytase is fungal, although bacterial and yeast phytase

have also been identified. Currently phytases from *Aspergillus* species account for the largest phytase market and are the best characterized phytases [795], and the majority of the literature on the use of phytase in animal feed is concerned with them.

The *assay for phytase* in the feed is among the most consistent between the different commercial producers. It is based on the method of [796] and is a colorimetric assay. The assay involves the incubation of the phytase with its substrate, sodium phytate, and the subsequent release of inorganic phosphate. This will then produce a yellow complex with an acid molybdate vanadate reagent, which can be measured spectrophotometrically at 415 nm. The main variation in the assay between producers is in terms of pH, with the optimum pH for the specific phytase being used: pH 5.5 (Novozymes and Roche) or pH 5.0 (Röhm).

5.2.4.2.3 **Protein-Degrading Enzymes (Proteases)**

Aside from the cereals used in animal feed the next largest component of the diets is the protein source. This can be vegetable or animal, and as with the cereals is of variable quality.

A number of proteases have been developed for animal feed, all of which are members of the peptidase family of enzymes (E.C. 3.4) and act on peptide bonds. The reactions of the proteases differ in their specificity for the peptide bonds. The most commonly used protease, subtilisin (E.C. 3.4.21.62), a serine endopeptidase produced by *Bacillus* species, has broad specificity and hydrolyzes peptide amides. Other proteases that are available are bacillolysin (E.C. 3.4.24.28), again produced by *Bacillus* species, but having a more specific reaction, preferring peptides that contain leucine or phenylalanine residues. Aspergillopepsin (E.C. 3.4.23.18), produced by species of *Aspergillus*, and oryzin (E.C. 3.4.21.63), also produced from fungal sources, both have broad specificity, but the latter does not hydrolyze peptide amines.

Generally the proteases are added to feed in combination with other enzymes in order to overcome antinutritional factors associated with the protein sources in the diet. The main protein source tends to be soybean meal, which contains lectins and protease inhibitors. These ANFs can reduce the digestion of the nutrients, and both compounds have also been implicated in physiological damage [797]. The proteases can break down the protein sources into smaller units that are easier for the animal to digest.

Proteases act by breaking down protein into its constituent peptides and amino acids. The animal can then more readily absorb these smaller molecules. It is not used as a single addition to animal feeds, but in combination with xylanase or β-glucanase for use in viscous diets, or with xylanase and amylase for use in nonviscous diets.

Due to the high concentration of protease inhibitors in cereals, especially wheat, the *analysis of protease* in feed preparations is very difficult. The inhibitors must be separated from the enzyme, and conditions for this are by necessity very harsh and can result in the denaturation of the protease. Commercial substrates are available for in-feed analysis, but these have proved to be very variable, and the results are not reproducible. New methods that overcome the problem of protease inhibitors still need to be developed.

5.2.4.2.4 Starch-Degrading Enzymes (Amylases)

α-Amylases (1,4-α-D-glucan glucanohydrolase; E.C. 3.2.1.1) are used in maize-based diets for poultry in combination with other enzymes to increase starch digestibility. Although maize is widely regarded as a highly digestible ingredient, evidence is emerging that there is great variability between different strains, and that starch digestibility is lower than previously thought [798]. The use of amylase helps to overcome these differences. Amylase is also an accepted addition to the diets of young pigs to help them adjust to a new feeding regime at weaning.

A typical broiler trial is shown in Table 33, in which the use of an enzyme product that contains amylase, protease, and xylanase (Avizyme 1500, Finnfeeds International) with a maize-based diet, gives an increased weight gain and an improved feed conversion ratio.

The main source of commercially used α-amylases in animal feed are *Bacillus* species. Amylases act by breaking down the starch present in the diet to produce dextrins and sugars. They catalyze the endohydrolysis of 1,4-α-D-glycosidic linkages in which three or more glucose units are present.

Table 33. The use of amylase, protease, and xylanase in broiler diets containing maize (Finnfeeds International)*

	Control	+ xylanase, amylase, protease
Weight gain, g	1702	1901
Feed intake, g	3557	3525
Feed:gain ratio	2.09	1.86

*A total of 1280 (sex-separated) Ross broilers were assigned to 16 pens of 80 chicks per pen, with 8 pen replicates (4 males, 4 females) per diet. Mash diets were fed for *ad libitum* consumption, the starter from 1 to 21, and the finisher from 22 to 42 d (Table 34).

Table 34. Diet composition for the trial reported in Table 33

Formulation	Starter (1 to 21 d)	Finisher (22 to 42 d)
Corn	570	606
Soybean meal-48	295	261
Fishmeal Peru-63	30	20
Wheat middlings, 15 % CP	50	50
Tallow	17	26
Vitamins & minerals	38	37
Avizyme 1500	0/1	0/1
AME,* MJ/kg	12.4	12.8
Crude protein, %	21.0	19.0
Lysine, %	1.21	1.07
Met & cys, %	0.90	0.82

*AME = apparent metabolisable energy.

The assay for α-amylase is based on the release of reducing sugars from a starch substrate. For *in-feed analysis* a colorimetric test is employed using insoluble dyed starch polymers as substrates.

5.2.4.3 Future Developments

The future developments in the feed enzyme area are likely to include a focus on the increased temperature stability needed to combat the processing regimes that feeds encounter. At present liquid application can be used to overcome the problems of a loss in enzyme activity, but this is expensive as it requires specialist equipment, and is not practical in many feed mills.

The major disadvantage for feed enzymes at present is the variability in the substrate and the interaction of the enzymes with other feed ingredients, both of which lead to a variation in the response. By increasing our understanding of the substrates, the enzymes can be targeted more successfully. Methods are available that optimize the enzyme dose in wheat-based diets, and these are also required for use with corn-based diets and for phytase dosing.

The production of feed enzymes is also an area in which new developments are to be found. At present the enzymes are produced commercially by traditional fermentation methods, through expression in a host system, usually bacterial or fungal. Researchers are looking at the possibility of expressing the enzymes in plants, although it is questionable whether such products would be accepted in the EU due to the current climate surrounding genetically modified organisms. If the enzymes were produced at sufficient levels of expression in plants already used in animal feed, then the costs of downstream processing compared to traditional methods would be greatly reduced.

To date phytase has been the enzyme of choice for research into production of enzymes from transgenic plants, although none have been commercialized. Plants investigated have included soybeans [799], wheat [800], tobacco [801], alfalfa, and oil seed rape.

Finally, at present the poultry and swine industries dominate the market but there is an increased profile of enzymes for use in the ruminant and aquatic sectors, and these markets have a potential for growth.

5.2.5 Enzymes in Textile Production

5.2.5.1 Introduction

The use of biotechnology in textile processing has been known for more than 2000 years. The first application known is the retting of bast fibers with the use of microorganisms [802]. Other early examples are the removal of starch by soaking starch-sized cloth with water liquor containing barley (1857) and the use of amylases in the same desizing process in 1912 [803]. Since ca. 1990, the use of enzyme technology has increased substantially, especially in the processing of natural fibers. A major reason for embracing this technology is the fact that application of enzymes is regarded as environmentally friendly and the reactions catalyzed are very specific with a focussed performance as a consequence. In contrast, chemical processes are less specific and

often result in side effects, mostly undesired, for example, a reduction in the degree of polymerization of cellulose. Other potential benefits of enzyme technology include cost reduction, energy and water savings, improved product quality and potential process integration. Application and potential of enzyme technology have been reviewed [804–808]. Table 35 gives an overview of enzymes used in textile processing. Applications treated in this chapter have not all been implemented on an industrial scale yet (ca. 2002), but this is expected within the next five years.

5.2.5.2 Cellulose Fibers

5.2.5.2.1 Desizing of Cotton Cellulose Fibers

The first application of enzymes in textile processing was the desizing of cotton fabric. Prior to weaving, the warp yarn is sized to strengthen the yarn and prevent breakage in the weaving process. The natural sizes, which are of starch origin, can be removed after enzymatic degradation with α-amylases (E.C. 3.2.1.1) by simply washing out the water-soluble products. Besides starch, several synthetic polymers are available for strengthening the warp yarn, for example, poly(vinyl alcohol), PVA, and poly(vinyl pyrolidone), PVP. Nevertheless, starch is still the favored sizing agent for economic reasons [809], and α-amylase is preferred for desizing, since the other available processes require harsh chemicals such as acids and oxidizing agents. The enzymes used are mainly of bacterial origin, especially from *Bacillus subtilis*. Their pH optimum is between 5 and 7, while the optimal operating temperature ranges from around 65 °C, for natural, unmodified enzymes to beyond 100 °C for engineered enzymes. The stability of those enzymes is improved when some calcium is present in the application system (ppm level).

5.2.5.2.2 Scouring of Cotton

Before gray cotton fabric can be dyed and finished in water-based processes, it must be cleaned in order to make it hydrophilic and white. The convential industrial method includes a treatment with sodium hydroxide at high temperature followed by extensive washing. In this process hydrophobic (waxes and fats) and other noncellulosic compounds (pectin, protein, and organic acids) are removed in a nonselective way. Much research has focused on replacing this process by an enzymatic one. Enzymes such as pectinases [lyases, E.C. 4.2.2.2; polygalacturonases, E.C. 3.2.1.15 (endo-acting type) and E.C. 3.2.1.67 (exo-acting type)], proteases (E.C. 3.4.21–25), cellulases (endoglucanases, E.C. 3.2.1.4; cellobiohydrolases, E.C. 3.2.1.91; xylanases, E.C. 3.2.1.8), and lipases (E.C. 3.1.1.3) have been examined for degradation and subsequent removal of the different natural components present in the outer layer of cotton fibers [810–816]. The best results have been obtained with a pectinase or a pectinase in combination with a cellulase. A bacterial alkaline pectinase (a lyase, E.C. 4.2.2.2) [817] proved to be especially effective. It is thought that pectin acts as cement in the primary cell wall of cotton fibers. After enzymatic destabilization of the pectin structure, the different components present in the primary cell wall layer can be removed easily in a subsequent washing procedure. Bioscouring processes, integrated in the desizing process in batch mode, have been performed successfully on an industrial scale [818, 819]. However, the bioscouring process based on pectinase does not remove motes or seed coat fragments,

Table 35. Enzymes used in textile processing. Applications mentioned are industrially implemented or in the research phase

Enzyme	Substrate	Application	Technical benefit	Performance
Amylase	amylose	desizing cotton	increased removal of starch	removal of starch from fiber
Cellulase	cellulose	1. biopolishing cotton	removal of fuzz	maintaining the new look
		2. defibrillation Lyocell	removal of microfibers	improve processibility/generate peach-skin feel
		3. stone washing of denim	removal of indigo selectively	creation of ageing effects
Pectinase	pectin	1. scouring cotton	destabilization of outer cell layer	removal of noncellulosics
		2. retting bast fibers	destabilization of outer fiber layer	improvement of fiber extraction
Catalase	hydrogen peroxide	bleaching	peroxide breakdown	neutralisation of bleaching agent
Laccase	mediator	1. bleaching	creation of bleaching agent	improvement of whiteness
	indigo dye	2. bleaching dye	degradation of chromophore	ageing of denim
	mediator	3. effluent treatment	oxidation of dyes	decolorization of effluent
Glucose oxidase	glucose	bleaching	creation of bleaching agent	improvement of whiteness
Protease	protein	1. scouring wool	removal of scales	improve anti-shrinkage
	sericin protein	2. degumming silk	degradation of sericin	removal of sericin
	cellulase protein	3. stone washing of denim	prevent indigo binding via protein	reduction of backstaining
Hemi-cellulase	hemi-cellulose	retting bast fibers	destabilization of outer fiber layer	improvement of fiber extraction
Xylanase	lignin	processing jute	degradation of lignin	improvement of bleachibility

whereas the nonspecific alkaline scouring process removes these fragments. This may be a benefit when a ''natural look'' is desired, but is a problem when dyeing in lighter shades. Enzymatic solutions for this problem are currently (ca. 2001) under investigation using cellulases [820, 821].

5.2.5.2.3 **Bleaching of Cotton**

Scoured cotton fabric must be bleached prior to dyeing, especially with lighter shades. This is conventually performed with chlorinating agents or hydrogen peroxide. Alternative enzymatic processes are being studied. One process is the enzymatic generation of hydrogen peroxide by using a glucose oxidase (E.C. 1.1.3.4), after which the bath is made alkaline for bleaching the fabric. This so-called indirect enzymatic bleaching process makes use of the glucose produced in the desizing bath when an α-amylase is used for starch degradation [822, 823]. This process of peroxide generation requires oxygen as reactant, so aeration is required in the process. Benefits of this enzymatic process are:

- Consumption of the glucose generated in the desizing bath results in reduced BOD in wastewater treatment
- The gluconic acid produced in this process is a good chelator and can be used as a sequestering agent for metal ions
- The process can be integrated easily in the enzymatic desizing and scouring process to form a single integrated cotton pretreatment process

The integrated enzymatic desizing, scouring, and bleaching process yields products with acceptable levels of hydrophilicity and whiteness [824], and a considerable reduction in the required quantity of rinse water has been realized. The glucose oxidase enzyme has been immobilized in order to make repeated use possible [825].

Attention is also focused on a direct enzymatic bleaching process using oxidoreductases (E.C. 1.10.3.2) and different mediators. Much work has been devoted to the development of a system in which the oxidative power of oxygen or peroxide is increased. However, so far an industrially feasible enzyme-based bleaching process is not available, although the technology is steadily improving [826].

5.2.5.2.4 **Removal of Hydrogen Peroxide**

After the bleaching process, excess peroxide must be removed prior to dyeing to prevent oxidation of the dye. Conventionally this is realized by washing the fabric with large amounts of water or by adding a reducing agent [827]. Catalases (E.C. 1.11.1.6) have been successfully introduced in this process phase. The excess peroxide is rapidly and effectively decomposed into oxygen and water. This method reduces both process time and the consumption of water and energy [828]. An additional advantage is that the subsequent dyeing process can be performed in the same bath when production is carried out in the batch mode. A case study [829] on an industrial scale in an Egyptian textile mill revealed that applying catalase technology achieved the following reductions:

- Energy consumption by 24 %
- Costs for chemicals by 83 %
- Water consumption by 50 %
- Processing time by 33 %

5.2.5.2.5 Cotton Finishing

Cellulases are used to create different effects on cotton. This class of enzymes can be used for introducing an aged look (denim) or a renewed look on fabrics. These apparent contradictionary performances can be realized by using the appropriate type of cellulase enzyme(s) in combination with a certain degree of agitation or shear force during application [830–835]. In a cotton fabric short (micro)fibers protrude from the fabric surface (fuzz). When these fiber ends become entangled, pills are formed. The process of renewing the look of cotton, known as biopolishing [836], [837] or depilling, is based on the removal of fuzz and pills to give an improved surface appearance [807], [838] which can be described as:

- Cleaner surface with a cooler feel
- Increased softness and improved drapebility
- Brighter colors (color revival or disappearance of the greyish look)
- Improved resistance to fuzzing and pilling

Additionally, a high dyeing yield is claimed [839], although a later study revealed no significant impact on uptake of Green 26 and Red 81 dyes [840]. This study also demonstrated only limited impact of cellulase action on cellulose pore size and volume. The biopolishing process has been introduced on industrial scale in the batch mode only, because of the high shear force required in the process and the lack thereof in continuous processing equipment. Besides the above-mentioned benefits of this enzymatic biopolishing process, a major disadvantage is the potential tensile strength loss (TSL). Much research has focused on minimizing TSL. It was found that use of different individual cellulases (endoglucanases and cellobiohydrolases) leads to different TSL values [841]. This observation is the key to the assumption that using the right mixture of different cellulases (especially certain endoglucanases) will lead to optimal biopolishing performance with a minimum TSL [842–845].

Since cellulases degrade cellulose, application of this type of enzyme on cotton fabric poses a risk of destroying the fabric itself. Therefore, attention has focused on the elucidation of the performance mechanism, especially in relation with the required shear force. It was suggested that high shear forces lead to higher rates of desorption of endoglucanases and hence more random cellulose degradation and shorter processing times for mechanical breakdown of the weakened fibers [728], [846]. Another suggestion is that cellulases first attack the cellulose in the microfibrils protruding from the surface of the fabric because they are more externally exposed than the cellulose of the original fabric structure [847]. Mechanical breakdown of the enzyme-weakened microfibrils can take place before the cellulose in the main fabric has been degraded, and thus the TSL is limited. A more plausible explanation for the relation between enzymatic

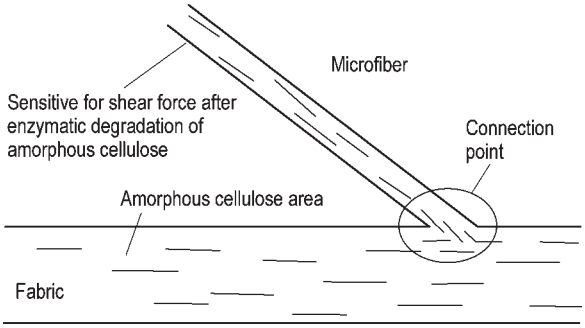

Fig. 89 Schematic presentation of a microfiber emerging from the planar surface as side-view.

cellulose degradation and shear force is based on the cellulose structure [848]. Cotton contains both crystalline and amorphous cellulose distributed in a homogenous fashion [849]. The amorphous structure is more flexible due to its lower ordering and water binding [850], while the crystalline structure is rather rigid and therefore sensitive to physical stress. Microfibrils protruding from the fabric surface have a damaged, and therefore amorphous, flexible cellulose structure at the point where they emerge from the fabric surface. Since cellulase activity is highest on amorphous cellulose, all amorphous cellulose will be degraded preferably, including the amorphous fraction at the connection point of the emerging microfibril. The consequence of this cellulase degradation is that crystalline cellulose remains mainly unmodified, and thus the connection point becomes a rather rigid physical structure. Because this cellulase-modified rigid microfibril protrudes from the fabric surface, it is most sensitive to shear force, which breaks it from the fabric (see Fig. 89). Based on this mechanism, guidelines have been published for minimizing TSL in the biopolishing process [851].

5.2.5.2.6 **Ageing of Denim**

Today, the old fashioned pumice stone process to obtain a well-worn appearance on denim jeans has largely been replaced by a cellulase process. In this enzymatic process, in which the shear force also plays an important role, both soluble (less than six residues) and insoluble sugars are produced with indigo dye trapped inside the fiber [852, 853]. This enzymatic process can be used to create a worn appearance of textile goods dyed with surface dyes like indigo, sulfur dyes, pigments, and vat dyes [804]. Different cellulases have been examined for performance, and an endoglucanase is the best performing enzyme. Especially the EGII from *Trichoderma reesei* is most effective, both in removing color from denim and producing a satisfying stone-washed effect with the lowest degree of hydrolysis [854]. Preference for this enzymatic process is due to the fact that the use of pumice stones is eliminated or reduced, thus protecting the machines and avoiding the occurrence of pumice dust in the laundry environment.

A major drawback of the enzymatic process is backstaining, that is, the redeposition of indigo dye on the undyed white weft yarns during washing. This results in an

undesired lower contrast between warp and weft yarns. A recent study showed that the adsorbed indigo can be washed out to an extent of about 80 % at maximum by using chemicals such as linear ethoxylated fatty acids and modified poly(acrylic acids) [855]. An enzymatic solution for this backstaining problem has also been developed. Adsorption of the indigo dye proceeds via enzyme binding to the fabric [856, 857]. Therefore, the use of cellulases with weak fabric-binding properties and a low affinity for indigo reduces the problem of backstaining substantially [699, 856]. Also the addition of a protease is of benefit in reducing this drawback [858].

In 2001 a new enzymatic process for indigo dye bleaching was introduced to the market, based on the use of a laccase (E.C. 1.10.3.2) and mediator [859, 860]. In this bleaching process, indigo — both attached to the fabric as well as in dispersion in water — is oxidized to soluble and colorless indigo degradation products [861]. In this way, an attractive bleaching effect with improved contrast is produced, while maintaining the original ecru color of the weft yarns. The overall look of laccase-treated denim is therefore darker than that obtained by bleaching with NaOCl. In this nonspecific chemical bleaching process, the weft yarns are also bleached. The following performance benefits of this enzymatic process over the conventional chemical bleaching process are reported:

- Creation of new looks, fashions, and finishes (e.g., grey cast/antique finishes)
- Enhancement of denim abrasion
- Maximal strength retention at high abrasion levels (maintenance of cellulose structure)
- Prevention of backstaining [862] and therefore improved garment contrast

No information is available on the penetration of this new process into the denim market.

5.2.5.2.7 **Processing of Man-Made Cellulose Fibers**

Man-made cellulose fibers are obtained from wood. A relatively new fiber is Lyocell, which entered the market in the late 1990s [863]. Total production of Lyocell fibers in 1998 was already over 100 000 t. In contrast to the production of viscose, for which a cellulose derivative is used, Lyocell is produced in a spinning process using a genuine solution of cellulose in *N*-methylmorpholine *N*-oxide (NMMO) monohydrate. Because of the totally dissolved state of the cellulose in the Lyocell process, used textiles can be also used as cellulose source [864]. Cellulase technology plays a key role in the Lyocell production process. Lyocell tends to fibrillate upon physical stress during production due to its high crystallinity index, swelling characteristics, and low degree of interfibril interaction [865]. Although this fibrillation results in a good hand performance, the attendant properties of high pilling tendency and perceived color changes make it essential to remove the fibrillation enzymatically. The raised long fibers and fibrils (the so-called primary fibrillation) are removed by the use of cellulases in equipment that also produces mechanical stress. After this removal, a uniform secondary fibrillation is produced, consisting of relatively short fibrils (the secondary fibrillation) which give the

fabric its so-called peach-skin feel [866]. While the enzymatic removal of the primary fibrillation is essential for Lyocell staple fibers, it is not for Lyocell filament [867].

Despite its essential role in Lyocell processing, only a relatively small number of studies have been published concerning the optimal cellulase for the defibrillation application. Studies with a whole acid cellulase composition from *Trichoderma reesei* and different endoglucanase-enriched compositions, using Lyocell and blends thereof with cotton and linen, revealed that no essential differences occur between the various cellulase compositions [868, 869]. The intensity of mechanical action of the garment-processing equipment plays a more significant role in removing the fibrillation. However, when the performance of these enzyme compositions is evaluated on related parameters such as TSL and fabric hand, the endoglucanase-enriched compositions are preferred.

5.2.5.2.8 **Processing of Bast Fibers**

Flax contains fibers in the cortex region of the bast stem [870]. These fibers are separated from nonfiber tissue by retting and subsequently mechanically cleaned to remove nonfiber materials. Retting is usually a microbial process and is a crucial step in the production of linen fibers. The oldest known retting processes are dew and water retting. Both processes suffer from many disadvantages, including high labor costs, variations in fiber quality, and limited suitable geographical area [871]. Therefore, many studies have focused on replacing the above-mentioned processes by a more controllable enzymatic one. Despite the progress made, at present use of an enzymatic method is hindered mainly by cost. Mixtures of pectinases, (hemi)cellulases (E.C. 3.2.1.78), and xylanases (E.C. 3.2.1.8) have been investigated [870, 872]. The presence of a substantial amount of an endopolygalacturonase appeared to be essential [870]. Apparently, degradation of the pectin structure is the most effective route to make the cellulosics (over 50 % of the stem material) available for harvesting, a process comparable to the bioscouring of cotton (see Section 5.2.5.2.2). The newer enzymatic spray-retting process uses pectinase-rich enzyme mixtures and crimp stems [873], and is claimed to have potential for reducing costs. Its economical viability must first be evaluated on a plant scale before implementation can occur.

5.2.5.3 **Proteinous Fibers**

5.2.5.3.1 **Wool Processing**

Wool is a natural fiber with a rather complex architecture. It is mainly composed of proteins (ca. 97 %) and lipids (1 %) [804]. Morphologically, two major parts can be distinguished: the cuticle composed of overlapping cells, and the cortex composed of spindle-shaped cells separated by a cell membrane complex. At the exo side of the cuticle, the fatty acid 18-methyleicosanoic acid is bound to the protein matrix. These modified proteins can be removed by means of alcoholic alkaline or chlorine solutions. The protein present in wool is keratin. Two types are known: the acidic type I and the more neutral to alkaline type II keratin, both with a molecular weight of 40–60 kDa. [874]. Especially in the cuticle, the protein is cross-linked by disulfde (between cystine residues) and isodipeptide [ε-(γ-glutamyl)lysine] bonds, and thus forms an effective

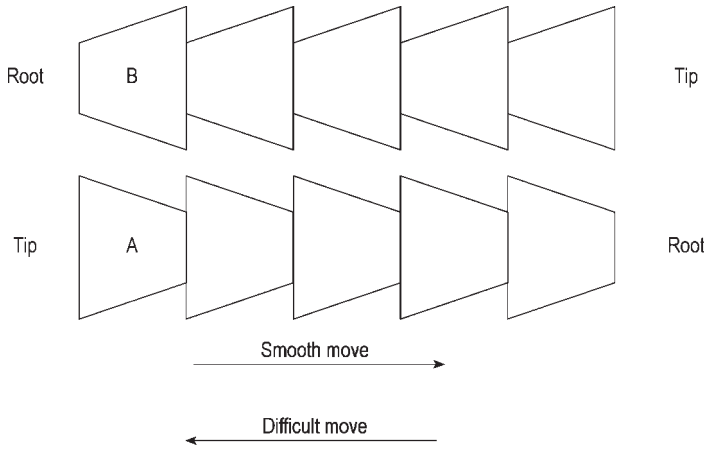

Fig. 90 Schematic presentation of wool fiber shapes. When fiber A is moved once to the right (smooth move direction), it cannot be moved back as a consequence of hooking in of the fibers A and B. This phenomenon is called felting.

barrier to the environment. Consequently, processing of wool must start with modification of the composition and morphology of the surface.

The intrinsic wool properties of felting and tendency to shrink are a consequence of the arrangement of the cuticle scales with their outer edges towards the fiber tip, so that the friction of a wool fiber in the scale direction is lower than that against the scale direction [875]. This phenomenon, known as the differential frictional effect, causes preferred fiber movement towards the fiber root when mechanical action is applied in the wet state (see Fig. 90).

To obtain machine-washable wool products, the wool is treated with an antifelt or antishrink finish. Conventional commercial shrinkproofing processes can be divided into three categories:

1. Chemical modification of the fiber surface by oxidative or reductive degradation
2. Application of a polymer resin to mask the scale structure
3. A combination of the first two methods

The best known and most frequently used commercial process is the Chlorine-Hercosett process, in which strongly acidic chlorine treatment is followed by neutralization and application of a polymer resin. Disadvantages of this process are that the polymer finishing changes the natural wool handle to a more synthetic one, and the formation of AOX, which release in wastewater is under pressure from environmental legislation.

Much effort has been devoted to developing alternative processes. Enzyme technology, especially protease technology, has been explored intensively [804, 806], as such or in combined processes. Protease activity towards wool is very slow due to the protection of the cross-linked cuticle cells. Once those cross-links have been eliminated by oxidation or reduction, the reaction rate is improved substantially [876, 877]. The

major problem in the application of protease to wool is damage caused by enzyme penetration between cuticle scales into the interior of the fibers and break down of the cell membrane complex. Diffusion of the protease into the interior of the wool fiber must be prevented. However, reaching sufficient levels of antishrinkage behavior to obtain machine-washable wool according to Woolmark TM 31 leads to severe damage to the fibers and nonuniform performance [878].

To improve the proteolytic antishrinkage process, different types of proteases have been studied. A thermo- and alkali-stable protease has been applied on an industrial scale in the prewashing step of a wool dyeing process [879]. Whereas the degree of whiteness is enhanced and improved dye uptake and penetration are obtained with a more even distribution, the felting tendency is still too high. A protease from the fungus *Aspergillus flavus*, isolated from wool, has been examined [880]. Although the enzyme aggressively attacks the cuticle proteins and modifies the scales, the decrease in mechanical properties that is also observed indicates penetration of the enzyme into the interior of the fiber. Application of another enzyme, protease SZ, in a process starting with a hydrogen peroxide pretreatment followed by enzymatic breakdown resulted in the degradation of the cuticle and pronounced antifelting behavior. This enzyme-treated wool is claimed to be machine-washable, while the loss of mechanical properties is limited. Additionally, excellent low-temperature dyeing behavior is noted [881].

In a newer combined enzymatic process the enzyme is incorporated in the alkaline peroxide treatment, after which chitosan is applied to the wool fiber [882]. The tight ionic bonding between sulfuric groups generated on the wool scales and chitosan resulted in good shrink resistance. It is claimed that the material treated in this way is shrink-resistant to the level of machine-washable. Additionally, it is observed that the whiteness and wettability are enhanced.

The above-mentioned enzymatic performance benefits of enhanced whiteness, dyeability, and shrinkage resistance to a machine-washable level, together with maintained handle and improved pilling resistance, are claimed for a different enzymatic process in which Perizym AFW, a product containing a protease of unknown origin, is used [875]. This process has been introduced on industrial scale. In combination with the subsequent application of a polymer resin, the obtained antishrinkage level meets the criteria of the Super-Wash Standard.

The use of enzyme technology in the wool carbonization process has been reviewed [804]. In the carbonization process natural soils on wool such as vegetable matter and skin flakes are removed by impregnation with sulfuric acid and subsequently baking in order to char the cellulosic impurities. Enzymes such as hydrolases, pectinases, and oxidoreductases have been studied in this process, with the objective of reducing wool-fiber damage, effluent load, and energy consumption. Although some enzyme performance has been observed, for example, weakening of the binding of burrs to the wool fiber, the expectation is that it will not come to an industrial process because of the required time window [806].

5.2.5.3.2 **Degumming of Silk**

Silk fibers consist of two fibroin filaments surrounded by the protein sericin. The diameter of silk fibers is in between 10 and 14 μm. Contrary to wool, the silk protein

contains hardly any methionine or cystein residues [883]. The molecular weight of the fibroin protein is ca. 370 kDa. Its primary structure contains the frequently repeated hexapeptide unit Ser-Gly-Ala-Gly-Ala-Gly. Fibroin contains mainly glycine (44 mol %), alanine (29 mol %), and serine (12 mol %) residues, whereas in cericin the main amino acid residues are serine (37 mol %), aspartic acid (15 mol %), and glycine (15 mol %). The content of sericin is around 17–25 wt %. The presence of sericin causes brittleness, a harsh handle, and yellowness of silk fibers [883]. Therefore, this protein is removed in a so-called degumming process to give the soft handle and typical lustre of silk. Removal of sericin leads to a tensile strength loss of ca. 20 %. Methods to remove sericin include the use of Marseilles soap at high temperature, an alkaline solution of sodium hydrogencarbonate, or enzymes (proteases). The ideal enzymatic method would selectively cleave proteins after aspartic acid and/or threonine residues, which are more than nine times more abundant in sericin than in fibroin.

Much effort has been invested in the search for the best performing protease enzyme in the degumming process [884–887]. In general, alkaline-operating proteases show a good performance with respect to sericin degradation and removal, handle, and lustre, while tensile strength loss is limited or not observed. The efficiency of degumming varies when different varieties of silk are used [886]. Proteolytic enzymes like trypsin, papain, and pepsin have a relative poor performance in this application. Also, a combination of a protease and a lipase has been tried with results better than were obtained with the conventual Marseilles soap [888]. The enzymatic degumming process is only used in China.

5.2.5.4 Textile Effluent Treatment and Recycling

Wet processing of textiles is a major source of water pollution. In general, starch or poly(vinyl alcohol) from the desizing process and a range of different dyes are found in mill effluents. Dyes are regarded as the most important contaminants to be removed from the effluent. The chromophoric compounds are quite stable, and conventional wastewater treatment is not efficient in removing recalcitrant dyes. For example, azo dyes, which are not easily biodegraded, are toxic and also become harmful for the environment upon degradation by formation of aromatic amines. However, most of the methods developed for the removal of dyes from wastewater, such as adsorption, ion exchange, electrokinetic coagulation, membrane filtration, and ozonation, are not implemented on a large scale due to high implementation cost and primarily because of the narrow application range: the methods are applicable to selected dyes only. Due to the emerging environmental awareness and the increasing pressure of legislative restrictions on colored effluent, the potential of biotechnology in this application area has been recognized (it is known that certain enzymes such as laccases, lignin peroxidases, and manganese peroxidases are capable of degrading aromatic rings [889]) and explored.

Several studies have reported complete or almost complete decolorization (dye degradation) using different fungal microorganisms. Among others, complete degradation of the dyes Acid Yellow 17, Reactive Blue 21, poly R-478, and Remazol Brilliant Blue R has been reported using the fungus *Pleurotus ostreatus* [890, 891] and of various azo dyes by *Phanerocheate chrysosporium* [892, 893]. In the latter study the fungus

is used in a bioreactor operated in a continuous mode with 95 % decolorization at 0.2 g/L dye load. In these studies a correlation was observed between decolorization and the presence of peroxidase in the effluent, which was secreted by the fungus. A laccase system has been developed for the decolorization of water polluted with the anthraquinone dye Remazol Brilliant Blue R [894]. This system makes use of a redox mediator, with violuric acid being the most effective. The above studies have been concentrated on the use of single enzymes or fungi and isolated dye classes. In a more practically oriented study, a microbial reactor containing different, but not identified, immobilized microbes has been used for treating a number of different textile effluent samples. The samples were collected from different mills, including bleacheries, bleaching and dyeing plants, screen-printing plants, and carpet dyeing mills, employing a variety of dyes, chemicals, and processes. In batch runs of 24 h for most samples a color removal of over 75 % was reached, together with a COD reduction of the same degree [895]. The study has been carried out on both bench and pilot scale, yielding water that can be reused at various points in the plant. Although the system has not been defined very well, the results are encouraging and is the basis for the expectation that implementation on relative broad industrial scale of a biotechnological system for textile effluent will be realized by ca. 2006.

The vat dye indigo, present in wastewater from stone-washing processes for denim, can be degraded by the use of different fungi [896], or the use of a laccase enzyme, both in the absence and presence of a redox mediator [897]. Laccase has also been immobilized prior to application in the decolorization process to reduce enzyme costs by reuse [898].

In the textile finishing industry, for processing of 1 kg of textiles over 100 L of water is consumed. Since the availability of water is a growing problem and the costs of disposal are increasing, new ways are being explored for reuse of water. One possibility is the reuse of (peroxide) bleaching process water in the dyeing process after degradation of the excess of hydrogen peroxide. The reuse of bleaching water after treatment with a catalase was studied in a dyeing process using Reactive Blue 198 [899]. Unacceptable color changes on the dyed fabrics were obtained. It appeared that the presence of the enzyme protein, which undergoes inactivation and unfolding, interferes in the dyeing procedure by interacting with the dye [900]. By immobilization of the catalase on a carrier, this drawback has been eliminated, and a dyed fabric is obtained with an acceptable color difference (not visually detectable) when compared to fabric dyed in fresh water [901]. Although no information is available on the possibility for multicycle reuse, the potential for reuse, and thus a substantial reduction of fresh water requirement, has been shown. The impact of accumulating processing agents may be a major hurdle for implementation of this water-recycling process. This development can be regarded as a first step towards a closed-loop production process.

As an example of achieving such a closed-loop water system, a study was performed for the desizing step in the textile production chain. In this model study, synthetic wastewater mimicking that from the desizing process was treated in an anaerobic (or methanogenic) bioreactor. This unit for biological wastewater treatment is integrated in the desizing phase of the textile production process to form a closed water loop

[902]. The feasibility of such integrated water system within a particular part of the overall production process has been demonstrated. Further research is required to identify possible drawbacks for other parts of the overall process before such process can be implemented. Nevertheless, it is predicted that in future textile production mills will have their own wastewater-purification systems to keep the costs of waste treatment low and to minimize the use of fresh water.

5.2.5.5 **Outlook**

First, the topics described in this chapter that are in the investigation phase will be brought to implementation on an industrial scale, for example, biofinishing of wool and enzymatic effluent treatment. Second, different bioprocesses, especially the pretreatment processes for fabrics made from natural fibers, will be integrated in a single process with benefits in time, energy, water and chemical consumption, as well as increased production capacity. Integration of enzyme-catalyzed processes is rather easily due to the comparatively mild process conditions and the fact that enzymes are very specific. One major prerequisite is that all integrated enzyme applications should be run in the same pH and temperature range. In case of disharmony, one can screen for an identical enzyme type with different pH and/or temperature profile. Third, enzymatic processes will also be developed for production in a continuous mode. At present, most implemented enzymatic processes are operated in the batch mode, often for reasons of time (most enzyme systems) and/or shear force (cellulase technology). For continuous application, the enzymatic incubation time should be reduced substantially to prevent fabric pile up. A different application opportunity for enzyme technology, and especially cellulase technology, is its use for subtle design development. When biopolishing can be applied in certain patterns by, for example, foam technology [851], different color shades can be realized.

Enzyme applications in the textile sector initially focused on natural fibers. It can be expected that the focus will be broaden in the near future to synthetic fibers like polyester and nylon. Already in 1998 positive performance results were reported for the use of lipases to increase the water-wetting and absorbent properties of regular polyester fabrics [903]. Likewise, it was shown that the culture filtrate of a white-rot fungus can substantially change the surface properties of polyamide fabrics made of PA66 and PA6, apparently via action of a manganese peroxidase (E.C. 1.11.1.13) [904]. Proteases were shown to have some impact (hydrolysis) on nylon oligomers [905]. Finally, the hydrolysis of polyacrylonitrile fibers and granulates by nitrilases (E.C. 3.5.5.1 & 7) or nitrile hydratase (E.C. 4.2.1.84)/nitrile amidase (EC 3.5.1.4) enzyme system has been proven [906]. In this study it was shown that enzymatic surface modification of acrylic fibers results in improved dyeing efficiency.

All these are initial studies. In general, the enzymatically catalyzed reactions on synthetic fibers are rather slow, and the methods as such cannot be implemented on large scale. Nevertheless, the potential of enzyme technology for synthetic fibers has been shown. A search for more catalytically active enzymes for these substrates, both by screening and genetic engineering, is necessary. Enzyme technology is also expected to become a valuable tool for environmentally friendly production of synthetic fabrics with improved comfort characteristics, comparable to those of natural fibers.

5.2.6 Enzymes in Pulp and Paper Processing

5.2.6.1 Introduction

Enzymatic applications in the pulp and paper industry have been developed since the 1980s, when enzymes were first introduced on a mill scale following the discovery of xylanase-aided bleaching. Most of the biotechnical applications proposed for the pulp and paper industry are based on the use of enzymes. The specificity of enzymes makes them unique tools for targeted modification of specific components in pulp or process waters and their catalytic nature makes them efficient even in small dosages. Enzymatic applications are generally considered to be environmentally benign. The limitations of the use of enzymes in pulping and papermaking processes are related to the size and properties of the enzymes, as well as to the relative high price of these catalysts. The conditions used in many process stages in pulping and papermaking have led to the development of enzyme preparations having pH and temperature ranges suitable for these target processes. Today (2006), the most important commercial applications of enzymes in the pulp and paper industry are bleaching, deinking, improvement of paper-machine runnability by hydrolysis of extractives or enhanced drainage, and fiber modification for speciality products.

Enzymatic applications for the main processes in the pulp and paper industry, i.e., pulping, bleaching, and papermaking, have been intensively studied and developed in the last 20 years. However, the implementation of enzymatic process stages into industrial processes has not been as extensive as expected in the 1990s. Nevertheless, xylanase-aided bleaching, currently (2006) the main biotechnical application in the pulp industry, developed unusually rapidly from laboratory to industrial practise. This was mainly because the implementation of an enzyme stage did not require any major changes in the process and because cost-efficient commercial enzyme preparations were rapidly developed for industrial-scale use. The successful introduction of enzymes into the pulp and paper processes apparently requires at least the following elements:

- Clear economical benefits should be obtainable from the enzyme stage,
- The quality and processing of fiber material should be maintained or improved,
- No unwanted changes in the process runnability should occur,
- A suitable enzyme preparation should be available in large quantities and at a reasonable price for mill scale trials and thereafter for industrial use.

Due to the complex chemistry involved in wood materials, a number of different enzymes can be used to improve the pulp and paper processes (Table 36). As effective and specific catalysts enzymes can be used in any process phase having appropriate conditions. The harsh process conditions, i.e., mainly high or low pH and high temperature, used in some process stages, focus the use of enzymes to less extreme process stages. To overcome the limits related to pH and temperature, novel thermophilic and alkalophilic enzymes are, however, being developed.

Table 36. Technical benefits obtained with enzymes acting on different components in pulp and papermaking. Process stage:

Component	Enzyme	Physicochemical modification	Process stage*	Technical benefit
Cellulose	cellobiohydrolase	microfibrillation	1	energy saving in tmp refining, increased flexibility of fibers
	endoglucanase	depolymerization	2	increased paper machine runnability, speciality products/high density paper
	mixed cellulases	depolymerization	3	increased release of ink particles
Xylan	endoxylanase	depolymerization	4	increased extractability of lignin
Glucomannan	endomannanase	depolymerization	4	increased extractability of lignin
		decreased colloidal stability	5	increased runnability of paper machine
	acetyl glucomannanesterase	decreased solubility of glucomannan	5	increased pulp yield, increased strength properties
Pectins	polygalacturonase	depolymerization	6	energy saving in debarking
			5	decreased cationic demand in papermaking
	galactanase	depolymerization of galactan	4	improved extractability of lignin
Lignin	laccase	polymerization	5	polymerization of lignans in process water, functionalization of fibers
	laccase mediator Mn-peroxidase	depolymerization	4	increased brightness in chemical pulps
Extractives	lipase	increased fiber hydrophilicity	5	increased strength properties of mechanical pulps, improved runnability of paper machine
Biofilm proteins and polysaccharides	mixed enzymes	depolymerization	1–5	slime control, improved runnability of paper machine

1. Mechanical pulping, 2. paper manufacturing, 3. deinking, 4. kraft pulp bleaching, 5. mechanical pulping/paper manufacturing, 6. debarking.

5.2.6.2 Enzymes

The enzymatic degradation of lignocellulosic material involves a set of different enzymes. Cellulases have been studied intensively for over two decades, and their reaction mechanisms have been revealed on the molecular level for many enzyme components. In spite of the long history, new interesting enzymes are still being found. The prices of commercial cellulases have decreased several fold during the last two decades, and further improvements can be expected. Today commercial, tailored cellulase preparations can be designed according to process needs. The enzymology of hemicellulases is also well established, with the same general trends. Oxidative enzymes have been studied as well, but detailed knowledge on their mechanisms of action in the fiber matrix is still needed.

5.2.6.2.1 Cellulases

Cellulose is the main carbohydrate in lignocellulosic materials. It is a chemically simple homopolymer consisting of up to 1000 β-1,4-linked anhydroglucopyranoside units [907]. However, its physical state makes it a challenging substrate for enzymes. Single glucose polymers are packed onto each other to form highly crystalline microfibrils in which the individual cellulose chains are held together by hydrogen bonds. Cellulose microfibrils also contain some amorphous regions. In wood fibers the winding direction of cellulose microfibrils varies in different cell wall layers, and this gives the fiber its unique strength and flexibility.

Efficient degradation of cellulose requires a mixture of different cellulases acting sequentially or in concert. Cellulases have traditionally been categorized into two different classes by the IUB. Endoglucanases (EG, E.C. 3.2.1.4) cleave the cellulose chains internally. Exoglucanases (cellobiohydrolases, CBH, E.C. 3.2.1.91) degrade cellulose starting from free chain ends, producing cellobiose. Endoglucanases act mainly on the amorphous parts of the cellulose fiber, whereas cellobiohydrolases are also able to degrade crystalline cellulose. However, this classification is not very satisfactory, as some exoglucanases have also been claimed to possess endo activity [908, 909]. The classification of cellulases becomes even more complicated, as some enzymes also have activity on other polysaccharides, such as xylan [910]. Therefore, a new classification of these enzymes has been adopted. Today, glycosyl hydrolases are grouped according to the structures of their catalytic domains into more than 70 families (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html).

Most cellulases have a multidomain structure consisting of a core domain separated from a cellulose-binding domain (CBD carbohydrate-binding module, CBM) by a linker peptide. The core domain contains the active site, whereas the CBD interacts with cellulose by binding the enzyme to it [911], [912]. The CBDs are particularly important in the hydrolysis of crystalline cellulose. It has been shown that the ability of cellobiohydrolases to degrade crystalline cellulose clearly decreases when the CBD is absent [913]. Attempts to direct the action of cellulases by adding or removing the CBMs or to modify fiber properties with the CBMs alone have been made [914, 915].

5.2.6.2.2 Hemicellulases

The two most common wood hemicelluloses are xylans and glucomannans. Hardwoods contain mainly xylan, whereas the amount of glucomannan in softwoods is

approximately twice the amount of xylan. Hardwood xylan is composed of β-D-xylopyranosyl units linked together with β-(1,4)-glycosidic bonds which may contain 4-*O*-methyl-α-D-glucuronic acid and acetyl side groups. 4-*O*-Methylglucuronic acid is linked to the xylan backbone by O-(1,2) glycosidic bonds and the acetic acid is esterified at the C2 and/or C3 hydroxyl group. Softwood xylan is arabino-4-*O*-methylglucuronoxylan in which the xylan backbone is substituted at C2 and C3 with 4-*O*-methyl-α-D-glucuronic acid and α-L-arabinofuranosyl residues, respectively. Softwood galactoglucomannan has a backbone of β-(1,4)-linked β-D-glucopyranosyl and β-D-mannopyranosyl units, which are partially substituted by α-D-galactopyranosyl and acetyl groups. The distribution of carbohydrates in the wood fibers varies depending on the wood species and growing conditions [907]. In softwoods, the xylan content of the innermost cell wall layer is generally very high, but otherwise xylan is relatively uniformly distributed throughout the fiber walls in native wood. In hardwoods, the outermost layers of fiber walls are rich in xylan. In softwoods the glucomannan content increases steadily from the outer parts to inner parts of cell walls. The amount and composition of hemicellulose components is drastically altered in kraft cooking. All acetyl groups are degraded, and most of the glucuronic acid groups in xylan are converted to hexenuronic acid groups [916]. Softwood glucomannan is deacetylated, and a considerable part is solubilized in the highly alkaline and hot cooking liquor [907].

Xylanases The majority of published work on hemicellulases deals with the production, properties, mode of action, and applications of xylanases [917–922]. Endoxylanases (E.C. 3.2.1.8) catalyze the random hydrolysis of β-D-1,4-xylosidic linkages in xylans. Most xylanases belong to the two structurally different glycosyl hydrolase groups (Families 10 and 11). The three-dimensional structures of xylanases in both of these families are available, and they show clear differences in size and structure. *Trichoderma reesei* xylanase from Family 11 is a small, ellipsoidal enzyme with a diameter between 32 and 42 . It does not have any separate substrate-binding domain [923]. Some xylanases have been reported to contain either a xylan-binding domain [924] or a cellulose-binding domain [925], [926]. Some of the binding domains have been found to increase the degree of hydrolysis of fiber bound-xylan, whereas others have no effect thereon. Neither a xylan- nor a cellulose-binding domain was found to have any significant role in the action of xylanases in pulp fibers [927]. Most of the xylanases characterized are able to hydrolyze different types of xylans, showing only differences in the spectrum of end products. From a practical point of view, the most important characteristics of xylanases are their pH and temperature stability and activity. A number of enzymes produced by extremophilic organisms have been characterized [928–930], but surprisingly few have reached commercial use, due to problems related to their efficient production in heterologous host strains.

Mannanases Endomannanases (E.C. 3.2.1.78) catalyze the random hydrolysis of β-D-1,4 mannopyranosyl linkages within the main chain of mannans and various polysaccharides consisting mainly of mannose, such as glucomannans, galactomannans, and galactoglucomannans. Mannanases seem to be a more heterogeneous group of enzymes than xylanases. The mannanase of *Trichoderma reesei* has a similar multidomain structure to several cellulolytic enzymes; i.e., the protein contains a catalytic

core domain which is connected by a linker to a cellulose-binding domain [933], [934]. The CBD increases the action of *T. reesei* mannanase on fiber-bound glucomannan, even though the catalytic domain can efficiently degrade crystalline mannan [935]. The hydrolysis yield of glucomannans is dependent on the degree of substitution and on the distribution of the substituents [936]. The hydrolysis of glucomannans is also affected by the glucose/mannose ratio. Some mannanases are able to hydrolyze not only the β-1,4-bond between two mannose units but also the bond between the mannose and glucose units [937], [938].

Other Hemicellulases The side groups connected to xylan and glucomannan main chains can be cleaved by α-glucuronidase (E.C. 3.2.1.131), α-arabinosidase (EC 3.2.1.55), and α-D-galactosidase (E.C. 3.2.1.22). Acetyl substituents bound to hemicellulose are removed by esterases (E.C. 3.1.1.72) [919]. Most of the side-group-cleaving enzymes are able to attack only oligomeric substrates produced by backbone-depolymerizing endo-enzymes, i.e., xylanases and mannanases. Only few enzymes can attack intact polymeric substrates. However, most accessory enzymes of the latter type prefer oligomeric substrates.

5.2.6.2.3 Lignin-Modifying, Oxidative Enzymes

In nature, lignin is mostly found as an integral part of the cell wall, embedded in a carbohydrate polymer matrix of cellulose and hemicellulose. The exact composition of lignin varies widely with wood species, but as a rule, softwood contains mainly guaiacyl units whereas hardwood also contains syringyl building blocks. It has been suggested that both the chemical and three-dimensional structure of lignin is determined by, or at least strongly influenced by, the polysaccharide matrix. For example, the lignin near the cellulose surface resembles a glassy or low-order structure, rather than a random one [939].

Reactions catalyzed by oxidative enzymes play a significant role in the complete degradation of lignocellulosic biomass. The enzymatic modification and degradation of lignin, the third major component of lignocellulosic materials, is still only partially understood, because of the oxidoreductive and radical reactions involved.

The enzymology of lignin has been the focus of enzymologists for more than 30 years, and has resulted in the identification of the major enzyme systems believed to participate in delignification; lignin peroxidase, (LiP, E.C. 1.11.1.14.), manganese-dependent peroxidase, (MnP, E.C. 1.11.1.13), and laccase (E.C. 1.10.3.2). In addition, other types of peroxidases have been identified, such as manganese-independent and LiP-like peroxidases [940]. In vitro the oxidative reactions of these enzymes lead mainly to polymerization of lignin. Purified ligninolytic enzymes have, however, been shown to cause limited delignification provided that additives, such as veratryl alcohol and H_2O_2 for LiP [941] and manganese, H_2O_2, organic acids, and surfactants for MnP [942], [943] are supplemented. Due to the promising results in using additives (coordinating/chelating compounds and electron mediators), laccases and manganese-dependent peroxidases have recently been the most extensively studied groups of enzymes in the area of lignin degradation.

Lignin peroxidase (LP) Fungal peroxidases are heme proteins which oxidize their substrates with hydrogen peroxide as electron acceptor in two one-electron steps, like all

other peroxidases [944]. LiPs have high redox potentials and can oxidize non-phenolic lignin model compounds, aromatic ethers, and polycyclic aromatics. Cation radicals formed by the action of the enzyme cause several different reactions and finally result in ring cleavages. LiPs have been characterized from several white-rot fungi. The three-dimentional structure of LiP from *Phanerochaete chrysosporium* has been solved and shows that the active-site environment is completely conserved, as in all peroxidases studied so far. Attempts to overproduce LiP in heterologuous hosts have mostly been unsuccessful, and this has hindered the commercial exploitation of LiP. The instability of LiP in the presence of an excess of hydrogen peroxide has been another limiting factor for the industrial use of LiP in delignification.

Manganese peroxidase (MnP) Manganese peroxidase (MnP) is a heme protein which oxidizes Mn^{2+} to Mn^{3+}. Chelation of Mn^{3+} by organic acids is necessary to stabilize the ion and to promote its release from the enzyme. The Mn^{3+} · chelate may function as a diffusible oxidant and degrade the phenolic moieties of lignin. Phenoxy radicals are formed and undergo a variety of reactions that result in products quite similar to those released by LiPs. Interestingly, MnP has been shown to promote peroxidation of unsaturated lipids without added H_2O_2. MnP genes have been isolated from several white rot fungi [945–948]. Multiple sequence alignments have revealed specific differences between LiPs and MnPs, in spite of the general sequence conservation. The three-dimensional structure of MnP from *Phanerochaete chrysosporium* has been published. MnP has also been successfully expressed in *Aspergillus oryzae*, and the secreted MnP was fully active and identical to the native enzyme [949].

Laccases Laccases belong to the copper metalloenzymes and to the blue oxidase subgroup. Laccases contain four copper atoms per molecule. Three types of copper atom can be distinguished by their spectroscopic and paramagnetic properties: type 1 (T1), type 2 (T2), and type 3 (T3). Laccases contain one T1 Cu bound as a mononuclear center, one T2, and two T3 Cu atoms. These three T2 and T3 Cu atoms form a trinuclear center. In the resting enzyme all four copper atoms are likely to exist in the Cu^{II} state. The three-dimensional structure of a laccase from *Coprinus cinereus* was first elucidated [950]. Numerous laccases, especially from fungal sources, have been recently isolated and characterized [951].

The typical reaction of laccase is oxidation of a phenolic compound with the concurrent reduction of molecular oxygen to water. After four cycles of single-electron oxidations forming free radicals, the enzyme reduces one molecule of oxygen, generating two molecules of water. This mechanism requires the protein to ''store'' four electrons like a ''molecular battery'' before reduction of oxygen. The mononuclear T1 Cu site functions as the primary electron acceptor, extracting electrons from the reducing substrate and delivering them to the trinuclear T2/T3 site. The trinuclear T2/T3 center, the binding site for the second substrate, dioxygen, accepts electrons from the T1 site for reduction. Reduction of oxygen most likely takes place in two steps, since bound oxygen intermediates are involved [950].

In laccase-catalyzed oxidation the substrate loses a single electron and forms a free radical. The unstable radical may undergo further laccase-catalyzed oxidation or nonenzymatic reactions such as hydration and polymerization. Aryloxy radicals formed

by laccases may undergo further nonenzymatic oxidation/reduction or couple to other phenolic structures and produce intensely colored products. Laccases have very broad substrate specificities and can couple four one-electron oxidations to a variety of substrates, such as di- and polyphenols, aromatic amines, and a considerable range of other compounds, to the irreversible four-electron reduction of dioxygen to water.

5.2.6.3 Enzymes in Pulp and Paper Processing

5.2.6.3.1 Mechanical Pulping

Mechanical pulping involves the use of mechanical force combined with pressure and temperature and in some cases also with chemicals to separate the wood fibers. Mechanical pulps, such as pressurized ground wood (PGW) or thermomechanical pulp (TMP) have a high yield (up to 95 %) and can be used to produce paper with high bulk, good opacity, and excellent printability. The drawbacks of these processes are the high energy intensity and the resulting fiber and paper quality, with lower strength, higher pitch content, and higher color reversion rate as compared to chemical pulps. Energy consumption is thus a key issue when developing new technical processes for high-yield pulping. The demand for improving paper quality has been the main reason for the increased specific energy consumption in thermomechanical pulping during the last few years. One way of reducing the high energy consumption of mechanical pulping is to modify the raw material by biotechnical means prior to refining.

The low accessibility of mechanical pulp fibers to enzymes limits fiber modification to the accessible pulp surfaces (outer fiber surface, fines) and to dissolved and colloidal material solubilized to process water [952]. The main biotechnical applications related to mechanical pulping investigated in various research institutes around the world have been biomechanical pulping [953], microbial reduction of pitch components prior to pulping [954], and enzyme-aided refining of coarse mechanical pulp fibers [955].

Recently, a new promising concept was introduced based on impregnation of wood chips with cellulases prior to primary refining, which results in a marked decrease in specific energy consumption [959]. In addition, pectinase pretreatment of wood chips prior to mechanical pulping has been reported to reduce the consumption of refining energy by 10 % in a defined freeness level of TMP [960].

Incorporation of an enzymatic step in a mechanical pulping process can be expected most successful only after primary refining A process concept for TMP based on the treatment of coarse mechanical pulp fibers with monocomponent cellulases has been developed [955], [956] [917]. Experiments with different enzymes have demonstrated that a slight modification of cellulose by the *T. reesei*cellobiohydrolase I (CBH I) results in energy saving of 20 % in a laboratory-or pilot scale disk refiner. Interestingly, when an unoptimized cellulase mixture was used, no positive effect on energy consumption was detected [956],[957]. The method was further verified in mill-scale trials [958]. Energy savings of 10–15 % were realized in a TMP production line with a capacity of 120 t/d, and these savings were not obtained at the expense of pulp quality. The enzymatic treatment was easy to combine with the prevailing process conditions without disturbances in the normal operation of the TMP line or the paper machine.

Neither the cellulase mixture nor CBH I induced evident morphological modifications in the coarse and rigid TMP fibers during a short incubation [914]. Based on the fibrillation index analyses it has been speculated that the action of CBH I possibly induces decreased interfibrillar cohesion inside the fiber wall and thus results in loosening and unraveling of fiber structure. Cellulase treatment with CBH I did not have any detrimental effects on pulp quality. In fact, the tensile index was even higher for the CBH I-treated pulp than for the reference. The increase in tensile index could be explained by the intensive fibrillation induced by the CBH I treatment. The good optical properties were also maintained after CBH I treatment.

5.2.6.3.2 **Chemical Pulping**

The main aim in chemical pulping is to remove lignin and to separate the wood fibers from each other to render them suitable for the papermaking process. In the pulping process, the lignified middle lamellae located between the wood fibers is solubilized by various chemicals. Today, the predominant pulping method is the kraft process, in which the cooking liquors are incinerated and the cooking chemicals recycled. Extensive modifications of hemicelluloses take place during pulping processes. During conventional kraft cooking, part of the hemicelluloses is first solubilized in the cooking liquor. In the later phases of the process, when the alkalinity of the cooking liquor decreases, part of the solubilized xylan is redeposited onto the cellulose fibers. Although glucomannan is the main hemicellulose in softwood, the bulk of glucomannans are dissolved and degraded during kraft pulping. Thus, the relative amount of xylan is increased in pine kraft pulp as compared to pinewood. In addition to xylan, lignin is also partially readsorbed on the fibers. Lignin has been reported to be linked to hemicelluloses in lignin–carbohydrate complexes (LCC). Furthermore, hemicelluloses seem to physically restrict the passage of high molecular mass lignin out of the cell walls of the pulp fiber, and hence the removal of hemicelluloses, especially xylan, can be expected to enhance the extractability of residual lignin from pulps.

Several methods have been studied for increasing the diffusion of cooking chemicals into wood to improve the efficiency of chemical pulping processes. A long-term goal would be to totally replace sulfur chemicals. Impregnation of chemicals into wood and removal of dissolved lignin are governed by diffusion and sorption phenomena, by the porosity and structure of the cell wall matrix, as well as by the molecular size of extractable molecules. In sulfate, or kraft, cooking wood fibers are liberated and partly delignified by combination of high alkalinity (pH 12–14), sulfidity, and high temperature (165–170 °C). The kraft fibers are more flexible and thus have more binding capacity than mechanical pulp fibers. The residual lignin that causes a dark brown color in kraft fibers must be removed by bleaching.

As in the case of mechanical pulping, prior to chemical pulping the ability of individual enzymes to penetrate into wood chips is limited by the low porosity of wood. It has, however, been reported that enzymes, including hemicellulases, pectinases, and cellulases, can increase the diffusivity of sodium hydroxide in Southern pine sapwood [959]. This result was attributed to the dissolution of pit membranes, which represent the main resistance to flow of liquids in wood. After acetone extraction and enzyme treatment the pulps were reported to be more uniform, to have higher viscosity and

yield, and lower rejects [961]. The enhanced pulp uniformity was attributed to more uniform delignification owing to improved diffusion. The role of the degree of hydrolysis, i.e., loss of carbohydrates during hydrolysis, and the need for acetone extraction in softwood remain to be clarified.

5.2.6.3.3 **Bleaching**

In bleaching, the primary goal is to remove the small amount of residual lignin present in the pulp after cooking without decreasing the molecular weight of cellulose. Lignin in unbleached pulps represents typically only about 1 % of the dry weight. During pulping, however, lignin is chemically modified and condensed into poorly degradable structures. Cooking and bleaching are separate process phases, differing from each other with respect to the selectivity of the chemicals used. In the bleaching processes, lignin is sequentially degraded and extracted in several phases. Bleaching sequences are generally composed of at least five phases. Previously, the bleaching of chemical pulps was carried out with elemental chlorine and chlorine dioxide. Today, in Europe, pulp is bleached by ECF (elemental-chlorine free) or TCF (totally chlorine free) bleaching processes, in which oxygen, ozone, or peroxides are used.

Xylanase-aided bleaching of chemical pulp is the main biotechnical application used today in the pulp and paper industry (2006). Xylanase-aided bleaching is an indirect method, which does not directly degrade lignin and has thus a limited effect. Another enzymatic process stage suggested for bleaching is laccase-mediator bleaching, which directly oxidizes and degrades lignin (Table 37). Other enzyme-assisted systems have also been studied [918], [919] .

Xylanase-Aided Bleaching The effect of xylanase in bleaching is based on the modification of pulp xylan to give enhanced extractability of lignin in subsequent bleaching stages [920]. Several alternative and perhaps concurrent mechanisms have been proposed to be involved in xylanase-aided bleaching. The enhanced leachability of lignin in the fiber wall has been suggested to be due to hydrolysis of reprecipitated xylan or to removal of xylan from the lignin–carbohydrate complexes (LCC) in fibers [965], [966]. Removal of xylan by xylanases from softwood kraft fibers was found to uncover surface lignin [967]. The action of xylanases on both reprecipitated and LC xylan suggests that it is probably not only the type but also the location of the xylan that is important in xylanase-aided bleaching. The role of hexenuronic acid in the effect of xylanase treatment on kappa number (measure of lignin content) can be estimated to be relatively small. The hexenuronic acid, containing a double bond, gives rise to the consumption of bleaching chemicals and permanganate, increasing the apparent kappa number of pulp [968]. The partial removal of hexenuronic acid substituted xylan by xylanase treatment consequently results in a slightly lower kappa number [969].

Xylanases seem to be efficient on all types of kraft fibers, whereas the effects of mannanases depend on the type of fibers used [970]. The effect of xylanase on bleachability in most cases was independent of the origin of the enzyme, and both fungal and bacterial xylanases have been reported to act on pulp xylan and result in enhanced bleachability [971]. The efficiency of xylanases from Family 10 and Family 11 in bleaching has been compared, and it has been proposed that some xylanases from

Table 37. Enzymatic bleaching concepts for chemical pulps

Concept	Mechanism	Benefits	Problems	Status
Xylanase-aided bleaching	degradation of redeposited xylan and lignin-carbohydrate complexes	10–20 % saving in chemical consumption, increased brightness	indirect method, chemical bleaching needed for delignification	commercialized
Mannanase-aided bleaching	degradation of residual glucomannan	savings in chemical consumption, increased brightness	effect limited to certain pulp types	commercialized (limited use)
Laccase-mediator system	degradation of lignin in the presence of mediators (nitrogen-containing aromatic compounds, ABTS, transition metal complexes)	up to 50 % delignification when combined with alkaline extraction stage	specificity, recyclability of mediator, bleaching costs higher than in conventional bleaching	not commercialized
Mn peroxidase bleaching (with additives)	degradation of lignin in the presence of additives	good performance	costs of enzymes, cost of additives	not commercialized
Other enzyme-assisted oxidative systems (e.g., HOS, lipase with ketone, fatty acid and H_2O_2)	degradation of lignin by active compounds, such as dioxirane (HOS)	good performance claimed	system needs to be verified, costs of components	not commercialized

Family 11 could be more effective in bleach boosting as compared with the Family 10 xylanases [972]. The effect of cellulose- and xylan-binding domains (CBD and XBD) of xylanases on bleaching efficiency has also been investigated, and so far neither an XBD nor a CBD has been reported to have any significant role in the bleach-boosting efficiency of xylanases [927].

The use of xylanases in different bleaching sequences leads to a reduction in chemical consumption. The benefits obtained by enzymes are dependent on the type of bleaching sequence used and on the residual lignin content of the pulp. In chlorine bleaching an average reduction of 25 % in active chlorine consumption in prebleaching or a reduction of about 15 % in total chlorine consumption has been reported both in laboratory-scale and in mill trials. As a result, the concentration of chlorinated compounds, measured as AOX, in the bleaching effluent during mill trials was reduced by 15–20 %. Today, xylanases are industrially used both in ECF and TCF sequences. In ECF sequences, the enzymatic step is often implemented due to the limiting chlorine dioxide production capacity. The use of enzymes allows bleaching to higher brightness values when chlorine gas is not used. In TCF sequences, the advantage of the enzymatic step is due to improved brightness, maintenance of fiber strength, and savings in bleaching costs [920]. About 20 mills in Northern America and Scandinavia use enzymes [973].

Thermostable xylanase products for pulp bleaching have been on the market since 1995 [974]. The thermostabilities of new enzyme products are continuously improving, and research on xylanases acting at both high pH and temperature (pH 10 and 90 °C) is in progress. The approximate price of xylanase treatment is less than $2 per tonne of pulp. Calculations of the economic benefits in an ECF sequence reveal that reduction in the chlorine dioxide consumption leads to savings of at least $2 per tonne of pulp. The costs of oxygen-based chemicals (ozone, peroxide) are even higher, and the respective savings even more pronounced. Thus, the potential economic benefits of enzyme-aided bleaching are significant to the user.

Laccase-mediator Concept in Bleaching In the laccase-mediator concept, the electron-transferring molecule (mediator) oxidized by laccase acts directly on lignin and results in efficient delignification. In the initial study, the common substrate of laccases, the diammonium salt of 2,2′-azino-di(3-ethylbenzothiazolinsulfonate), ABTS, was used as the mediator [975]. The search for more suitable mediators resulted in the discovery of 1-hydroxybenzotriazole (HBT), violuric acid (VIO), and *N*-hydroxy-*N*-phenylacetamide, (NHA) [976–978]. The most effective mediators in delignification usually contain N—OH— functional groups [979]. The high cost due to the mediator dosage needed and the potential of generating toxic byproducts in a batch process are, however, concerns when the system is incorporated in mill-scale bleaching. Therefore, a continuous method for slow-dosing of the mediator using a precursor of NHA (i.e., DiAc, *N*-Acetoxy-*N*-phenylacetamide) has been developed [980]. In addition to nitrogen-based mediators, inorganic mediators such as transition metal complexes, preferably containing molybdenum, have recently been successfully tested for laccase-mediator bleaching [981].

Several studies on the mechanisms of laccase-mediated delignification of pulps have been published [982–986]. The LMS system has been shown to be able to replace either the oxygen delignification or ozone stage [985], [987].

The combination of xylanase and laccase-mediator bleaching systems in sequence has been shown to result in additional enhancement of pulp bleachability [969]. The application of the LMS system employing HBT as mediator with xylanase treatment in a single stage was, however, found to be ineffective, apparently due to the inactivation of xylanase by HBT. This inactivating effect of HBT has also been observed towards laccases [978], as HBT radicals undergo chemical reactions with the aromatic amino acid side chains of many laccases. Studies on new mediators have revealed that NHA caused less damage to enzymes [969], [988]. In practice, it would be beneficial to combine delignifying laccase-mediator treatment and indirect xylanase treatment, as the target substrate of the treatments are different and thus the maximum effect of both treatments could be gained.

5.2.6.3.4 **Papermaking**

In papermaking, paper is formed from pulp, various papermaking chemicals, and pigments in a paper machine. Following stock preparation the paper machine normally performs the unit operations of web formation, pressing, drying, sizing, and calendering. A large amount of water is needed for the formation of a uniform paper web from the pulp slurry in the paper machine. High efficiency is required from all unit operations due to the high speed of the paper machine.

Papermakers are currently searching for options to fulfill the legislative and environmental demands to reduce fresh water usage. One major problem in water-circuit closure is the accumulation of dissolved and colloidal substances (DCS) in the process waters. These substances consist mainly of hemicelluloses, pectins, dispersed wood resin, lignans, and dissolved lignin. The DCS composition has a direct impact on paper-machine runnability and paper quality, as well as on the demand for and efficiency of purification of process water.

The composition and structure of extractives, glucomannan, pectin, and lignans can be modified by suitable enzymes such as lipases, mannanases, pectinases, and oxidative enzymes [989]. The small chemical changes achieved by enzymes may result in significant modifications in the behavior of DCS in white waters and, more significantly, may lead to potential improvements in technical parameters such as yield, strength, and brightness of pulp.

Pitch problems originating from lipophilic extractives can be reduced by lipases hydrolyzing triglycerides to glycerol and free fatty acids in mechanical pulps. A commercial lipase preparation, Resinase (Novozymes), has been industrially used in production of groundwood pulps for several years in Japan [990]. Triglycerides from various types of pulps have been reported to be efficiently hydrolyzed by lipases, reducing the stickiness and pitch problems [991–993]. Lipase treatment allows savings in the consumption of additives and surface-active chemicals and results in improved fiber properties [990], [993]. The improved tensile strength apparently is due to the increased hydrophilicity of the fibers [993]. In addition to hydrolytic enzymes, oxidative enzymes such as laccases have been used to modify the composition and structure of lipophilic and hydrophilic extractives [994–996]. Laccase treatment polymerizes lignans to fibers and could also slightly modify lipophilic extractives [1003].

Cationic demand in peroxide bleached mechanical pulps due to galacturonic acids in pectin can be considerably reduced with pectinases. Pectinase treatment has been

reported to lower cationic demand of the white water by up to 60 % [997–1000]. Consequently, savings in cationic chemicals (alum, retention aids, strength agents, starch) and improved runnability of the paper machine in mill-scale trials with pectinase have been obtained [1001, 1002].

Enzymatic modification of the papermaking properties of chemical, mechanical, and recycled pulps were widely studied in the late 1990s. The main ideas have been to enhance the beatability and thus the strength properties of fibers through increased fibrillation or to affect the amount of fines in pulp in order to increase its drainage or water retention. ''Fiber engineering'', i.e., the directed modification of fiber surface components for improved fiber properties, mainly binding, has been carried out with chemical pulps. Successful fiber engineering of mechanical pulps would, however, probably result in clearer changes in pulp properties than in the case of chemical pulps, as fewer chemical additives are involved in mechanical pulping.

The effects of individual cellulases on the properties of unbleached or bleached kraft pulp have been studied in detail [1004–1006]. *T. reesei* cellobiohydrolases (CBH) have been found to have only a modest effect on pulp viscosity, whereas endoglucanases (EG), and especially EG II dramatically decrease pulp viscosity and thus the strength properties after refining. Treatment of ECF-bleached pine kraft pulp with *T. reesei* endoglucanases EG I and EG II has been reported to enhance the beatability considerably [1005], [1006]. The strength properties of the pulp were simultaneously impaired, presumably due to the attack of the endoglucanase on the amorphous cellulose present, especially in the defects and irregular zones of the fibers [1004]. However, in Douglas fir chemical pulp, cellulases and cellulase–hemicellulase mixtures have been reported to enhance the beatability of coarse fibers and thus to improve the paper properties [1007]. The positive effect of *T. reesei* CBH I on beatability and thus on the development of binding properties of ECF-bleached spruce kraft pulp in refining was reported [1006].

Commercial cellulase–hemicellulase mixtures are available for improving the drainage (freeness) and runnability of paper machines. This is, however, an application area suitable for speciality paper grades or occasional special needs in the paper mills. The successful use of cellulase preparations also needs special attention regarding the enzyme dosage and treatment time.

5.2.6.3.5 **Deinking**

Recycled pulp is increasingly used in newsprint, tissue paper, and in higher grades of graphic papers. Fibers in recovered paper must be deinked, i.e., repulped and cleaned from dirt and ink before they can be used again in papermaking. In deinking ink particles are detached and removed from fibers by combined mechanical and chemical action. When aiming at more efficient and environmentally friendly deinking processes, enzyme-aided deinking is a potential alternative. The application of cellulase and hemicellulase mixtures in deinking has been studied on laboratory, pilot, and mill scales [1008–1017]. The use of enzymes in deinking of recovered paper is one of the largest potential enzymatic applications in the pulp and paper industry and is already used on the mill scale.

There are two principal approaches for the use of enzymes in deinking: the enzymatic liberation of ink particles from fiber surfaces by carbohydrate-hydrolyzing enzymes such as cellulases, hemicellulases, or pectinases, or the hydrolysis of the ink carrier or

coating layer. The suitability of enzymes acting on soya-oil-based ink carriers, lignin, and starch has been tested. The hypothesis on enzyme-aided deinking is that the enzymatic hydrolysis of the ink carrier, starch coating, or fiber surface liberates ink particles that are large enough to be removed by flotation deinking. Enzyme mixtures designed for target paper grades are, however, needed to increase ink detachment and flotation of ink particles.

5.3
Development of New Industrial Enzyme Applications

5.3.1
Introduction

In addition to the long-established fields of technical enzyme applications such as detergents, cleansers, textiles, foods, feeds, and pulp and paper, a number of new applications have been developed since ca. 1990. Since most of these applications result in smaller market volumes for the enzyme industry and are less well-known as enzyme use in laundry detergents, they are often neglected. Whenever a new enzyme application is developed it must be elaborated for optimum enzyme effect, safety, economy, and stability of the product. Safety evaluation and risk assessment must take into account the fact that enzymes act as sensitizers when inhaled as dust or aerosol. This safety aspect especially limits the application of enzymes in cosmetic products and hard-surface cleaning, where their performance potential is clear, but where aerosol or dust generation must be avoided even under foreseeable conditions of misuse. As of ca. 2002 the application of enzymes in cosmetics is just emerging from development and the future is not yet clear. In a series of other industrial application fields enzymes are already established, e.g., in specific areas of hard-surface and membrane cleaning. Enzymes are also being used in quite exotic industrial processes, such as cork treatment, and the patent literature indicates that many processes are under evaluation which might in future be carried out by enzymes.

This chapter focuses on both new applications for known enzymes and on new enzymes in existing but less well known application fields. The industrial application potential of enzymes is mainly based on their specific reaction mechanisms and good ecological compatibility. Nevertheless, many good ideas for enzyme applications have not been realized. The reasons why these ideas did not go to market must be discussed against the background of the requirements for enzyme-based products: In the eyes of the customer the enzyme-containing product must show improved or unique performance compared to a conventional product without enzymes. It must be price competitive with existing products and safe to humans and the environment. From the point of view of the manufacturer the enzymatic product must be cost competitive with existing products, display a unique performance characteristic, be stable during handling, and must be compatible with other components and conditions of the product. It must be safe to humans during industrial handling of large quantities. All these parameters must be considered during product development.

Enzyme-containing products or applications have shown in the past that they can result in unique performance characteristics and that their use can be made economically attractive once an application is established. In all cases of new application ideas the

safety aspect is probably the most important. Enzymes have been known as sensitizing agents since the 1970s, when Findt discovered that enzyme dust leads to allergic symptoms in workers who inhaled it during production of biological detergents [1018]. These symptoms range from rhinitis or other hay-fever symptoms to asthma. In the area of detergents this discovery lead to technical measures such as ventilation in the production areas and enzyme granulation and coating procedures, which in combination with other precautionary measures resulted in safe enzyme usage in detergent production [1018]. An additional consequence was the declaration of enzyme products under risk phrase R42 (causing sensitization by inhalation).

Certain new ideas for enzyme application, for example, carpet cleaning and malodor removal by spraying enzymes into the air, are not in compliance with these requirements of user safety and avoidance of human contact with the treated product unless additional measures could ensure this safety target is met. A special requirement of the safety evaluation for industrial products is the need to consider potential false application or a foreseeable misuse of the product. Safety evaluations have been performed for enzyme applications in institutional dish washing and in enzymatic stain pretreatments. These were organized by the U.S. and European detergent trade associations (SDA, AISE). The industry association of enzyme manufactures and formulators (AMFEP) informs on its web site on the necessary safety evaluations for enzyme-containing consumer products (see www.amfep.org). In 2005 the American Soap and Detergent Association (SDA) published detailed guidelines on risk assessment and risk management of enzyme-containing products [1019].

Apart from the most essential safety evaluations and precautions, a number of additional enzyme-specific parameters must be considered by the manufacturer of enzymatic products: for all industrial applications enzyme formulations must be stable, both with respect to storage and process stability. Enzymes, as proteins, are easily degradable, and their degradation by microbes, proteases, and other chemical or physical influences such oxidation, light, heat, removal of essential metal ions or coenzymes must be prevented during industrial application. Starting with the stability aspect, the search for an optimal formulation represents a crucial step in product development.

A further critical aspect of technical enzyme use is the amount of time available for an application. Compared to most technical processes the time needed for enzyme-catalyzed reactions is long, ranging from a minimum of 15 min up to several hours. It depends on other application parameters such as temperature, pH, and the presence of chelating or chaotropic agents. In many cases such a prolonged reaction time is tolerated neither in technical nor in household applications.

Whereas good stability and short application time are a target for all technical products, there is, especially in the consumer product area, the additional requirement for an appearance of the enzyme product that is in compliance with the expectations of consumers, especially concerning color and odor. The crude enzyme material is usually associated with specific color and odor of a fermented product. The purification of an enzyme to fulfill these requirements is technically feasible for most enzymes, but it also is associated with higher production costs.

Finally, but generally of extreme importance in the industrial field, intellectual property rights must be observed, and in many cases these restrict the transfer of known enzymes and their reaction mechanisms to novel product applications.

5.3.2 Enzymes in Cosmetics

Enzymes have been investigated for their potential in hair dyeing, tooth cleaning, and skin treatment [1021–1027]. Cosmetic products containing enzymes are still relatively rare but their number has increased since ca. 1990. Due to the higher price of enzymes compared to conventional synthetic raw materials their use is restricted to products with unique performance characteristics which cannot be achieved by conventional compounds, or to products with a ''natural'' image relying on bio-ingredients and promising gentle and nature-compatible cosmetic efficacy.

In general, the number of patent applications claiming the use of enzymes in cosmetics still exceeds the number of corresponding enzymatic products. The safety aspect is especially important in cosmetic products. Depending on the application form (rinse off, leave on) the risk of inhalation varies and must be assessed for each product category.

5.3.2.1 Hair Dyeing

Permanent hair dyeing processes are based on the oxidative polymerization of small aromatic dye precursors [1028]. The initial oxidation is usually performed by hydrogen peroxide. The dye precursors are colorless and consist of two components, the so-called developers and couplers. Commercial developers used in hair dyeing products are phenols or aminophenols which are oxidized in the initial step of the hair dyeing process to the corresponding quinones or imines. At alkaline pH the developers and their initial oxidation products diffuse effectively into the hair, where they polymerize with the couplers to give higher molecular weight oxidation products. These polymerization products remain permanently in the hair. Both the chemical nature of the developers and couplers and their composition leads to the colors and shades offered to customers. Hydrogen peroxide not only initiates the polymerization reaction of dye precursors but also bleaches the natural hair pigment melanin, so that changes from darker to lighter colors can be achieved and the color of the polymerized dye precursors is not shifted by the natural hair color. Commercial hair dyeing products both for home and professional use are usually formulated as two-component systems, i.e., the dye precursors and the hydrogen peroxide are packaged separately to guarantee a stable product.

The concentrations of hydrogen peroxide needed for oxidation of dye precursors are in the range of 3 % in the final application formulation. Special bleaching products contain up to 6 % hydrogen peroxide to achieve bright blond shades. These concentrations, when applied repeatedly, can cause hair damage. The alkaline pH used to swell the hair and make it more accessible for the dye precursors may enhance this effect.

To achieve more gentle dyeing, a milder oxidation process is of interest, even if such products so far are only suitable for hair dyes that change the color to a darker color tone, because they are not strong enough to bleach natural melanin.

Since the 1960s a broad patent literature has claimed the application of several oxidative enzymes such as oxidases, peroxidases, and tyrosinases as substitutes for hydrogen peroxide to give a milder hair coloring process. Mildness is related to avoiding

hydrogen peroxide and working at neutral pH, which is compatible with the enzymes. The oldest patent application in this field appeared as early as 1917 [1029].

However, up to now no enzymatic hair dyeing product has appeared on the market. The main reason is that the performance, even of the more recent enzyme hair dyes, cannot compete with conventional dyes with respect to permanence, antigraying performance, and color diversity. Since 1999 on average more than 40 new patent applications concerning enzymatic hair dyeing processes appeared each year, and this can be considered to be a sign that the applying companies (e.g., L'Oréal [1030], Wella [1031], Lion [1032], Henkel [1033], Kao [1034]) are continuously working on the improvement of enzyme performance in their products.

Several different types of oxidative enzymes have a potential for different types of dyeing products. Figure 91 summarizes schematically the different enzymatic mechanisms.

5.3.2.1.1 **Oxidases**

Oxidases such as glucose oxidase and uricase generate hydrogen peroxide in the presence of oxygen by oxidizing their substrates in situ. When these enzymes are used for hair dyeing the conventional oxidizing agent hydrogen peroxide is produced directly on the

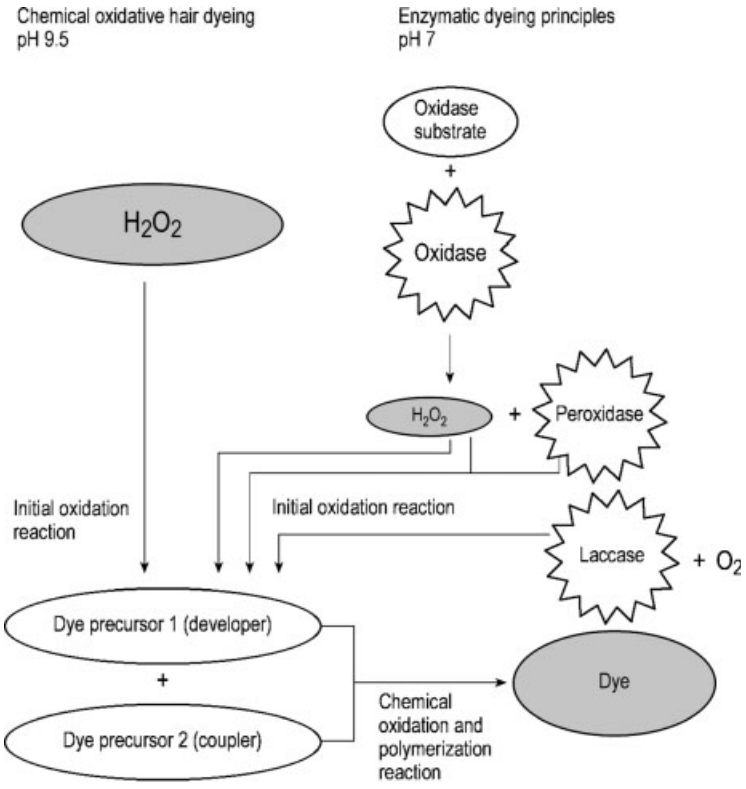

Fig. 91 Principles of chemical and enzymatic oxidative hair dyeing processes

hair, i.e., the location where it is needed. The process is claimed to be milder than the conventional process due to the lower concentrations of hydrogen peroxide needed. In addition the oxidizing agent is produced continuously at its location of action and consumed by the dyeing process, so no excess of hydrogen peroxide can damage the hair. The coloring of hair with such oxidases was shown to be satisfactory with a number of aromatic amines, e.g., *p*-phenylendiamine [1035]. Uricase produced hydrogen peroxide concentrations up to 0.06 % after 5 min of reaction at pH 7 [1036]. Since L'Oréal's patent applications from 1999, the hydrogen peroxide generating oxidases are referred to as two-electron transfer oxidases due to their reaction mechanism, which transfers two electrons from the substrate to molecular oxygen with formation of hydrogen peroxide [1037].

5.3.2.1.2 **Peroxidases**

Peroxidases (e.g., isolated from horseradish or soy bean) generate more potent oxygen species from hydrogen peroxide, which are able to oxidize dye precursors at a significantly lower concentration than hydrogen peroxide itself. Patent applications claim the combined use of oxidases and peroxidases to achieve dye formation with very small amounts of hydrogen peroxide [1038–1040]. These two enzyme types perform under identical reaction conditions (ionic strength, pH, temperature, reaction time). The disadvantage is that the price of a dye product would be increased not only by one additional component but by two, both of which are more expensive than hydrogen peroxide.

5.3.2.1.3 **Polyphenol Oxidases**

Polyphenol oxidases are copper-containing enzymes that reduce molecular oxygen to water and oxidize a polyphenolic substrate directly by a radical mechanism to the corresponding quinone [1041]. They are divided into four subclasses: laccases (E.C. 1.10.3.2; [80498-15-3]), catechol oxidases (E.C. 1.10.3.1), ascorbate oxidases (E.C. 1.10.3.3), and tyrosinases (E.C. 1.14.18.1 and 1.10.3.1). The E.C. nomenclature is not totally consistent concerning the subclasses; for example, it classifies laccases and ascorbate oxidases differently due to their substrate profiles, although both enzyme types oxidize all kinds of polyphenols and also ABTS, albeit with different affinity. The E.C. system also classifies tyrosinases both as E.C. 1.14.18.1 and E.C. 1.10.3.1. The characterization of tyrosinases as E.C. 1.14.18.1 is due to their hydroxylation activity, which is not observed in laccases and which is catalyzed by the same copper centers as the polyphenol/quinone oxidation.

Whereas tyrosinases have long been considered for enzymatic hair dyeing processes, laccases have been the focus of more recent patent literature. Both enzymes display the above-mentioned rather complex oxidation mechanism of polyphenols by transfering a total of four electrons in two oxidation cycles from their corresponding substrates to molecular oxygen, which is thereby reduced to water. Due to this mechanism laccases and tyrosinases can use commercial dye precursors as substrates, which is an advantage over the two-electron-transfering oxidases. The latter need additional substrates such as glucose or uric acid for the catalytic cycle, which leads to a more complex end formulation.

For dye formation from polyphenolic precursors laccases do not need a so-called mediator, as is usually required for bleaching reactions in well-established laccase-based processes such as denim treatment.

Laccases that are useful for hair dyeing have first been isolated from the fungus *Myceliophthora* by Novozymes [1042]. L'Oréal has claimed a series of laccases in various patent applications for use in a broad variety of hair dyeing products (see e.g., [1043]).

Up to now only few data on safety evaluation and risk assessment of oxidative enzymes in hair dyes have been published [1044]. As soon as studies with nondusting and non-aerosol-forming enzyme formulations result in safe products, enzymatic hair dyes are likely to appear on the market.

5.3.2.2 **Hair Waving**

A number of patent applications deal with the use of enzymes belonging to the classes of oxidases, transferases, or isomerases for hair waving [1045–1049]. Hair waving is conventionally achieved by forming hair waves mechanically on rolls, then reducing disulfide bonds in hair keratin (e.g., with thioglycolic acid) to break the natural hair structure, and finally oxidizing thiol groups to new disulfide bonds with hydrogen peroxide. The oxidation step fixes the new structure of hair keratin induced by the rolls. This process damages the hair by swelling it and by disturbing the natural structure of disulfide bonds without completely rearranging them. In addition an unpleasant odor results from the reduction step with thioglycolic acid. The enzyme mentioned in the patent literature for waving by rearranging disulfide bonds without using thioglycolic acid or hydrogen peroxide is protein disulfide isomerase (E.C. 5.3.4.1) [1050]. For oxidizing thiol groups in hair keratin the enzyme glutathione sulfhydryl oxidase (E.C. 1.8.3.3) is mentioned [1049]. Transglutaminases (E.C. 2.3.2.13) are also described as potential catalysts for waving [1048]. These enzymes cross-link peptide chains by forming isopeptide bonds between glutamin and lysin residues, which are present in the keratin fibers. A recent patent also describes laccases as potential catalysts in waving [1051]. The radical intermediates produced by the laccase reaction are claimed to be able to oxidize thiol groups in hair keratin to form new disulfide bonds.

Although at least transglutaminase is available on a bulk scale, due to its applications in the food industry for meat treatment, none of these enzymes has reached the product phase in hair-waving applications. The main reason seems to be the small market segment represented by waving products. Waving went out of fashion in the western world in the 1990s, and enzymes would represent a smaller niche of this small segment, i.e., milder or odorless waving products. In addition enzymes would increase the price of the product, which makes the acceptance of a niche product in the market even more difficult.

5.3.2.3 **Skin Care**

Enzyme baths containing bacteria and/or enzymes are well known in traditional Japanese culture for giving smooth skin. As there is no major market potential for bathing products in the western world, enzyme applications are restricted to leave-on products of the mid to premium price range. Proteases from plant origin such as papaya (papain, E.C. 3.4.22.2) and pineapple (bromelain, E.C. 3.4.22.4) are used in western face-care products to provide gentle peeling effects. Up to the early 1990s such products were used mainly in professional cosmetic applications, but more recently an increasing number of consumer products have emerged containing plant and bacterial

proteases for peeling of facial skin, for example, Juvena and Eucerin (Beiersdorf) in Germany. Cosmetic manufacturers seem to be switching from plant to bacterial proteases, which can be produced more economically in fermentation processes as opposed to plant-extraction processes. The bacterial enzyme in this context is subtilisin (E.C. 3.4.21.62), an enzyme type well known for its use in laundry detergent. These proteases hydrolyze to a limited extent proteins of epidermal cells to give a peeling effect similar to that of fruit acids, which are also used for this treatment.

The application of enzymes in shower gels, which have a much bigger market potential than bathing products, was not realized, due to safety assessments performed by Procter & Gamble in the 1990s [1052]. The enzyme formulation tested in this study on atopic persons led to sensitization to the enzymes. A possible explanation was based on the idea, that under the conditions of the shower application, the water formed an enzyme-containing aerosol after impinging on the soap-covered skin. The inhalation of the formed aerosol would then lead to the observed sensitization.

5.3.2.4 **Toothpastes and Mouthwashes**

The use of enzymes in toothpastes or dentifrice has been an existing field of enzyme application in personal care products for several decades. Some products use a combination of oxidative enzymes to fight pathogenic bacteria causing plaque, caries, and gingivitis in the mouth. Others use starch- or protein-hydrolyzing enzymes to remove food residues and prevent tooth staining. Yet most of the experience is based on the product Zendium, produced under the umbrella label ''Braun Oral-B'' from The Gillette Company. This toothpaste uses the enzymes amyloglucosidase (E.C. 3.2.1.3) and glucose oxidase (E.C. 1.1.3.4). Amyloglucosidase splits starch into glucose, and glucose oxidase oxidizes glucose to gluconolactone and hydrogen peroxide. Hydrogen peroxide is then used by saliva peroxidase to produce hypothiocyanite, which is a strong antibacterial agent. This hypothiocyanite-mediated antibacterial mechanism is also part of the physiological protection system of human saliva and is claimed to be enforced by the Zendium enzyme system.

The toothpastes and dry mouth treatment products Infa-Dent, Bioténe, and Oralbalance (Laclede, USA) contain the enzymes glucose oxidase, lactoperoxidase, and lysozyme as well as the protein lactoferrin as antibacterial system. All these types of proteins are also part of natural saliva and develop their antibacterial effects by the production of hypothiocyanite ions from glucose, similar to the Zendium mechanism. In addition lysozyme can hydrolyze the cell walls of pathogenic bacteria such as *Streptococcus mutans* and other gram-positive bacteria involved in formation of plaque, gingivitis, and even periodontitis. The hydrolytic action of lysozyme on cell walls is considered to be facilitated by the hypothiocyanite ions. The fourth protein of the Laclede system is lactoferrin, which is an iron-binding protein able to inhibit pathogenic bacteria by depleting them of essential iron.

The toothpaste ''Rembrandt'' contains the protease papain in its whitening product. Papain is isolated from papaya fruit and is claimed to remove stains from teeth together with citric acid in the patented complex Citroxain [1053].

As the oldest enzyme-containing brand Zendium represents a benchmark for the use of enzymes in dentifrice. Many patents have since claimed further fields of enzyme application. The patent literature refers to the use of proteases, amylases, and cellulases

to degrade food residues [1054], the application of lytic or cell-wall-degrading enzymes like lysozyme and enzyme preparations from *Streptomyces* strains [1055], and the application of mutanase and dextranase enzymes to degrade the mutane or dextrane matrices which protect *Streptococcus mutans* on the teeth [1056, 1057].

Some additional beneficial effects for the Zendium system were described in the literature with respect to prevention of recurrent aphthous ulcers when applied as mouthwash or dentifrice [1058]. Although there are clearly established oxidative and hydrolytic enzyme systems on the market and a number of additional ideas published, so far no major dentifrice product lines have been launched by companies such as Colgate and Procter & Gamble.

This also holds true for mouthwashes and chewing gums, which are included in a number of the above-mentioned patents. One reason might be that a number of enzymes with beneficial effects have been identified in the meantime in human saliva, among them lactoperoxidase, lysozyme, and lactoferrin. These findings lead to a critical evaluation of the benefit of added enzymes, unless a clear indication is given that an individual is not producing sufficient amounts of saliva or the corresponding enzymes [1059].

In recent years the prevention of halitosis has become another topic in oral-care products such as mouthwash, toothpaste, mints, and gums. The application of laccases such as the enzyme originating from *Myceliophthora thermophila* has been described as a means to reduce halitosis. The toxicological evaluation of this enzyme for this application represents a good example for the typical approach in the case of a new enzyme [1060].

With regard to safety aspects the use of enzymes in mouthwash or dentifrice can be seen as a benchmark of safe enzyme usage, where no consumer problems have become known so far.

5.3.2.5 Enzymes in Cleaning of Artificial Dentures

Considering the patent literature, cleaning of artificial teeth or dentures is a known field of applications for proteases, amylases, and other glucosidases, as well as oxidative enzymes. In analogy to dishwashing products proteases, amylases, and endoglucanases hydrolyze food residues and help dissolve them from the dentures [1061, 1062]. Dextranases, mutanases, and levanases are claimed to dissolve the special sugar components of dental plaque [1063, 1064]. Oxidases are considered to help bleaching stained dentures [1065]. The denture cleaning process usually takes place for several hours or overnight at moderate temperatures, that is, good conditions for enzymatic reactions. As the enzymes do not come into direct contact with the human body, adequate safety is achieved by a careful rinsing step. For the formulation of an enzyme-containing denture-cleaning product the same safety measures apply as for formulation of enzymatic automatic dishwashing detergents.

However, apparently Corega Tabs (GlaxoSmithKline, USA) is the only product on the market at present (2002) which contains a protease to support the cleaning step.

5.3.3
Enzymes for Preservation

The use of enzymes for preservation is a known field of application in the food area. The use of lactoperoxidase (E.C. 1.11.1.7) is a typical example. Lactoperoxidase is a natural antimicrobial system in bovine milk. The structure, functions, and applications of this

enzyme system have been described in detail [1066]. The interest in using such natural systems that are harmless to mammalian cells has grown beyond the use in the food area [1067]. The number of products in the industrial field is still small, where such systems are used for prevention of proliferation of contaminating microorganisms, but their importance is expected to increase.

5.3.4 Enzymes in Hard-Surface Cleaning

The use of enzymes in hard-surface cleaning is limited by safety considerations, since most of the obvious application fields for enzymatic hard-surface cleaning, e.g., in slaughterhouses, would be associated with release of enzyme aerosols. But even with these limitations some enzyme applications could be shown to be safe and feasible, because the enzymes are used in closed systems or in baths.

In the field of meat treatment, equipment for personal protection can be treated with enzymatic cleaning baths, where the equipment is dipped into or soaked in the enzyme-containing bath. The enzymes relevant for such treatment are usually proteases of the subtilisin type (E.C. 3.4.21.62), which can be identical with enzymes used in automatic dish washing detergents.

Clogging of sinks can be avoided or cleared by using proteases in combination with surfactants and potentially reducing agents in the household setting. In restaurant kitchens the use of lipases is accepted in some countries to clean fat separators by degradation of the fat into free fatty acids [1068].

5.3.4.1 Enzymes in Membrane Cleaning

With increasing use of membrane technology in the food industry and biotechnology, an increasing number of enzyme applications has arisen. In many applications enzymes were used directly in food processing to improve the membrane filtration performance or yield. The applications in membrane cleaning are focused only on the separate membrane cleaning step, with no contact between enzyme and filtered material or product. The fruit juice industry is the preferred target for such processes [1069], but over the whole range of food industries enzymes are described in cleaning of cross-flow filtration membranes [1070]. In some cases, e.g., milk or beer, contact could even lead to negative sensoric evaluation. Thus, the process has to guarantee the effectiveness of the enzymatic cleaning product and at the same time the complete absence of contact of the relevant product with the cleaning agent. Initially, the benefits of enzymatic cleaners were primarily seen on organic ultrafiltration membranes with their limited stability towards harsh cleaning conditions. In the meantime even for more stable inorganic membranes the use of enzymes like the subtilisin protease Alcalase has been described [1071]. The efficiency of detergent - enzyme cleaning solution versus enzyme-free detergents in controlling biofouling of membrane bioreactors has been demonstrated [1072].

When the process is not linked to treatment of product streams, but to wastewater treatment it must meet different requirements [1073] (see Section 5.3.8).

5.3.4.1.1 Proteases

Subtilisin proteases (E.C. 3.4.21.62) clearly improve the performance of alkaline membrane cleaning formulations when protein is involved in fouling or blocking films on membranes. Such applications include quite different fields ranging from the food industry to biotechnological downstream processing [1074].

5.3.4.1.2 Hemicellulases

The term hemicellulases describes enzymes with activity on polysaccharides like xylan, xyloglucan, arabinan, arabinogalactan, and galactomannan. Hemicellulases are thus a group of enzymes able to degrade these gel-forming polysaccharides from plants. The most common hemicellulases are galactanases (β-1,4-galactanases, E.C. 3.2.1.89; β-1,3-galactanases, E.C. 3.2.1.90), xylanases (endoxylanases, E.C. 3.2.1.8; exoxylanases, E.C. 3.2.1.37), xyloglucanases, and mannanases (E.C. 3.2.1.78) [1075]. Hemicellulases are used in the food industry for processing of fruit and vegetables to produce juice or concentrates. They are also used for maceration of plant material for improved filtration processes.

The same enzyme preparations used for direct fruit treatment can also be used to clean membranes in processes in which plant-derived material or juices are filtered. Large molecules of hemicellulosic or pectic nature can be associated with polyphenols or proteins. Because of their size and physicochemical properties they can block the ultrafiltration membranes used for concentration of fruit juices and thus reduce the flux rates. By hydrolyzing these polymers, enzymes can result in increased filtration periods, higher flux rates, and decreased costs. Examples of such membrane cleaning applications are known in fruit juice filtration [1075] and beer filtration before bottling. Especially the beer industry offers a broad field for applications of proteases, cellulases, and hemicellulases for cleaning of membranes used for filtration of beer. In contrast to other applications, the enzymatic treatment of beer-filtration membranes profits from the fact that there is little to no variation in the composition of the membrane-blocking material [1076]. Thus, an optimized enzymatic composition for this application can be used for extensive periods of time and over a broad geographic range.

5.3.5 Enzymes Generating a pH Shift

The activity of many enzymes is linked with pH changes based on consumption or release of acid or base by the reaction catalyzed. The consequent change of pH is often an unwanted side effect with respect to the application and is normally counterweighed by carrying out the reaction under buffered conditions. In some cases this side effect is considered the primary effect that changes the pH situ during the reaction to modify the conditions for the next reaction step. In most industrial processes which require pH shifts (e.g., pulp and paper treatment) acids or bases are added due to their low price and high effectiveness. pH-shifting enzymes include hydrolases such as protease, lipase, and, most effectively, urease. The last-named hydrolyzes urea and releases ammonia, leading to a pH increase, whereas esterases usually release acids from esters, resulting in a drop in pH. The efficiency of the enzymatic reactions is often limited to shifts of 1 or

2 pH units. pH-shifting enzymes have therefore only been investigated for hair treatment processes requiring relatively gentle pH shifts.

In hair waving, urease (E.C. 3.5.1.5) is claimed to increase the pH from neutral up to 9 over several minutes. Alkaline conditions improve the reducing efficacy of thioglycolic acid, which is used for reducing disulfide bonds [1077]. To stop this reducing reaction in hair waving, lipases have been described. They hydrolyze esters and release acids, which leads to a drop in pH. At pH values below 8 the reduction is no longer effective [1078]. Although patent literature concerning pH shifts in hair waving appeared as early as 1957 no commercial waving product contains an enzymatic pH shifting system to date. As mentioned in Section 5.3.2.2 the hair-waving market at this moment is rather small and would need further development to allow a quite expensive enzymatic system to be introduced into products.

5.3.6
Enzymes in Cork Treatment

The traditional stopper of wine bottles consists of cork made from the bark of *Quercus suber* L. Since the supply of high-quality material is limited, an increasing quality problem with cork is observed in the market. The problem is the generation of a specific cork taste leading to a wine quality that is rejected by the consumer. The replacement of cork by artificial material is possible, but is not generally accepted by customers, who prefer the traditional methods.

Chemically the cork taste is based on the generation of trichloroanisole (TCA). TCA is not a natural compound of cork, but is derived from natural polyphenols that become chlorinated to 2,4,6-trichlorophenols during chlorine bleaching of cork. The trichlorophenol is methylated to TCA by the metabolic action of bacteria in the cork. The TCA is finally extracted from the cork by the wine during storage. This extraction efficiency depends on the type of wine and a number of physicochemical parameters related to the wine.

Although the ability to identify TCA is not evenly distributed among consumers, the substance is usually smelled and thus tasted at extremely low concentrations in the range of 3 ng/L. There are more than hundred patents and patent applications claiming to prevent the generation of TCA in cork.

The application of laccases from *Myceliophthora* (Suberase, Novozymes, Denmark) leads to oxidation and subsequent polymerization of the polyphenols and thus removes them as reaction partners for the further steps of TCA generation [1079]. The oxidative polymerization also modifies the surface of the cork, generating an increasingly hydrophobic surface with increased surface tension. Thus, the water-repellent properties of the cork are increased, and extraction of substances into the wine reduced. By this method it is possible to significantly reduce the number of bottles with wine tasting of cork.

The potential of using the polymerization reaction of phenol-oxidizing enzymes in processing and preparation of cork material has been evaluated with a focus on replacing environmentally harmful binders. This technology is closely related to the use of laccase in fiberboard production (see Section 5.3.9) [1080].

The bleaching of cork with hydrogen peroxide and subsequent removal of the hydrogen peroxide with catalase has also been described [1081]. In the context of this article this must be considered a classical application of enzymes in production processes.

5.3.7 Enzymes in Oil-Field Applications

Polysaccharides such as starch, β-glucan, galactomannan, mannan, or polyanionic cellulose derivatives are used in oil fields for improved removal of drilled material by the drilling equipment. The filtration-control polymer solution is designed for low viscosity. The control of the viscosity of these drilling fluids is the important factor for achieving a rapid decrease in viscosity of the resulting filter cakes. This step is often catalyzed by enzymes. The specific requirement for this application is the high temperature, which can reach 100 °C. Thus, high-temperature stability of enzymes is required for this application.

The use of cellulases and amylases has been described for drilling fluid based on polyanionic cellulose and starch [1082]. Galactomannan and mannan are also used for drilling fluids. Hence, mannanases, with their ability to hydrolyze these polysaccharides, have also been tested for oil field application [1083]. With increasing industrial and food relevance of guar gums, an increasing number of mannanases (E.C. 3.2.1.25, E.C. 3.2.1.78) has become available, and for these enzymes an increasing number of applications is claimed in patents [1084].

In deep and high-temperature wells, as well as in water-based reservoir drilling fluids, thermostable mannanases have shown their potential for control of viscosity of mannan-based formulations, thus allowing for improved extraction of oil and gas from existing wells, resulting in greater production and increased revenue per well. A typical example are the mannanases distributed under the trade name Pyrolase. They were isolated from natural diversity and screened for high-temperature stability by Diversa (San Diego, USA). Diversa has claimed a whole range of thermostable glycosidases from *Thermococcus*, *Staphylotermus*, and *Pyrococcus* organisms for a broad range of applications [1085].

5.3.8 Enzymes in Wastewater Treatment

In many wastewater treatment processes the use of intact microorganisms producing the relevant enzymes in situ is the preferred solution over the use of isolated enzymes. Only in industrial processes with specific wastewater problems is the use of enzymes relevant. A typical example is the treatment of industrial wastewater containing hydrogen peroxide, for which catalase (E.C. 1.11.1.6) treatment is the method of choice to degrade the hydrogen peroxide fast and efficiently.

Another typical example is the use of lipases in grease separators, which is not generally allowed in all countries, since it leads to the inflow of long-chain fatty acids into the biological sewage treatment plants with consequent formation and the

stabilization of foam in activated-sludge treatment tanks [1068]. The application of enzyme-containing surfactants in grease traps in restaurants also showed that such products can have detrimental effects [1086]. This leads to a situation in which lipase-containing products must be seen not as a general solution, but as a product with the need for a case by case evaluation. Industrial processes, with their more stable and reliable wastewater situation, thus seem to be the preferred site for enzyme application.

Membrane cleaning has become increasingly relevant in ultrafiltration of wastewater treatment plant effluents, where irreversible membrane fouling otherwise reduces the lifetime of the membranes significantly. The effectiveness of enzyme-containing cleaners has been described in comparison to traditional methods. If, due to coagulant dosing, metal complexes are formed with proteins and exopolysaccharides in the fouling layer of prefiltered wastewater effluent an additional acid cleaning step prior to enzyme treatment is beneficial [1087].

5.3.9
Enzymes for Polymerisation: Wood Fiberboard Production

Polyphenol oxidases such as laccases catalyze the oxidation of a phenolic substrates by formation of a free radical. This radical can itself represent a further substrate of laccase action, but it can also react nonenzymatically with water or other phenols with formation of polymers. The ability to form polymers has been exploited to use laccases in fiberboard production from wood.

State-of-the-art fiberboard production is based on pressing wood chips with binders like urea, melamine, or phenol–formaldehyde resin to achieve a stable structure. This process is regarded as unfavorable for humans due to the emission of formaldehyde. The phenol oxidase based process uses enzymatic oxidation of lignin phenolic hydroxyl groups to form free radicals in lignin. By further reaction of these free radicals the auto-adhesion between wood fibers can be enhanced significantly to form fiberboards of good mechanical stability [1088]. Beech fibers were suspended in an aqueous solution of laccase, then dried, and pressed into fiberboards. The amount of water introduced in the enzymatic process was described as the critical parameter for transfer into an industrial process.

The pretreatment of wood chips with peroxidase and hydrogen peroxide before conventional pressing has also been described as effective for obtaining fiberboards with a stability close to medium quality standards [1089].

5.3.10
Enzymes in Composting

Composting is a complex process which, similar to a fermentation, is very much dependent on the starting material and the process conditions. Enzymes are used in composting as processing aids, often in combination with microbial starter cultures. The microorganisms are the major source of enzymes in composting, but externally added enzymes such as hydrolases may support the microorganisms, especially in the adaptation or lag phase. Although enzymes are often described and used in composting, their role in

the process has not been investigated in great detail. Enzymes have also been described as additives in odor control of composting processes. In these cases the enzymes are normally sprayed onto the surface or into the air space above the compost. Very little has been published about the biochemical nature of enzymes used in odor control.

5.3.11
Application of Bacteriorhodopsin in Security Printing and Data Storage

Bacteriorhodopsin is a photochromic retinal pigment from the archeon *Halobacterium salinarum* that converts energy from sunlight into chemical energy. This protein shows an interesting side effect easily observable by the naked eye: When exposed to visible light the initial purple color of bacteriorhodopsin turns to yellow and returns to purple in the dark. This property of light-induced color switching (photochromism) provides an effective tool for copy protection, for instance, in anti-counterfeiting applications.

The combination of properties on the molecular level, such as light-inducible color change, photochemical data storage, and traceability due to molecular markers in the protein sequences, with the high thermal ($> 100^{\circ}C$) and chemical stability of the crystalline protein makes bacteriorhodopsin a promising material for security applications.

Photochromic bacteriorhodopsin-based inks have been developed for screen, pad, and ink-jet printing. Through chemical modification of the natural pigment a whole range of different colored photochromic pigments for printing inks were synthesized. Due to the high photochromic sensitivity towards varying illumination, it is not possible to reproduce the dynamic color by photoprinting (copy protection of documents) [1090].

Furthermore, bacteriorhodopsin can be used as an information-storage medium able to store several megabytes of optical information on a few square centimeters. The impact of high-energy laser light pulses in the pico- to nanosecond range generates a stable state of the pigment that stores the polarization of the actinic beam. Data can be written onto a memory strip in a write-once read-many (WORM) way. Stored information cannot be copied by common scanners and can only be read by means of suitable terminals [1091].

The bacteriorhodopsin protein consists of 248 amino acids. By using genetic and protein engineering tools the amino acid sequence can be changed without any alteration of the protein function. Particularly in the C-terminal segment of the protein, which functions in the natural environment as a membrane anchor, amino acid exchanges can be carried out easily, but they can only be detected by elaborate analytical equipment. Therefore, it is possible to prepare important documents with a specific peptide which makes them traceable up to the producer of the material. The analysis of molecular recognition sides (tags) allows the origin of each single tagged batch of the pigment to be identified reliably.

5.4
Overview of Industrial Enzyme Applications

This chapter summarizes enzyme use in industrial applications, as presented in Sections 5.1 to 5.3 . Table 38 gives an overview of enzymes which are commercially

Table 38. Enzyme applications as described in Sections 5.1, 5.2, and 5.3

E.C. number	Name	Section	Application area	Function
Class 1: Oxidoreductases				
1.1.3.4	glucose oxidase	5.1.1	5.1.1 baking	increase gluten strength
		5.1.3	5.1.3 brewing	shelf life improvement
		5.1.4	dairy	milk preservation
		5.1.5	textile	indirect enzymatic bleaching
		5.3	new industrial use	tooth paste
1.1.3.5	hexose oxidase	5.1.1	baking	increase gluten strength
1.10.3.2	laccase	5.2.5	textile	prevention of backstaining in enzymatic stone washing
		5.2.6	pulp & paper	pulp bleaching
		5.3	new industrial use	cork treatment
		5.3	new industrial use	polymerization of lignin for production of wood fiberboards
1.11.1.6	catalase	5.1.3	brewing	shelf life improvement
		5.1.4	dairy	milk preservation
		5.2.5	textile	hydrogen peroxide removal
		5.3	new industrial use	wastewater treatment, hydrogen peroxide removal
1.11.1.7	peroxidase, lactoperoxidase	5.1.1	baking	dough improvement
		5.1.4	dairy	milk preservation
1.13.11.12	lipoxygenase	5.1.1	baking	whitening of breadcrumb
Class 2: Transferases				
2.3.2.13	transglutaminase	5.1.4	dairy	texture improvement in yoghurt, whipped cream
2.4.1.5	dextransucrase	5.1.3	brewing	production of isomalto-oligosaccharide beer
Class 3: Hydrolases				
3.1.1.3	triacylglycerol lipase	5.1.1	baking	bread improvement
		5.1.4	dairy	cheese clotting
		5.2.1	laundry detergent	removal of greasy stains
		5.2.6	pulp & paper	pitch removal
3.1.1.11	pectin methylesterase	5.1.2	fruit juice	apple and red berry juice, citrus fuit peeling
3.1.1.26	galactolipase	5.1.1	baking	in situ formation of surfactants in dough for better gas retention
3.1.3.8	3-phytase	5.1.3	brewing	mashing
		5.2.4	animal feed	phosphate release from phytic acid in animal feed
3.1.3.26	6-phytase	5.1.3	brewing	mashing
		5.2.4	animal feed	phosphate release from phytic acid in animal feed
3.2.1.1	α-amylase	5.1.1	baking	antistaling in dough

Table 38. (continued)

E.C. number	Name	Section	Application area	Function
		5.1.2	fruit juice	apple juice production
		5.1.3	brewing	mashing, fermentation
		5.2.1	laundry detergent	removal of starch containing stains
		5.2.2	automatic dishwashing	removal of starch containing stains
		5.2.3	grain wet milling	starch hydrolysis
		5.2.4	animal feed	improved digestion of starch in maize feed
		5.3	new industrial use	viscosity control in oil drilling
		5.2.5	textile	textile desizing
3.2.1.2	β-amylase	5.1.3	brewing	mashing
3.2.1.3	glucoamylase, amyloglucosidase	5.1.2	fruit juice	apple juice production
		5.1.3	brewing	mashing
		5.2.3	grain wet milling	hydrolysis of maltooligosaccharides
		5.3	new industrial use	toothpaste
3.2.1.4	endo-1,4-β-glucanase, cellulase	5.1.3	brewing	fermentation
		5.2.1	laundry detergent	removal of particular soils, softening, improved color brightness
		5.2.5	textile	cotton finishing, denim ageing
3.2.1.6	endo-1,4(3)-β-glucanase, cellulase	5.2.4	animal feed	improved weight gain and feed efficiency in poultry and swine
3.2.1.8	endo-1,4-β-xylanase	5.1.1	baking	improved dough handling, dough stability
		5.2.4	animal feed	increase digestibility of cereals
		5.2.5	textile	flax retting
		5.2.6	pulp & paper	pulp bleaching
3.2.1.55	arabinosidase	5.1.2	fruit juice	apple juice production
3.2.1.60	glucan 1,4-α-maltotetraohydrolase	5.2.4	baking	antistaling in dough
3.2.1.67	exopolygalacturonase	5.2.5	textile	cotton scouring
3.2.1.78	endo-1,4-β-mannanase	5.2.1	laundry detergent	removal of guar gum containing stains
		5.2.5	textile	flax retting
		5.2.6	pulp & paper	pulp bleaching
		5.3	new industrial use	viscosity control in oil drilling
3.2.1.91	exo-cellobiohydrolase	5.2.1	laundry detergent	removal of particular soils, softening, improved color brightness
		5.2.5	textile	cotton finishing
		5.2.6	pulp & paper	mechanical pulping
3.4.X.X	protease	5.2.5	textile	silk degumming, wool antishrinking

Table 38. (continued)

E.C. number	Name	Section	Application area	Function
		5.3	new industrial use	artificial-denture cleaning
3.4.21.62	subtilisin	5.2.1	laundry detergent	removal of proteinaceous stains
		5.2.2	automatic dishwashing	removal of proteinaceous stains
		5.2.4	animal feed	Improved digestibility of proteins in animals feed
		5.3	new industrial use	membrane cleaning
3.4.21.63	oryzin	5.2.4	animal feed	improved digestibility of proteins in animals feed
3.4.22.X	cysteine endopeptidases	5.1.3	brewing	filtration aid
		5.3	new industrial use	skin care
3.4.23.4	chymosin	5.1.4	dairy	cheese clotting
3.4.23.18	aspergillopepsin I	5.2.4	animal feed	improved digestibility of proteins in animals feed
3.4.23.22	endothiapepsin	5.4.4	dairy	cheese clotting
3.4.23.23	mucorpepsin	5.1.4	dairy	cheese clotting
3.4.24.28	bacillolysin	5.2.4	animal feed	improved digestibility of proteins in animals feed
Class 4: Lyases				
4.1.1.5	α-acetolacetate decarboxylase	5.1.3	brewing	diacetyl removal for flavor enhancement
4.2.2.2	pectate lyase	5.2.5	textile	cotton scouring
4.2.2.10	pectin lyase	5.1.2	fruit juice	apple and red berry juice, citrus fruit peeling
Class 5: Isomerases				
5.3.1.5	xylose isomerase	5.2.3	grain wet milling	fructose production
5.3.4.1	protein disulfide isomerase	5.3.2	new industrial use	cosmetics, hair waving

used in the applications described in these Sections. It is limited to commercial applications presented by the authors and does not include applications which are under development in 2003 or for which development has ceased. Although this limits the scope, the table gives a good overview of the industrial enzyme usage as presented in these chapters. A closer look at the table and analysis of enzyme usage in industrial applications in more detail reveals a total of 35 different enzyme functionalities, which are used in almost 80 different technical applications. The 35 enzyme functionalities account for numerous different products, whose number goes far into the hundreds or even thousands, worldwide. Each enzyme company certainly has more than 35 products in its portfolio.

The discrepancy in number of enzyme functionalities and enzyme products can be explained by the fact that most of the enzymes are tailored for one specific application.

Enzyme products appear in different formulations, each designed so that the enzyme performance is optimized for usage in a particular application.

The differentiation of a small number of functionalities into a broad range of products is also achieved by selecting and producing enzymes from different sources (e.g., animals, bacteria, yeast, fungi) with a wide variety in intrinsic properties such as pH/activity profile or stability under a certain set of conditions. The need for such differentiation becomes evident when looking at the different conditions used in the industrial enzyme field. The fruit juice industry uses processes in which enzymes have to operate at an acidic pH and rather low temperature. On the other hand, the detergent industry uses alkaline conditions up to pH 11, a temperature range between 40 °C and 60 °C, and a denaturing regime of surfactants and sequestrants that would be fatal for most enzymes. So far, the most extreme temperature stability has been obtained for α-amylases in the starch liquefaction, which is performed under pressure above the boiling point of water. The α-amylase has to operate for a few minutes at around 106–108 °C followed by a 2 h cooling period to 90–95 °C.

Table 38 also shows the wide chemical diversity in terms of the catalyzed reactions, as illustrated in Figure 92: the majority of the industrial enzymes that are described in the previous chapters are from the class of the hydrolytic enzymes. Almost 70 % of industrial enzymes belong to this group, class 3 of the IUB enzyme classification system.

The oxidoreductases (class 1) are the second largest contributor to the group of industrial enzymes (15 %). Classes 2, 4, and 5 are almost unused in industrial

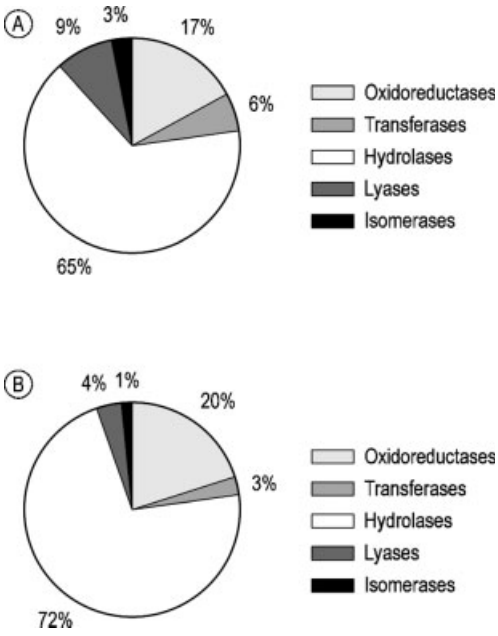

Fig. 92 A) Distribution of technical enzyme functionalities over IUB enzyme classes. B) Distribution of technical enzyme applications over IUB enzyme classes.

applications, and there is no example yet of an industrial enzyme of class 6. From this it is clear that the huge diversity of catalysts for chemical reactions which is available in nature's laboratory is completely under-utilized in industrial enzyme applications. There is thus a huge range of natural catalytic potency that awaits exploitation by humans.

A closer look at new but as-yet uncommercialized applications reveals a change in trend. The need to use new, unexploited functionalities, especially in new developments, is evident. The use of oxidoreductases in particular seem to be receiving more attention.

Another observation is that some enzymes are used in mixtures rather than as individual enzyme preparations. A good example of these is cellulases, which are used in many textile applications as complex mixtures of different types of cellulases. The basic chemical reaction is of course the same in all cellulases: the cleavage of the β-1,4 bonds between the sugar moieties. The difference lies, however, in the regioselectivity (exo or endo) and in the difference in enzyme binding to different regions of the variety of complex cellulase substrates. In most textile applications of cellulases, the synergistic action of the enzyme mixtures is needed to obtain the desired effect.

α-Amylases are good example for the use of one catalytic chemical reaction in numerous industrial applications. α-Amylases seem to be the most versatile enzymes in the industrial enzyme sector, with eight different applications, no doubt due to the abundance of starch. The widespread occurrence of this biopolymer and the ability of α-amylases to catalyze the hydrolytic degradation of amylose or amylopectin by cleavage of the α-1,4 bond between the glucose molecules results in a multitude of applications for this enzyme. Applications of α-amylase include starch degradation in the liquefaction step of glucose, maltose, or high-fructose corn syrup production; the degradation of starch-containing stains in detergent and dishwashing applications; and the clarification of fruit juice and beer. The use of α-amylase as an antistaling agent in baking is slightly different. In this application, the action of α-amylase modifies the three-dimensional polymer structure of amylose and thus changes the material properties of bread by influencing the crumb structure. Since starch is also used to service oil wells, thermostable α-amylases are used to control the viscosity of oil well fluids.

The beauty of the selectivity of enzyme use in industrial enzyme applications is again best exemplified by the use of α-amylase in textile desizing. In this particular application α-amylases are used to remove a protective layer of starch which is applied to the cellulose fibers to avoid damage during mechanical treatment in the manufacturing process. α-Amylases break down the amylose and amylopectin of starch, in which the glucose monomers are linked together by α-1,4 bonds, whereas the glucose moieties of cellulose, which are linked through β-1,4 bonds, are not hydrolyzed. In effect the starch size can be removed in a mild process without disrupting the integrity of the cellulose polymer. The cellulose polymer can subsequently be attacked by cellulases to give, for example, a stonewashed look. In chemical terms the stereochemistry of one carbon atom in the monomer is enough to direct the selectivity of the enzymes which attack the polymers.

It is impossible for this chapter to cover all uses of all enzymes in all industrial areas: many of the enzymes listed in Table 38 are also used as digestives, nutraceuticals, and probiotics; in the upgrading of waste materials; in the bating of hides; to accelerate silage; or in the recovery of silver from spent films, just to summarize some of the more exotic uses of enzymes in industrial applications.

6
Nonindustrial Enzyme Usage

6.1
Enzymes in Organic Synthesis

6.1.1
Introduction

A modern survey of catalysis would not be complete without a discussion of enzyme-catalyzed transformations. The fact that enzymes represent the first chiral catalysts should ensure them a place next to the most sophisticated man-made catalysts. Although it may seem odd that the catalyst must be isolated from cells, tissue, or organs and that sometimes whole organisms are employed, the rapid development and success of biocatalytic processes [1092–1112] for the synthesis of natural products, pharmaceuticals, and agrochemicals [1113] has immensely increased their acceptance among organic chemists. They have in part found industrial applications, especially since many technical problems, such as enzyme stabilization, enzyme immobilization, and cofactor regeneration, have been solved. Also, the availability of enzymes has been greatly improved: of the more than 3000 enzymes known, several hundred are commercially accessible or can be obtained in sufficient purity by easy, well-developed procedures. Moreover, enzymes can in principle be produced in large quantities by recombinant DNA techniques, and their properties can be manipulated in favor of a desired performance by directed evolution or DNA shuffling [1114], [1115]. Furthermore, enzyme techniques can be combined with high-throughput screening [1114] to give very powerful tools for testing for improved efficacy.

Due to the characteristic properties of enzymes compared with the chemical reactions usually employed, enzymatic transformations are exceptional in several respects:

1. They operate under mild conditions in a pH range of 5–8 at temperatures around 20–40 °C in aqueous media.
2. Many of them tolerate organic solvents [1101], [1108], [1116–1118].
3. Enzymes are highly efficient catalysts, accelerating reactions by factors of 10^5–10^{12} compared to the corresponding uncatalyzed reactions.

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4. Enzymes often combine a high selectivity for the reactions they promote and the structures they recognize with a broad substrate tolerance.
5. Besides their chemoselectivity many enzymes exhibit a high degree of diastereo-, regio-, and/or enantioselectivity.

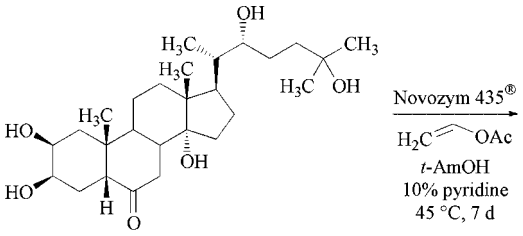
As a result of the increasing demand for complex, biologically relevant substances and the problems connected with their efficient preparation, it is mainly the enantiodiscriminating properties of enzymes that have led to the exponential development of the use of such complex molecules in organic synthesis.

6.1.2 Examples of Enzymatic Conversions

6.1.2.1 Syntheses by Means of Hydrolases

Due to their ready availability and the ease with which they can be handled, hydrolytic enzymes have been widely applied in organic synthesis. They do not require coenzymes, are reasonably stable, and often tolerate organic solvents. Their potential for regioselective and especially for enantioselective synthesis makes them valuable tools [1106], [1111], [1113], [1116–1118].

Acylation Regioselective acylations of polyhydroxylated compounds such as carbohydrates, glycerols, steroids, or alkaloids have been carried out with lipases, esterases, and proteases [1109, 1110], [1119-1123]. One example is the *Candida antarctica* lipase B (CALB, E.C. 3.1.1.3, immobilized on acrylic resin) catalyzed monoacylation of the



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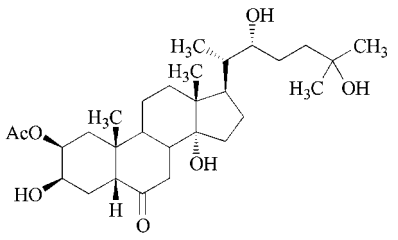


2

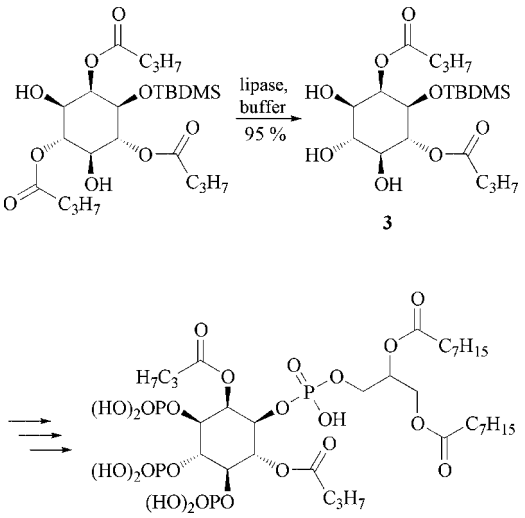
95%

signalling steroid ectysone (**1**) giving selectively the 2-*O*-acetate **2**. Using vinyl acetate for this transesterification irreversibly pushed the reaction to the product side, since the liberated enol instantaneously isomerizes to acetaldehyde [1124]. The sometimes unfavorable aldehyde is avoided when 1-ethoxyvinyl acetates [1125], [1126], trichloro- or fluoroethyl esters [1127], [1128], oxime esters [1129], or thioesters [1130] are employed for the ''quasi-irreversible'' reaction courses.

Deacylation In the synthesis of highly phosphorylated phosphoinositide derivatives, regioselective hydrolysis of one of three butyrate groups by a lipase from *Candida rugosa* (CRL, formerly *C. cylindracea*, E.C. 3.1.1.3) in pH 7.8 buffer containing 5 % methanol gave the key intermediate **3**, unfortunately without yielding enantiomeric excess (Scheme 1) [1131].

Enantioseparation An intensively utilized strategy is the kinetic resolution of racemic mixtures [1116-1118], either by stereoselective (*trans*)-esterification or -amidation reactions in organic solvents or hydrolysis in aqueous media. This approach is particularly attractive when the unconverted enantiomer is racemized in the process and reconsumed. In the example shown in Scheme 2 ethyl acetate was used as the acylating agent and triethylamine as the solvent [1132]. The enzyme was again the immobilized lipase from *Candida antarctica* B (Novozym SP 435). Racemization of the unconverted enantiomer (*S*)-**4** was achieved with palladium on charcoal. Isolated yields of **5** were 64 % with an ee of 99 %.

Another advantageous use of hydrolytic enzymes is the enantioselective hydrolysis of prochiral substrates, making use of the ability of these biocatalysts to discriminate



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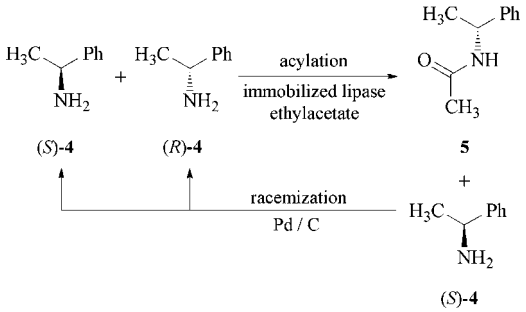
Scheme 1. Regioselective hydrolysis of the less hindered butyrate by a lipase



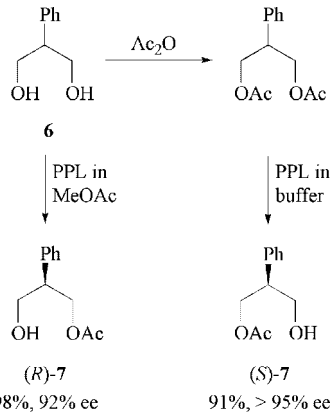
Scheme 2. Enantioselective acylation of racemic amines and in situ racemization of the unconverted enantiomer

between enantiotopic groups. An elegant way to obtain both enantiomers (*R*)-**7** and (*S*)-**7** from the prochiral diol **6** is the combination of porcine pancreatic lipase (PPL, E.C. 3.1.1.3) mediated hydrolysis and esterification (Scheme 3) [1133].

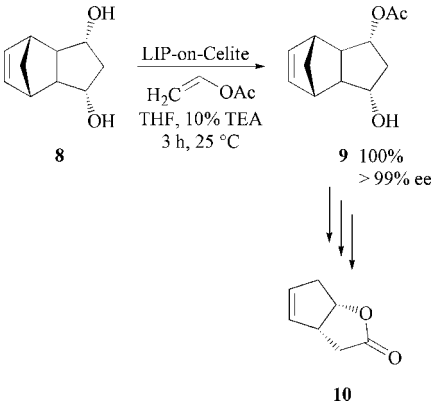
Pig liver esterase (PLE, E.C. 3.1.1.1) has found extensive use for the hydrolytic cleavage of methyl or ethyl esters of prochiral carboxylic acids.

The usefulness of hydrolases such as the immobilized lipase from *Pseudomonas species* (LIP, E.C. 3.1.1.3) to provide chiral substances for elaborated synthetic strategies is demonstrated by the quantitative acetylation of *meso*-Diol **8** to the monoacetate **9**, an important intermediate in the preparation of synthon **10** (Scheme 4) used for prostaglandin syntheses [1134].

As can be deduced from the examples above, availability and use of immobilized enzymes has significantly increased over the past years. When used in organic solvents immobilized enzymes often show a greatly augmented catalytic activity due to



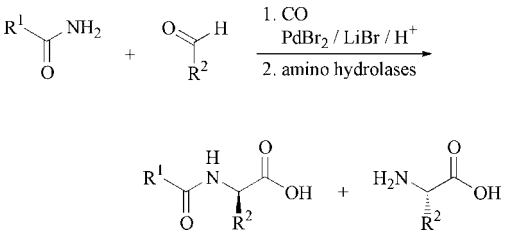
Scheme 3. Preparation of 2-phenylpropane-1,3-diol monoacetates using enantiotopic hydrolysis versus transesterification



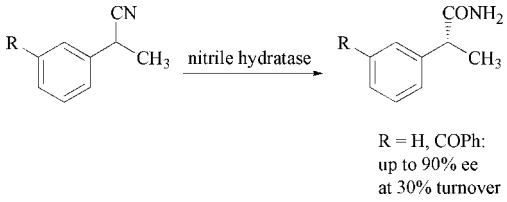
Scheme 4. Efficient enantioselective monoacetylation of a meso-diol by an immobilized lipase in organic media

increased surface area and reduced denaturation [1117]. Three techniques are frequently employed to immobilize enzymes: simple absorption, entrapment, and covalent immobilization. Especially lipases, with their need for an aqueous–lipid interface proved to be favorably altered in their activities. Lipases entrapped in hydrophobic sol–gels made from alkylsilanes and $Si(OMe)_4$ showed up to 100-fold increased activity in organic solvents [1135-1137]. Their use in water has also been demonstrated [1138]. Covalent fixation of enzymes to a matrix is often achieved by cross-linking an absorbed biocatalyst with glutaraldehyde [1139], [1140]. Surprisingly, this methodology is particularly powerful when small cross-linked enzyme crystals (CLEC) are formed [1141–1143].

Several hydrolytic enzymes other than esterases have been applied for synthetic purposes. One important subject is the chemoenzymatic preparation of amino acids. An industrial method for the synthesis of non-natural D- or L-amino acids employs the enzymatic hydrolysis of hydantoins, prepared by Bucherer–Bergs condensation using D- or L-hydantoinase (see Section 6.1.4) [1144], [1145]. Another efficient method to prepare natural and non-natural amino acids is a two-step synthesis involving a palladium-catalyzed amidocarbonylation to afford racemic *N*-acyl amino acids, followed by enantioselective hydrolysis with various acylases [1146].



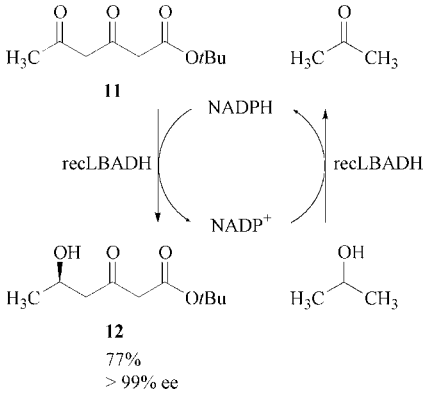
The enzymatic hydrolysis of a broad range of nitriles to the corresponding amides and acids is documented [1147-1149]. These conversions are effected directly by nitrilases (E.C. 3.5.5.1) or by successive action of a nitrile hydratase (E.C. 4.2.1.84) and an amidase (E.C. 3.5.1.4). Most of these enzymes are unstable, and whole-cell preparations are preferred. However, recently a purified nitrile hydratase preparation without amidase activity was shown to convert several 2-arylpropionitriles enantioselectively to the corresponding optically active amides (Eq. 2) [1150].



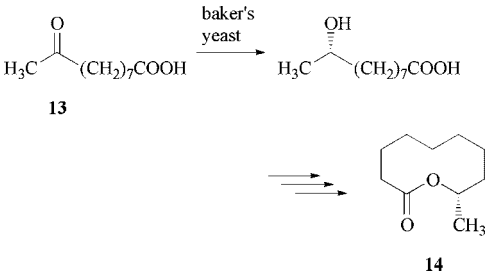
The important use of penicillin acylases (E.C. 3.5.1.11) is discussed in Section 6.1.4.

6.1.2.2 Reduction of C=O and C=C Bonds

Carbonyl reductases utilizing NADH or NADPH as cofactor have been successfully applied for the asymmetric reduction of carbonyl groups. These enzymes deliver a hydride from the reduced cofactor in accordance with Prelog's rule to the *re* face or in rare cases to the *si* face of the carbonyl group. The most commonly used alcohol dehydrogenase for this purpose is that from horse liver (HLADH, E.C. 1.1.1.1), which preferentially reduces cyclic carbonyl compounds [1151]. The completely regioselective reduction of the 3,5-dioxocarboxylate **11** using the alcohol dehydrogenase from



Scheme 5. Recombinant *Lactobacillus brevis* alcohol dehydrogenase (recLBADH) catalyzes the stereoselective reduction of 3,5-dioxocarboxylates with in situ NADPH regeneration



Scheme 6. Ketone reduction using baker's yeast as the key step in the synthesis of phorocantholide 1

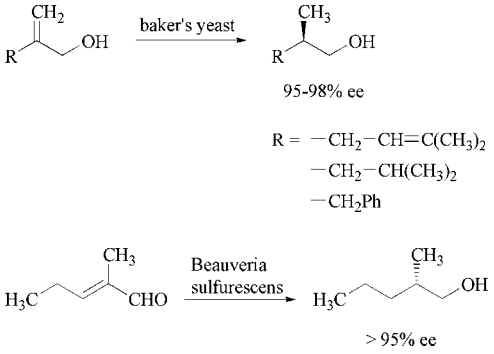
Lactobacillus brevis (E.C. 1.1.1.2) and employing substrate-coupled recycling of the cofactor gave the alcohol **12** (Scheme 5) [1152] in excellent enantiomeric excess and good yields.

Lactate dehydrogenases are another important subgroup of carbonyl reductases. They reduce α-oxo acids enantiospecifically to α-hydroxy acids. Both L- and D-selective enzymes (E.C. 1.1.1.27 and E.C. 1.1.1.28, respectively) are available, giving access to both enantiomers of various α-hydroxy acids [1153–1155].

The use of purified nicotinamide cofactor-dependent dehydrogenases in preparative reduction reactions requires an efficient regeneration of the expensive and unstable NADPH cosubstrate. For the recycling of NADH the formate/formate dehydrogenase system is well established, which combines the use of an inexpensive substrate (HCOONa) with the formation of the volatile coproduct CO_2[1156], [1157]. The use of cofactor-bound CLECs is an elegant way to avoid the instabilities of enzyme and cofactor [1143]. An example for an alternative single-enzyme system is shown in Scheme 5. Through oxidation of the added propan-2-ol the dehydrogenase (E.C. 1.1.1.2), which also catalyzes the reduction of the carbonyl compound, regenerates NADPH [1158], [1159]. To avoid troublesome and often problematic cofactor regeneration, whole-cell-mediated reductions are currently utilized extensively. By this means the necessary cofactors and their regeneration is provided by immanent pathways. For example, baker's yeast catalyzed reduction of ketone **13** was a key step for the preparation of phorocantholide 1 **14** (Scheme 6) [1160].

Baker's yeast has also been used for the highly stereoselective reduction of prochiral 2,2-dimethyl-1,3-cyclohexanedione [1161]. The resulting *S*-configured hydroxycyclohexanone served as a versatile starting material for the synthesis of various natural products [1106], [1162].

NADH-dependent enoate reductases are also interesting tools for synthetic applications. They stereoselectively reduce conjugated carbonyl double bonds and those activated by electron-withdrawing substituents, as shown above [1163–1165].

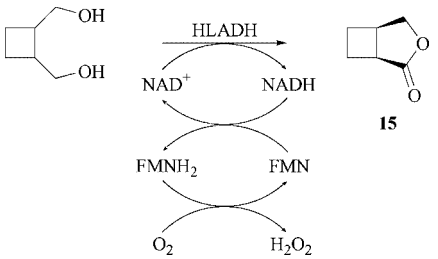


6.1.2.3 Oxidation of Alcohols and Oxygenation of C–H and C=C Bonds

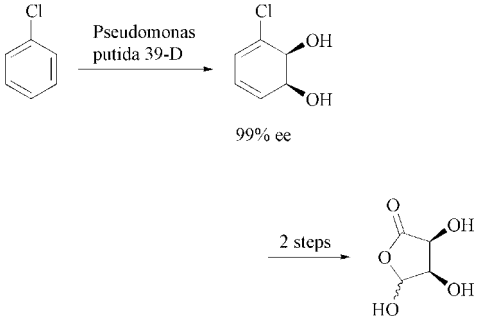
Horse liver alcohol dehydrogenase (HLADH, E.C. 1.1.1.1) has been used for the enantiotopic oxidation of *meso*-diols, preferably by applying an ammonium α-ketoglutarate/glutamate dehydrogenase recycling system to regenerate NAD^+. The initially produced asymmetric hydroxyaldehyde cyclizes to the hemiacetal, which is further oxidized to the lactone **15** [1166]. Scheme 7 presents an illustrative reaction with an alternative NAD^+ regeneration path [1167].

Mutants of *Pseudomonas putida* were found to exhibit arene dioxygenase activity, which has been exploited in whole-cell reactions for the regio- and enantioselective preparation of cis-dihydrodiols starting from benzene, substituted benzenes, and polycyclic or heteroaromatic compounds [1168–1171]. The products are invaluable precursors for natural product synthesis, as exemplified in Scheme 8 [1172], [1173].

The enzymatic counterpart of the Baeyer–Villiger reaction is catalyzed by a flavine-containing monooxygenase (cyclooxygenase) in the presence of oxygen and NAD(P)H. Using whole cells, racemic mixtures of cyclic ketones were kinetically resolved or enantio- and regioselectively oxidized [1174]. Recently, a cell-free system with cyclohexanone monooxygenase (CHMO, E.C. 1.14.13.22) and recombinant $NADP^+$-

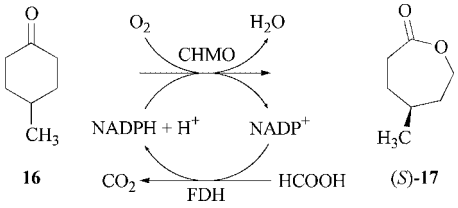


Scheme 7. Horse-liver alcohol dehydrogenase (HLADH) catalyzed stereoselective oxidation of a meso-diol with in situ NAD^+ regeneration through a flavin mononucleotide (FMN) oxygen recycling system



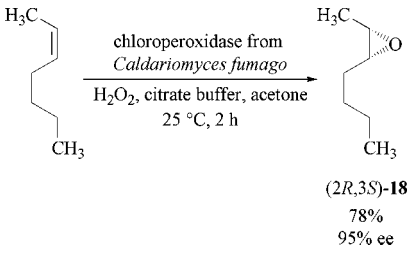
Scheme 8. Enzymatic dihydroxylation of chlorobenzene using whole cells give access to enantiomerically pure *cis*-dihydrodiols

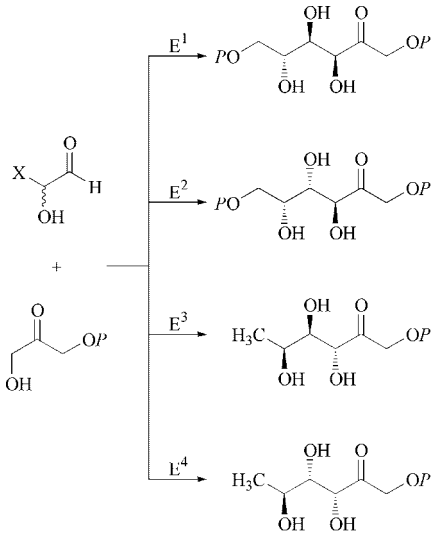
dependent formate dehydrogenase (FDH, E.C. 1.2.1.4) for cofactor regeneration was successfully introduced to prepare the chiral β-lactone **17** from prochiral 4-methylhexanone **16** (Scheme 9) [1175].



Scheme 9. Baeyer–Villiger oxidation of a prochiral cyclic ketone to a lactone with in situ cofactor regeneration (CHMO = cyclohexanone monooxygenase, FDH = formate dehydrogenase)

Regio- and stereospecific monohydroxylations of elaborate molecules have been achieved with monooxygenases that are in most cases membrane-bound cytochrome P450 enzymes. Because these are coupled with a complex redox system for electron transport from NADPH, the reactions are preferably carried out with whole cells [1176], [1177]. The same is true for enantioselective enzymatic epoxidations. An impressive example is the preparation of **18** in 78 % yield and high enantiomeric excess [1178].





E^1 = D-fructose-1,6-bisphosphate-aldolase (EC 4.1.2.13)

E^2 = D-tagatose-1,6-bisphosphate-aldolase (EC 4.1.2.40)

E^3 = L-fuculose-1-phosphate-aldolase (EC 4.1.2.17)

E^4 = L-rhamnulose-1-phosphate-aldolase (EC 4.1.2.19)

$P = PO_3^{2-}$

X = $-CH_2-OP$ (for E^1, E^2)

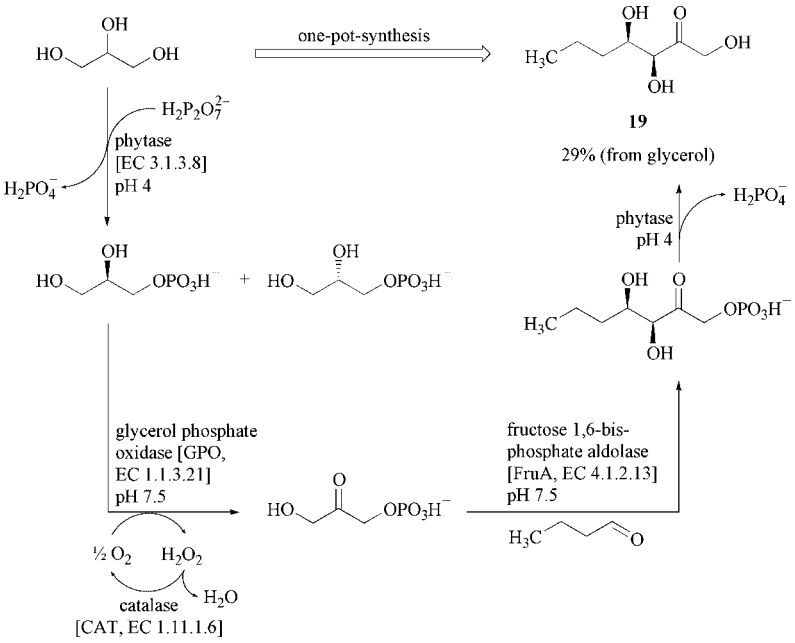
X = $-CH_3$ (for E^3, E^4)

Scheme 10. Stereoselective C–C bond formation by means of DHAP-utilizing aldolases

6.1.2.4 **C–C Coupling**

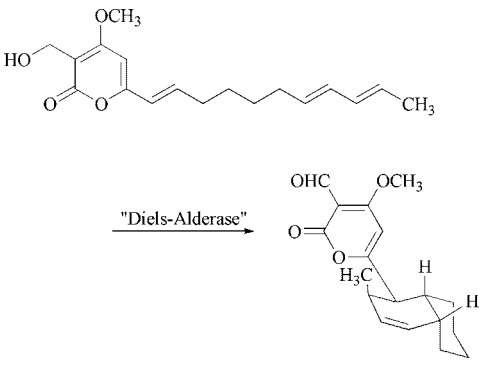
A wide range of natural and non-natural monosaccharides has been generated by exploiting the catalytic capacity of aldolases, which perform reactions equivalent to nonenzymatic aldol additions [1179-1184]. More than 20 aldolases have been identified so far and can be divided into three main groups, accepting either dihydroxyacetone phosphate (DHAP), acetaldehyde, or pyruvic acid, and phosphoenolpyruvate as nucleophilic methylene component. A common feature is their high stereocontrol in the formation of the new C–C bond. As presented in Scheme 10 all four possible *vic* diols are accessible by selection of the appropriate DHAP aldolase [1093], [1094], [1185–1187], all of which show a distinct preference for the two stereocenters and a broad substrate tolerance for the aldehyde component.

In a particularly elegant example (Scheme 11), 5-deoxy-5-ethyl-D-xylulose (**19**) was prepared from glycerol by a four-step, one-pot chemoenzymatic reaction sequence. The key step was the aldol reaction catalyzed by fructose 1,6-bisphosphate aldolase (FruA, E.C. 4.1.2.13) from *Staphylococcus carnosus* [1188].



Scheme 11. Careful pH control can switch the enzyme activities for phosphorylation, oxidation/aldol addition and dephosphorylation. Only readily available starting materials are necessary

Other C–C coupling reactions have been published [1092–1094]. A prominent example is the enzymatic Diels–Alder reaction which affords (−)-solanapyrone A (**20**). The enzyme solanapyrone synthase from extracts of the phytopathogenic fungus *Alternaria solana* has hence been named "Diels-Alderase" [1189].



20
51%, > 98% ee

6.1.2.5 Formation of Glycosidic Bonds

The most frequently applied concepts for the enzymatic formation of oligosaccharides and glycoproteins involve the utilization of glycosyl transferases or *exo*- and *endo*-glycosyl hydrolases (glycosidases) [1190–1194]. Glycosyl transferases require expensive and often unstable sugar nucleotides as activated donor substrates, a major problem besides the poor availability of the enzymes. However, transferase-catalyzed glycoside synthesis leads to regio- and stereochemically pure products in high yields. Glycosidases, on the other hand, are inexpensive and easy to handle. The reactions are mostly carried out under kinetic control using simple glycosides as donors. Generally, one or more regiomers are obtained in moderate yields, all of them having the same anomeric configuration of the newly formed glycosidic linkage.

For instance α-fucosides and α-sialosides, which are both difficult to produce by means of classical carbohydrate chemistry, have been synthesized by use of the corresponding nitrophenyl glycoside donors by applying pig liver α-fucosidase (E.C. 3.2.1.51) [1195] and the sialidase (E.C. 3.2.1.18) from *Vibrio cholerae* [1196], respectively.

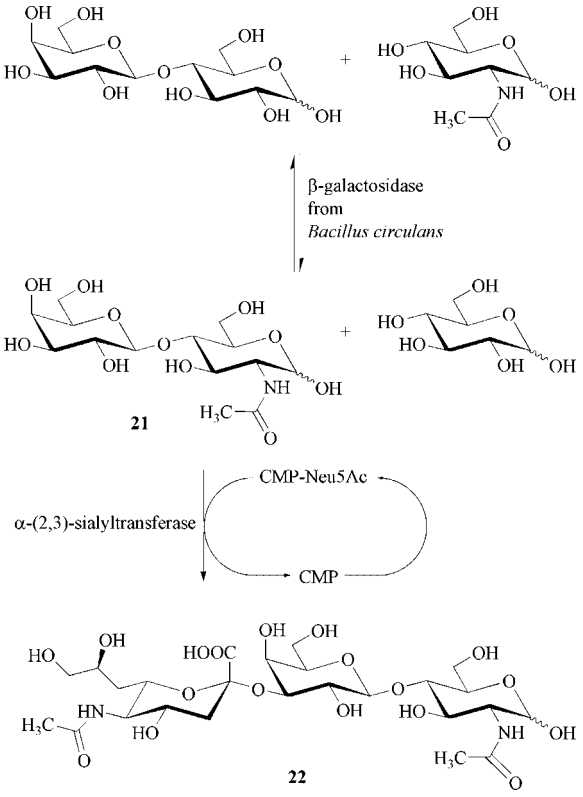
Starting from lactose as donor and *N*-acetyl glucose as acceptor substrate the β-galactosidase from *Bacillus circulans* (E.C. 3.2.1.23) gives exclusively *N*-acetyl lactosamine **21** [1197], which has been subjected to sialyltransferase (E.C. 2.4.99.6) catalyzed conversion to the trisaccharide Neu5Ac-α(2,3)Gal- β(1,4)GlcNAc (**22**; Scheme 12). By combining these two enzymatic steps the reaction is pushed toward the product, and the problem of low yields related with the galactosidase-mediated reaction could be overcome [1198].

To avoid problems associated with transferase-catalyzed reactions, i.e., the high price of the sugar nucleotides and possible inhibition by the released nucleotide phosphates, schemes for the regeneration of the sugar nucleotides were developed [1199–1201]. Thus, multienzyme systems have been successfully used in one-pot reactions for the large-scale synthesis of several oligosaccharides [1190–1194], [1199–1201].

A particular challenge is enzymatic in vitro glycoprotein synthesis. Initially, the way was paved for the preparation of several glycoforms of ribonuclease B (RNase B) (**24** and **25**). First, a monoglycosylated form of RNase B (**23**) was prepared by deglycosylation of the heterogeneous sugar moities [1202]. Subsequently, the homogeneously modified sialyl Lewis X RNase B was generated with the help of a series of glycosyl transferases (Scheme 13). In an alternative approach *endo*-glycosidase A (*endo* A, E.C. 3.2.1.96) transferred a mannose-rich glycoside core from an *N*-linked aspartate donor to the mono-glycoside of RNase B to give the homogeneous protein [1203].

6.1.2.6 Enzymatic Protecting Proup Techniques

Often, not only highly selective but also extremely mild methods to introduce and remove protecting groups are needed. This applies in particular for the construction of complex, polyfunctional molecules, e.g., oligosaccharides, peptides, nucleotides and their conjugates, as well as for the synthesis of alkaloids, macrolides, and other natural products. In this respect enzymes offer valuable opportunities complementing the already elaborated classical techniques. The chemo-, regio-, and stereoselectivity of enzymes allows them to establish otherwise unattainable ''quasi-orthogonal'' protecting group arrangements.

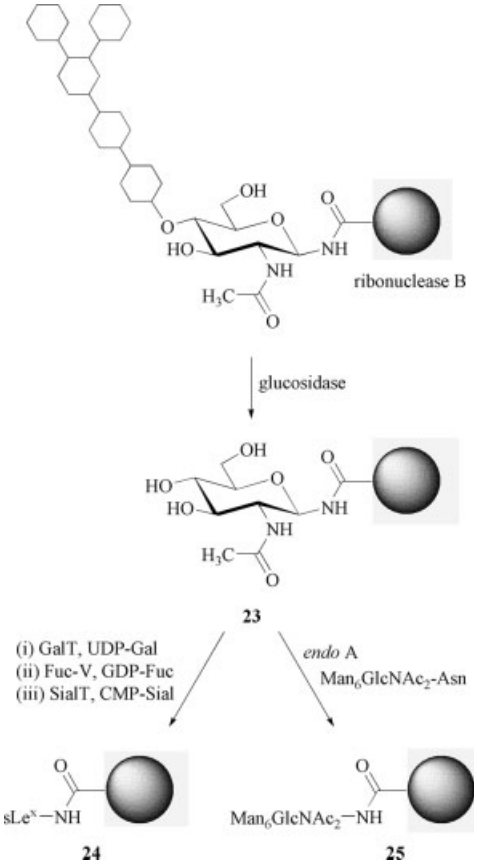


Scheme 12. Enzymatic synthesis of a sialyl trisaccharide by combination of glycosidase- and glycosyl-transferase-catalyzed reactions

The aforementioned hydrolase-catalyzed regioselective acylations and deacylations of a wide array of polyhydroxylated compounds has been addressed in numerous cases [1109], [1110].

In a recent advanced application of enzymatic protecting group techniques for the construction of complex multifunctional and sensitive targets, the synthesis of the characteristic *S*-palmitoylated and *S*-farnesylated lipohexapeptide of human *N-Ras* protein **26** was constructed. Compound **26**, which would not withstand standard procedures under basic or acidic conditions, could be obtained by following two different routes, both employing enzyme-labile protecting groups. The choline ester group was employed for C-terminal chain elongation [1204], while the *p*-acetoxybenzyloxycarbonyl (AcOZ) urethane group served well in a N-terminal coupling strategy [1205] (Scheme 14).

The spectrum of enzymatic deprotecting tools (Fig. 93) was further broadened by the introduction of the *p*-phenylacetoxybenzyloxycarbonyl (PhAcOZ) group for N-terminal protection [1206], [1207] and the *p*-phenylacetoxybenzyl ester (PAOB) group for C-terminal protection of peptides [1208], respectively. Both groups are readily cleavable



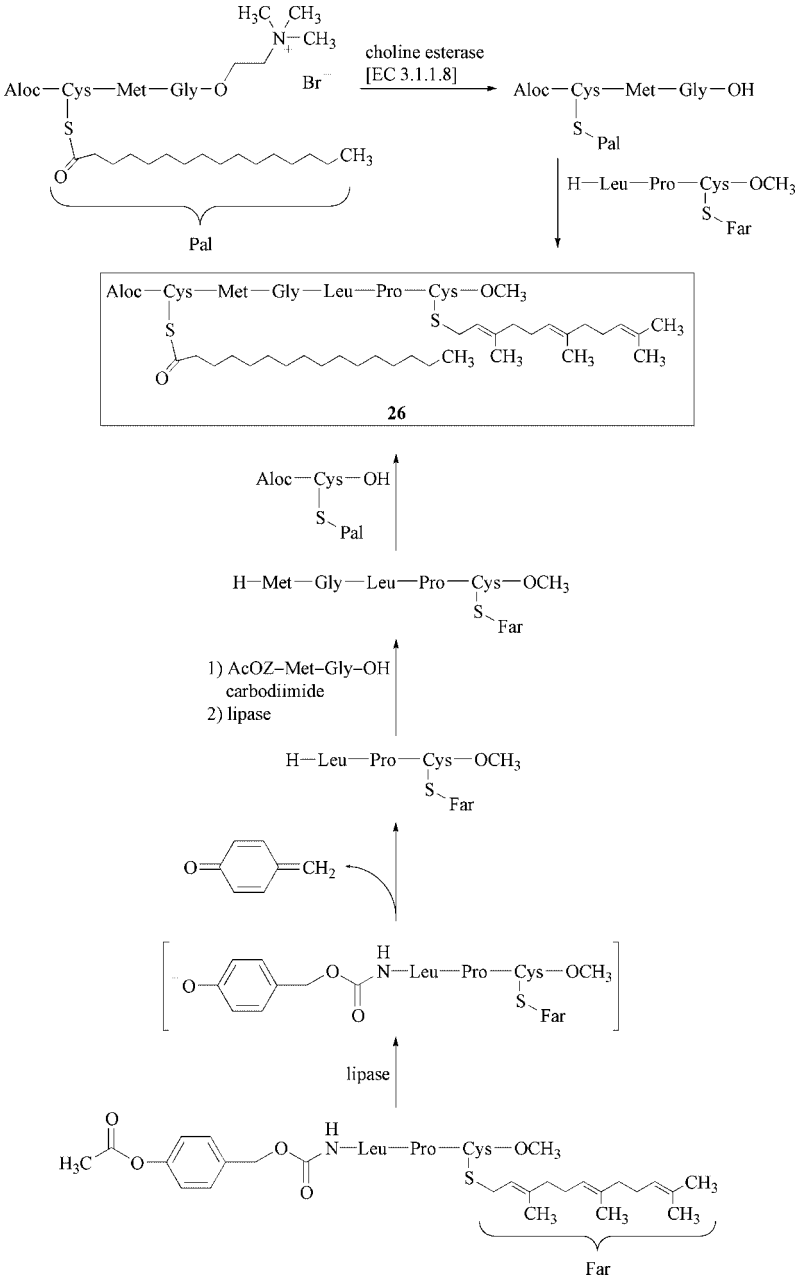
Scheme 13. Glycosylation of ribonuclease B using either glycosyltransferases or endo-glucosidase (endo-A) systems results in unnatural or homogeneously glycosylated proteins, respectively

by penicillin G acylase (E.C. 3.5.1.11) and have been successfully used in lipopeptide and glycophosphopeptide synthesis [1208], [1209]. The glucosyloxycarbonyl (Gloc) group is a suitable substrate for α- and β-glucosidases (E.C. 3.2.1.20 and 3.2.1.21) [1210].

6.1.3
Enzyme-Analogous Catalysts

The capability of enzymes to transform organic substrates smoothly, in good yields, and highly selectivity, have stimulated the desire of organic chemists to tailor catalysts with similar features for specific reactions.

In this respect, reactions catalyzed by monoclonal antibodies have been the focus of much interest [1211–1213]. One of the first antibody-supported syntheses on a gram



Scheme 14. Biocatalytic syntheses of a *S*-farnesylated and *S*-palmitoylated *N*-Ras fragment

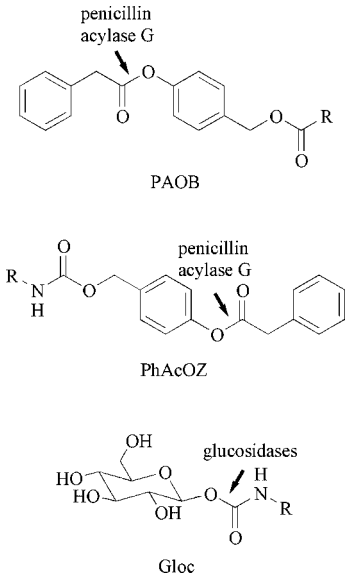


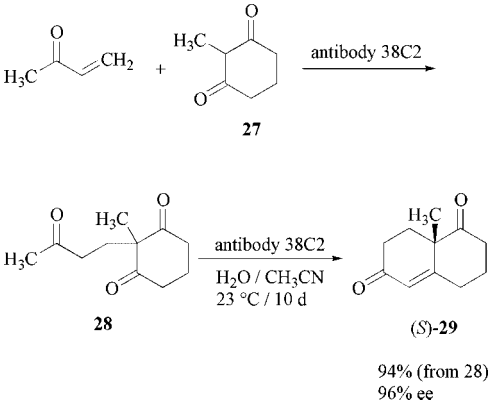
Fig. 93 Enzyme-cleavable protecting groups for carboxy acids (PAOB) and amines (PhAcOZ and Gloc)

scale and with an enantiomeric access of up to 99 % was the kinetic resolution of aldol adducts. The products resulting from the retro-aldol reaction served as precursors in the synthesis of epothilones A and B [1214], [1215].

For the production of an effective monoclonal antibody it is crucial to raise it from a well-designed transition-state analogue. Besides not resembling the product too much in order to avoid product inhibition, this hapten should mimic the transition state as closely as possible to maximize its stabilization by the antibody generated. Utilizing the extraordinary specificity of the immune system by this means, the combining site of the antibody is programmed to exhibit a specific catalytic activity. This technique has been directed toward the development of selective reactions which are chemically difficult to achieve, for example, ''disfavored'', and for which no suitable enzyme is available.

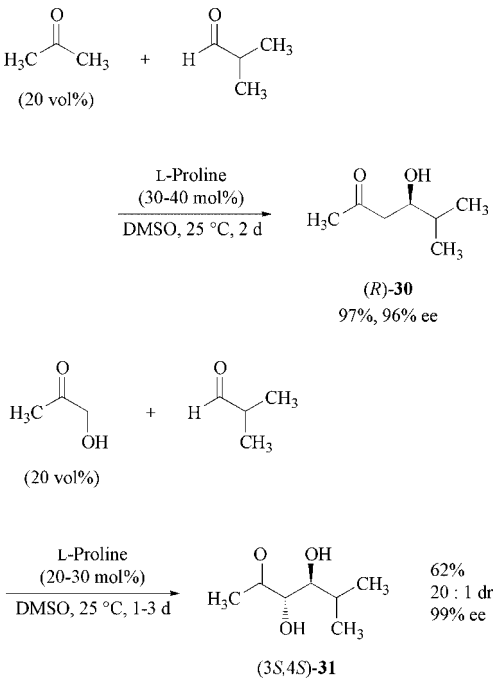
Therefore, several reactions were subjected to various antibody catalyzes, e.g., ester and enol ether cleavage, transesterification, ketone reduction, Cope rearrangement, ring-closure reaction via epoxide opening, and Diels–Alder cycloaddition [1216–1218], [1219]. An exceptional reaction is the antibody-catalyzed Robinson annulation of triketone **28** to the Wieland–Miescher ketone **29** on a preparative scale. Surprisingly, even the alkylation of diketone **27** with methyl vinyl ketone was catalyzed by the same antibody, but at moderate rates (Scheme 15) [1220].

Ribonucleic acids (RNAs) were employed as catalysts in the synthesis of amides and esters, peptide bond formation, and Diels–Alder reactions. The approach suffers from the fact that it requires the reactants to be either RNA itself or a compound covalently tethered to RNA. However, there is one prominent example in which RNA acts as a true catalyst in a bimolecular Diels–Alder cycloaddition reaction without tethering the substrates [1221–1223].



Scheme 15. Preparative enantioselective synthesis of a Wieland–Miescher ketone by an antibody

The chemical modeling of active sites of enzymes can help to gain deeper insight into their catalytic mechanisms and might also lead to enzyme substitutes which are reliable and easy-to-handle tools for organic synthesis. Recently, it was discovered that a single proline can mimick the active site of an aldolase. High ee values were obtained with acetone or hydroxyacetone, with generation of one (e.g., **30**) or two (e.g., **31**) new stereocenters in fair to excellent yields, respectively. This reaction seems to be well suited to industrial application, because of its simplicity and low cost [1224–1228].



A more recent topic has so far received little attention, but will most probably develop to great importance in bioorganic chemistry: the use of enzymes for ligating peptides [1229], [1230]. Peptide cyclization to form the antibiotics tyrocidine A and gramicidin S was achieved with the thioesterase domain of tyrocidine synthetase [1229]. α-Chemotrypsin (E.C. 3.4.21.1) catalyzed the condensation of larger peptide fragments to form an active sequence of the human thyroid anchoring protein Ht31 [1230]. The synthetic peptide was able to abolish forskolin-induced outward chloride currents in guinea pig ventricular myocytes.

6.1.4 Commercial Applications

6.1.4.1 General

Since the mid-1970s biotransformations have become a well-established tool in the fine-chemicals industry. Biocatalytic systems, including crude and purified enzymes as well as whole-cell systems performing highly selective reactions under mild conditions, are widely used, especially in synthesis and production of biologically active compounds in the agrochemical and pharmaceutical sectors.

Biocatalysis has already been proven in many cases to overcome specific synthetic problems. Thus, the need for hazardous reagents or the formation of specific impurities or byproducts can be avoided. Furthermore, biocatalysis can provide excellent regio- and stereoselectivity, and much higher turnover numbers than with chemical catalysts are possible. Therefore, it is not surprising that biocatalysis has also found an important place among industrial methods for the production of enantiomerically pure compounds (EPC; see Figure 94). The reported sales of EPCs exceeded $\$100 \times 10^9$ in 1999 for the first time.

In large-scale synthesis, cost-effectiveness and minimization of environmental problems are the most important factors in successful process development. The costs for the catalytic system, its separation, and in many cases its recovery are very important and have to be optimized. A critical figure in cost calculation is the ''catalyst consumption number'' (comparable with the TON in pure homogeneous catalysis) per kilogram of product produced. In the case of an enzyme, this number expresses how many units of enzyme or grams of biomass are deactivated and have to be replaced during production of 1 kg of product. Therefore, stability is often much more important than activity. The most suitable biocatalyst, which can be a native or an immobilized enzyme, or a whole-cell system, must be chosen carefully. Investigation of the total reaction engineering (including the reaction system) for optimal reaction conditions, reaction and reactor kinetics, as well as good downstream processing, offers the highest chance for success.

The examples selected here are commercially relevant and focus on specific topics discussed above. They demonstrate the advantage of biocatalysis in the conversion of functional groups and configuration, resolution of racemates, and asymmetric synthesis.

6.1.4.2 Nonstereoselective Biocatalytic Reactions

Even for simple, nonstereoselective conversions of functional groups, biocatalytic methods can be highly competitive (and superior) compared with chemical standard processes.

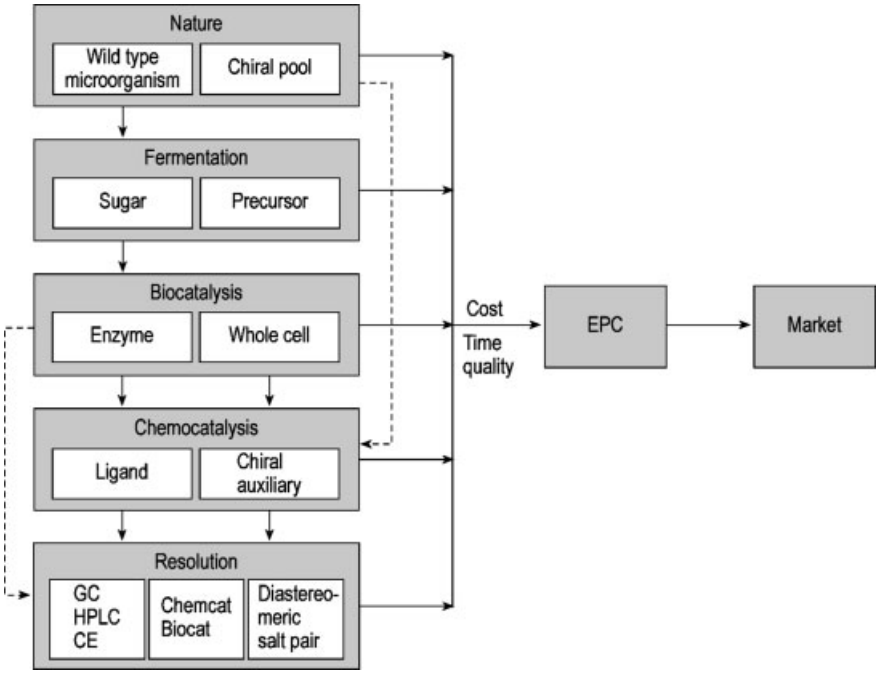
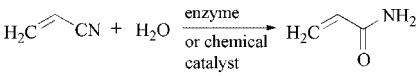


Fig. 94 Industrially applied approaches to enantiomerically pure compounds (EPCs)

This has been impressively demonstrated by Nitto Chemical Industries (now Mitsubishi Rayon) with the biocatalytic manufacture of acrylamide, an important building block for polymers and copolymers, produced in quantities of over 200 000 t/a [1231]. The chemocatalytic route to acrylamide (**32**) uses a reduced Raney copper catalyst for hydration. This metal-catalyzed process has been shown to be superior to the acid-catalyzed hydration, but catalyst poisoning and wastewater problems due to heavy-metal content cause some problems [1232], [1233].



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Nitto Chemical Industries developed and established an alternative way to produce acrylamide by an enzymatically catalyzed addition of water to acrylonitrile, using immobilized cells of *Rhodococcus* sp. N-774 and later an optimized mutant strain of *Pseudomonas chloraphis* B 23 [1232], [1234], [1235]. Using a highly improved cobalt-containing nitrile hydratase from *Rhodococcus rhodochrous* J1, it is possible to reach up to 700 g L^{-1} acrylamide in the reaction solution. The reaction temperature for the copper-catalyzed hydration is around 100 °C, whereas the enzymatic process is carried out below 10 °C. Nitto acrylamide production using bacterial nitrile hydratase is estimated

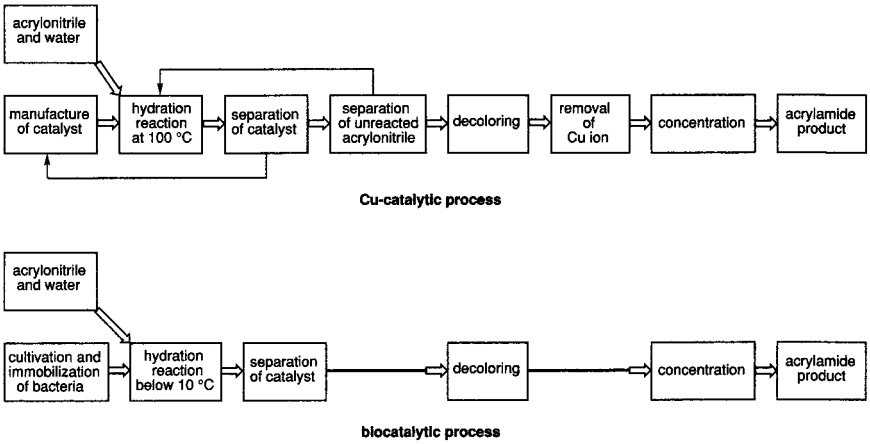


Fig. 95 Comparison of the microbial and conventional chemical process for acrylamide production

to produce around 30 000 t/a, consumes less energy, and avoids heavy-metal problems in the wastewater. In Figure 95, the chemo- and biocatalytic production processes for acrylamide are compared.

In addition, the conversion of aromatic nitriles to carboxylic acids or amides has been applied for the production of fine chemicals. For example, Lonza reported the biocatalytic manufacture of niacinamide starting from 3-cyanopyridine [1236]. It is said that this process will be carried out in the future on a multithousand tonnes per year scale.

Another interesting commercial nonstereoselective application is the biocatalytic conversion of methyl groups to carboxylic acids. This new technology, also developed at Lonza, is already successfully applied on an industrial scale in the production of 5-methylpyrazine-2-carboxylic acid, a versatile building block for pharmaceuticals [1236].

It is noteworthy that the biocatalytic formation of a methyl group via decarboxylation can also be realized on a technical scale. Decarboxylation of L-asparagine in the presence of an aspartate-4-decarboxylase (E.C. 4.1.1.12) allows an efficent synthesis of L-alanine [1237].

6.1.4.3 Biocatalytic Resolution Processes

On an industrial scale, resolution of racemates is still in many cases the preferred way of producing enantiomerically pure compounds. If chiral-pool compounds are not available as optically active starting materials, a cost comparison of asymmetric and resolution processes often reveals advantages for the latter. Among the top 15 optically active pharmaceuticals and their intermediates, a chemical asymmetric process could be established only in the case of naproxen. For all others, diastereomeric and biocatalytic resolution are the methods of choice, apart from fermentation, for creating optically pure compounds.

Normally, only one enantiomer is needed; therefore, highly effective methods for racemizing the undesired one must be integrated into the process. Besides the well-known examples of separating diastereomeric salt pairs by crystallization, biocatalytic resolution is gaining more and more interest. There are a number of key factors which must be optimized for successful industrial development; for example, substrate and enzyme costs (including recovery); chemical, optical, and space-time yields; number of reaction steps and positioning of the resolution step within the whole process; and downstream processing, together with the necessary equipment.

In biocatalysis, hydrolases are the most important class of enzymes for carrying out enzymatic resolutions. Many hydrolases, such as esterases, lipases, epoxide hydrolases, proteases, peptidases, acylases, and amidases, are commercially available; a substantial number of them are bulk enzymes [1238–1240]. Resting-cell systems, if they are not immobilized, are used in dilute suspensions and could be handled as quasi homogeneous catalysts.

In the following examples some industrial applications of biocatalytic resolutions are decribed.

Enzymatic Hydrolysis of Acyclic Amides Production of Amino Acids Using Amino Acylases: The Degussa Process Enantiospecific hydrolysis of *N*-acylamino acids by amino acylase I from *Aspergillus oryzae* (E.C. 3.5.1.14) is the best established large-scale process for L-amino acids. Both L- and D-amino acids could be produced according to this technology. Tanabe has developed a process using an immobilized enzyme [1241], [1242], whereas at Degussa a process using native amino acylase I from *Aspergillus oryzae* in homogeneous solution is applied. For this purpose, a new technology using an enzyme membrane reactor (EMR) has been designed, based mainly on the work of Kula and Wandrey [1243–1245].

The substrate specificity of the amino acylase from *Aspergillus oryzae* is very broad, and a wide range of proteinogenic and nonproteinogenic *N*-acetyl and *N*-chloroacetyl amino acids are transformed by the enzyme. The enzyme membrane reactor (Figure 96) is operated continuously as a recycle reactor, and the enzyme is retained by a hollow-fiber ultrafiltration membrane (molecular weight cut off: 10 000 Da).

Since the early 1980s this process has been scaled up to a production level of many hundreds of tonnes per year. Scheme 16 shows the Degussa process for manufacturing L-methionine (**33**). The biocatalyst is produced in bulk quantities, and its operational stability is high, so this continuous EMR acylase process demonstrates high efficiency, especially on a large scale [1246], [1247].

Production of Amino Acids Using Amidases: The DSM Process DSM has developed a widely applicable industrial process for production of enantiomerically pure amino acids by enantioselective hydrolysis of racemic amino acid amides. These precursor compounds can easily be obtained by alkaline hydrolysis of α-aminonitriles. The L-specific amidase (aminopeptidase) was found in *Pseudomonas putida* (ATCC 12633), and shows a broad substrate specificity. Methods for racemizing the undesired enantiomer are also integrated in the process (Scheme 17) [1248].

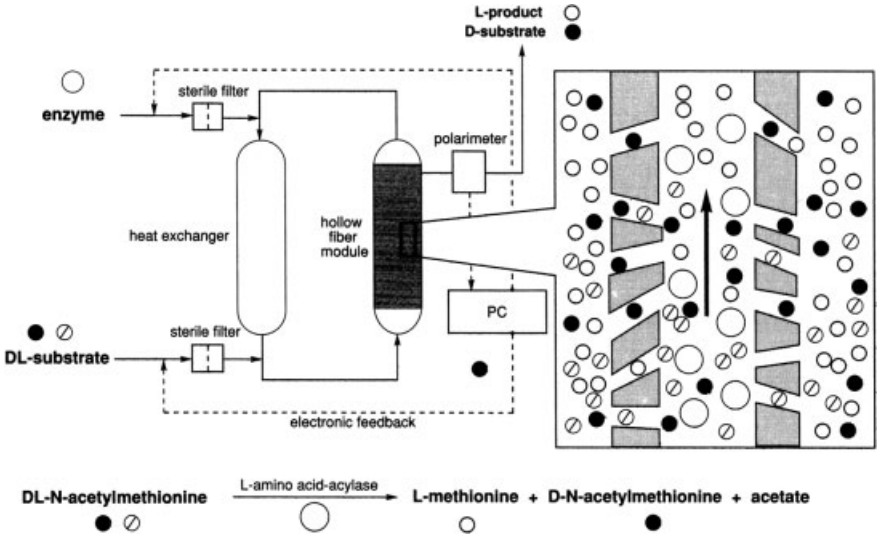


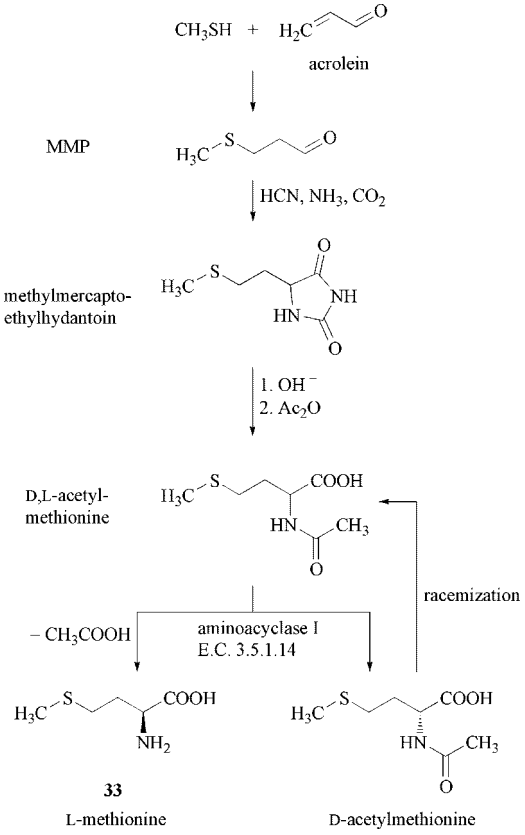
Fig. 96 Recycle reactor used for amino acylase resolution of *N*-acetyl-D,L-amino acids

Production of Carboxylic Acids Using Amidases: (S)-2,2-Dimethylcyclopropanecarboxylic Acid [(S)-Ibuprofen] (*S*)-2,2-Dimethylcyclopropanecarboxylic acid is an intermediate for the synthesis of cilastatin, a dehydropeptidase I inhibitor. Lonza has developed an industrial two-step biotransformation process starting from racemic 2,2-dimethylcyclopropane nitrile (**34**; Scheme 18). The stereoselective amidase was found in *Comomonas acidovorans* and cloned into a fast-growing *E. coli* strain which produces large amounts of the biocatalyst [1249], [1250].

For the production of the analgesic (*S*)-ibuprofen from racemic ibuprofen amide, (*S*)-selective amidase-containing microorganisms belonging to the genera *Brevibacterium* and *Corynebacterium*, in particular the species *Brevibacterium* R312, *Corynebacterium* N771, and *Corynebacterium* N774, have been found to be suitable biocatalysts [1251].

Production of 6-APA, 7-ADCA, and 7-ACA Using Amidases 6-Aminopenicillanic acid (6-APA, **35**), 7-aminocephalosporanic acid (7-ACA, **36**), and 7-aminodesacetoxycephalosporanic acid (7-ADCA, **37**) are the most important precursors for the highly active semisynthetic β-lactam antibiotics. The worldwide capacity thereof exceeds 20 000 t/a.

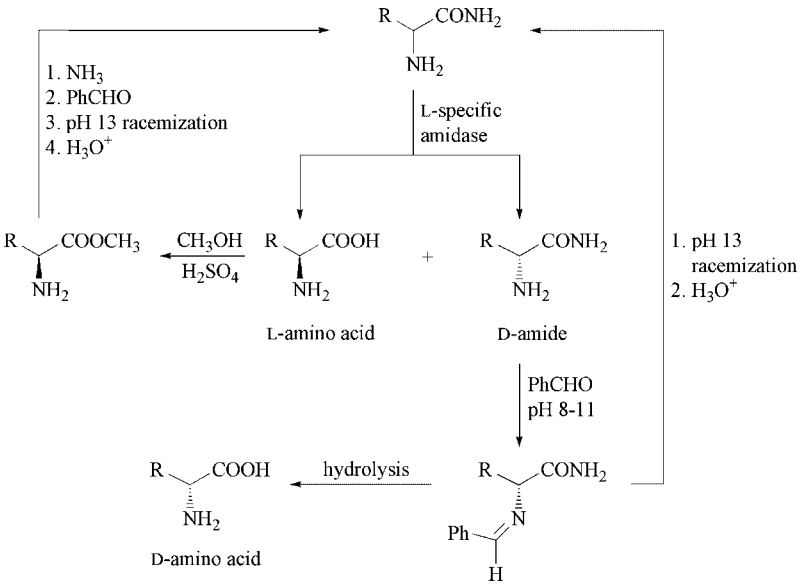
Before 1980, 6-APA and 7-ADCA were produced only by chemical deacylation of penicillin G and phenacetyl-7-ADCA, which could be obtained by chemical ring expansion of penicillin G (Scheme 19). Since 1980, enzymatic processes avoiding the use of environmentally critical reagents in large amounts have been developed [1252–1254]. It is noteworthy that, in addition to environmental advantages, the enzymatic route was found to be considerably cheaper than the chemical process [1255]. The most prominent enzyme is penicillin G amidase (Pen G amidase,



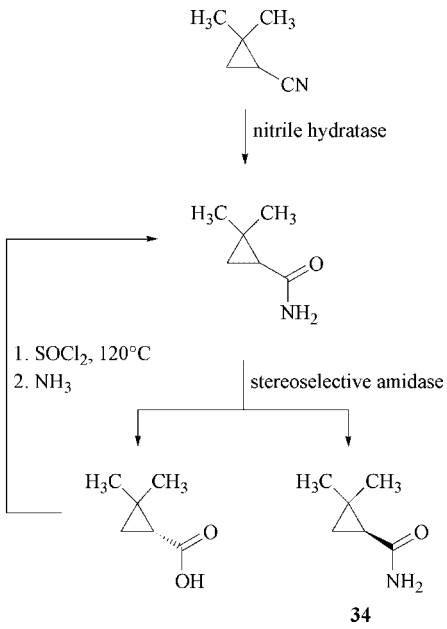
Scheme 16. Degussa L-methionine process using acylase

E.C. 3.5.1.11) [1255], [1256], which preferentially hydrolyzes penicillin G and penicillin V as well as phenacetyl-7-ADCA without affecting the β-lactam ring. A broad variety of cultures can be used for large-scale production of this enzyme, including *E. coli* [1257]. The penicillin G amidase from *E. coli* enzyme is one of the best studied acylases, and has been characterized as a heterodimer with molecular weights of 19 500 and 60 000 Da for the two subunits α and β, respectively [1258]. Nowadays, Pen G amidase (E.C. 3.5.1.11) is applied in immobilized form. A preferred solid support is Eupergit C, a bead polymer containing reactive epoxide functions which covalently bind to the enzyme [1259]. It is noteworthy that Eupergit-immobilized penicillin G amidase can be reused up to 600 times. The enzymatic hydrolytic reactions on an industrial scale are performed at 30–40 °C and pH 7.5–8.0 [1260].

For the industrial production of 6-APA and 7-ADCA (see Scheme 19) excellent conversion rates of at least 98 % were reported. In addition, yield of isolated product and selectivity are high. Typically the industrial reaction is carried out batchwise. Detailed summaries of production data are available in the literature [1260].



Scheme 17. DSM amidase process for enantiomerically pure α-amino acids



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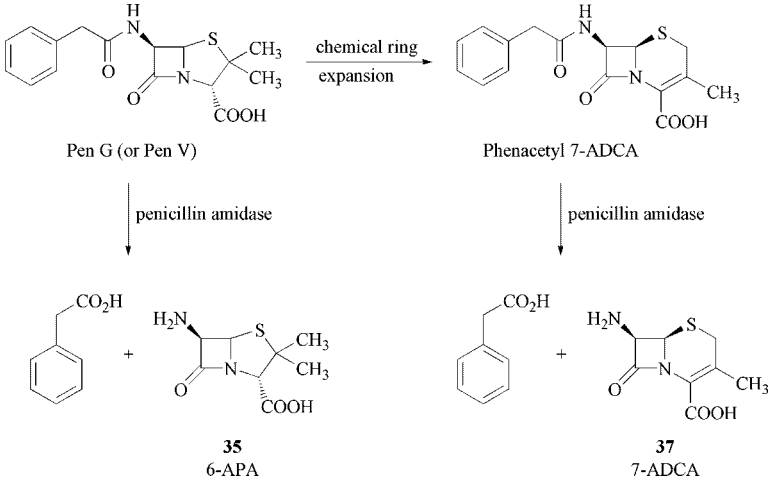
Scheme 18. Enzymatic production of (S)–2,2–dimethylcyclopropane carboxylic acid



Scheme 19. Enzymatic production of 6–APA and 7–ADCA

7-ACA (**36**) [1261] is produced in a three-step process using two enzymes, starting from cephalosporin C (Scheme 20). After deamination by a D-amino acid oxidase, the α-ketoadipyl-7-ACA spontaneously decarboxylates, and the remaining glutaryl side chain is hydrolyzed by glutaryl-7-ACA-acylase.

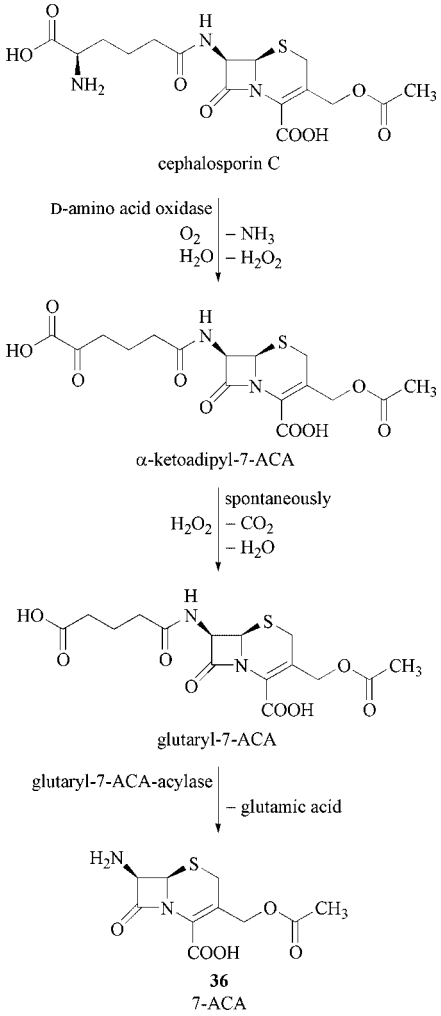
6.1.4.3.1 Enzymatic Acylation of Amino Groups

Production of Ampicillin and Cephalexin Besides being used for the production of the precursors 6-APA and 7-ADCA, penicillin amidases are also able to couple 6-APA and 7-ADCA with D-phenylglycine ester or amide in a kinetically controlled enzymatic peptide synthesis to form ampicillin or cephalexin (**38**,**39**). These reactions (Scheme 21) have a great potential for being commercialized in the near future [1262].

Production of Amines Using Lipases: The BASF Process Biocatalytic resolution has been applied efficiently by BASF for the manufacture of optically active amines, such as phenylethylamine [1263], [1264]. The process is based on a highly stereoselective resolution of racemic amines by means of an acylation reaction in the presence of a lipase as catalyst (Scheme 22). The products are obtained in high yields and with excellent enantioselectivities. The undesired enantiomer can be racemized subsequently. Thus, starting from a racemate, efficient access (with theoretically 100 % yield) to the (*R*)- and (*S*)-amines is available. This technology, which is said to be carried out on a greater than 1000 t scale, has been extended recently by BASF to the production of chiral alcohols.

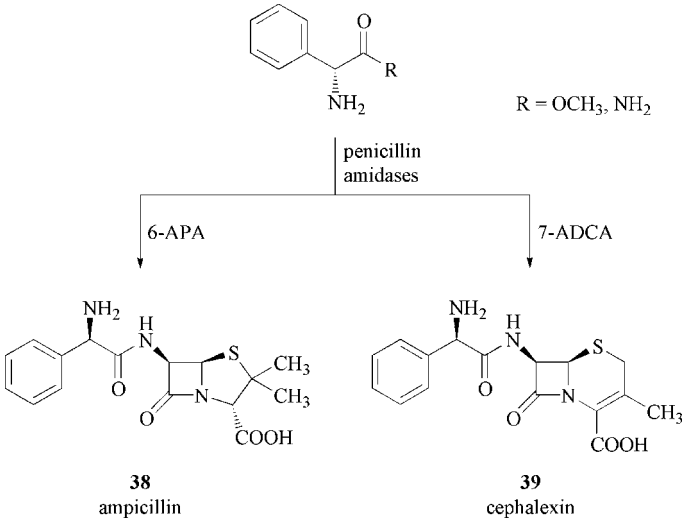
6.1.4.3.2 Enzymatic Hydrolysis of Hydantoins

Production of D-Amino Acids Amino acids with D configuration play an important role in synthesis and production of biologically active compounds. The most prominent D-amino acids are D-phenylglycine and 4-hydroxyphenylglycine, which form the

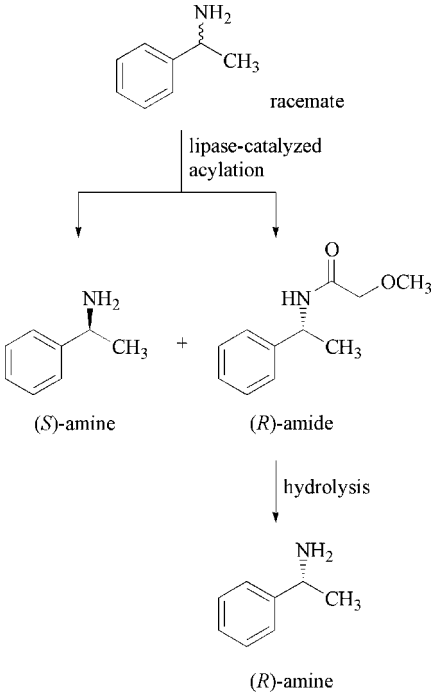


Scheme 20. 7–ACA production starting from cephalosporin C

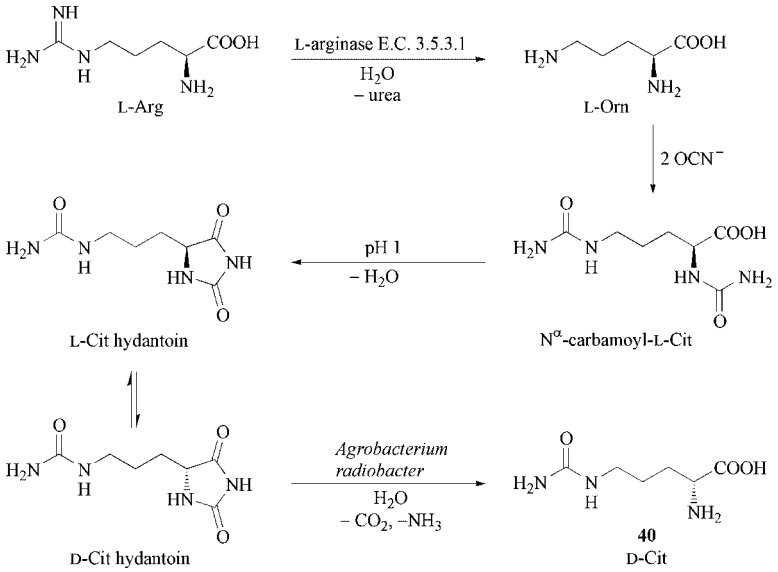
side-chain of the semisynthetic β-lactam antibiotics ampicillin, amoxicillin, and cephalexin. For production of both of these amino acids, resting-cell biocatalysts have been developed, containing a D-hydantoinase and sometimes also a D-carbamoylase and a racemase [1265]. The D-hydantoinase is able to transform a racemic 5-monosubstituted hydantoin into the D-hydantoic acid; the L-hydantoin can racemize at the alkaline pH of the reaction mixture. When the carbamoylase and racemase are both present, a complete transformation to the D-amino acid occurs. The racemase can assist hydantoin racemization if the process itself is too slow under the prevailing reaction conditions. Without the carbamoylase the liberation of the D-amino acid must be



Scheme 21. Enzymatic synthesis of ampicillin and cephalexin



Scheme 22. BASF process for the production of amines

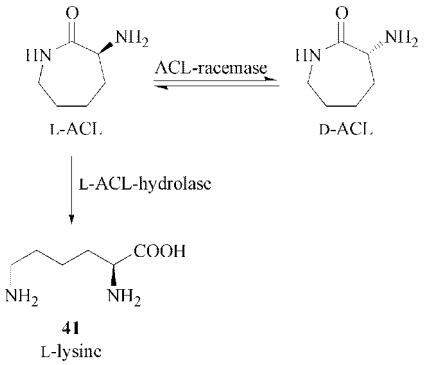


Scheme 23. Production of D-Cit starting from L-Arg

performed by nitrosation of the hydantoic acid. This is not a highly desirable reaction; additionally, HNO_2-sensitive functional groups are degraded.

Another example of the biocatalytic production of D-amino acids involves the ureido amino acid D-citrulline (D-Cit, **40**), which is needed for the luteinizing hormone-releasing hormone (LHRH) antagonist Cetrorelix, first synthesized by A. SCHALLY *et al.* and further developed by Asta Medica [1266-1268]. Cetrorelix consists of five D-amino acids, among which D-citrulline (D-Cit) is particularly difficult to synthesize. For the synthesis of D-Cit (**40**), a hydantoinase/carbamoylase system from *Agrobacterium radiobacter* was chosen. The synthesis starts from the inexpensive proteinogenic amino acid, L-arginine (L-Arg, see Scheme 23), which has the appropriate carbon chain length and also a nitrogen atom attached in the 5-position. In the presence of arginase, L-Arg is deguanylated quantitatively to L-ornithine (L-Orn). This reaction is much more effective than any chemical hydrolysis.

L-Orn then is then treated with two equivalents of cyanate to give dicarbamoylated L-Cit, which can easily be cyclized to give the L-Cit hydantoin. When the *Agrobacterium* biocatalyst with hydantoinase and carbamyolase activity is added to an aqueous alkaline solution of this hydantoin, complete transformation to D-Cit takes place. The ee of 99.8 % demonstrates that the enzymes act stereospecifically [1269]. At Degussa D-Cit (**40**) has been produced in quantities of several tens of kilograms. This hydantoinase/carbamoylase system offers an excellent method for producing D-amino acids starting from racemic or suitable L-precursors without additional racemization or subsequent chemical steps. Particularly for synthesis of side-chain-functionalized D-amino acids, this method is superior to other alternatives [1270], [1271].



Scheme 24. Toray enzymatic L-lysine process using α-amino-caprolactam as starting material

6.1.4.3.3 Enzymatic Hydrolysis of Lactams

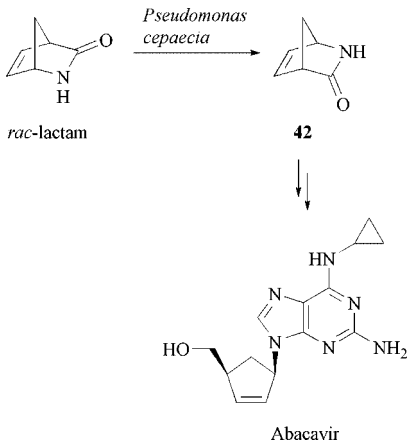
Production of L-Lysine: The Toray-Process For some years Toray's enzymatic process for L-lysine (L-Lys, **41**) was competitive with fermentation. This chemoenzymatic L-Lys production was established with a capacity of 5000–10 000 t/a. The key intermediate is α-amino-ε-caprolactam (ACL), produced from cyclohexanone in a modified Beckmann rearrangement. The enantiospecific hydrolysis forming L-Lys is based on two enzymes: L-ACL hydrolase opens the ring of ACL to give L-Lys, and in the presence of the ACL racemase D-ACL is racemized. By incubating D,L-ACL with cells of *Cryptococcus laurentii* having L-ACL lactamase activity together with cells of *Achromobacter obae* with ACL racemase activity, L-Lys could be obtained in a yield of nearly 100 % (Scheme 24) [1272–1275].

This process has now been abandoned because, on a 100 000 t/a scale, fermentation of L-Lys based on sugars has proven to be superior to the Toray process.

Production of 2-Azobicyclo[6.2.2.1]hept-5-en-3-one A highly selective enzymatic hydrolysis of racemic 2-azobicyclo[6.2.2.1]hept-5-en-3-one has been developed by Chirotech [1276], [1277]. The selective hydrolytic cleavage of the (+)-lactam led to the desired, unhydrolyzed (−) enantiomer with a high ee of >98 %. The single (−) enantiomer (**42**) functions as an intermediate in the synthesis of the Glaxo Wellcome anti-HIV drug Abacavir (Scheme 25). It is noteworthy that this process operates at a substrate concentration of >500 g/L.

6.1.4.3.4 Enzymatic Hydrolysis of C–O Bonds

Esterases, lipases, and proteases are widely used in enzymatic resolution of racemic carboxylic ester substrates. Industrially relevant examples are the manufacture of (D)-β-acetylmercaptoisobutyric acid, the optically active side chain of the angiotensin-converting enzyme (ACE) inhibitor captopril (**43**; Scheme 26) [1278], [1279], and synthesis and production of (*S*)-2-arylpropionic acids such as (*S*)-naproxen and (*S*)-ibuprofen, which are very important nonsteroidal antiphlogistic and analgesic drugs [1280–1284].



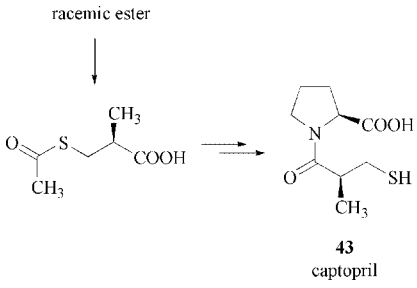
Scheme 25. Resolution of a racemic lactam

In Andeno's diltiazem synthesis (Scheme 27), an enantioselective enzymatic hydrolysis of an epoxy ester by a lipase is the key step, creating the necessary optically active intermediate **44** [1285–1287].

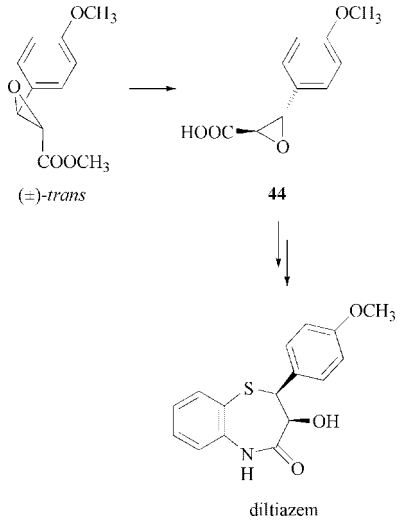
The enzymatic aspartame process of Tosoh can also be regarded as a reactive deracemization of D,L-phenylalanine methyl ester (D,L-Phe-OCH_3). In the key step the metalloproteinase thermolysin (E.C. 3.4.24.27) couples only the L isomer with (*Z*)-aspartic acid in a thermodynamically controlled peptide synthesis; the remaining D-Phe-OCH_3 is racemized and reused [1288].

Many more examples can be found in the literature [1289], [1290]. Another esterase process is described in more detail here. It is the optical resolution of D,L-pantoyl lactone (D,L-PL) by a fungal lactonase. The D isomer is a key component in the vitamin pantothenic acid and in coenzyme A. D,L-PL is easily produced by adding HCN to the aldol product of formaldehyde and isobutanal with subsequent acidic lactonization. For commercial production of D-pantoyl lactone (D-PL) the racemate can be resolved by chiral amines.

Recovery of the chiral auxiliary and racemization of the L isomer are troublesome. Therefore, much work has been done on developing more efficient resolution



Scheme 26. Production of captopril



Scheme 27. Production of diltiazem

processes or asymmetric syntheses. YAMADA *et al.* screened new fungal aldonolactonases which are able to hydrolyze D-PL specifically to D-pantoic acid (D-PA, see Figure 97).

This D-acid can easily be separated from the remaining L-PL, which can be racemized and recycled. Relactonization of D-PA yields the desired D-PL.

Among various lactonase-containing fungal microorganism, *Fusarium oxysporum* AKU 302 showed the highest activity. Compared with asymmetric chemical or microbial reduction methods or conventional chemical resolution, this lactonase process exhibits superior performance [1291–1293].

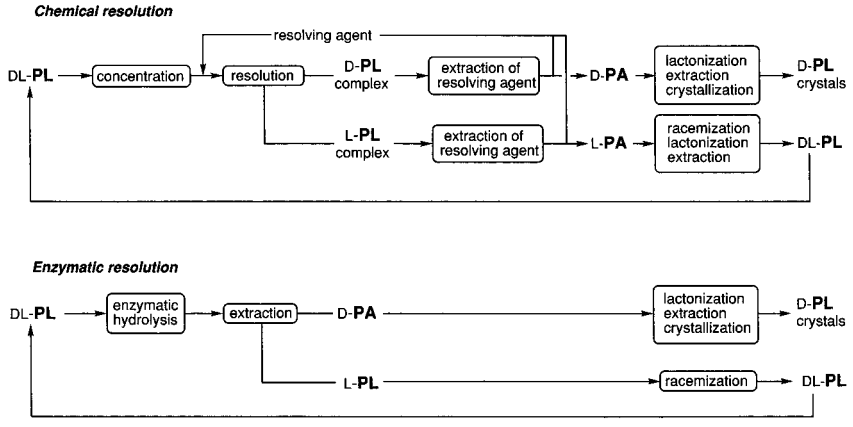
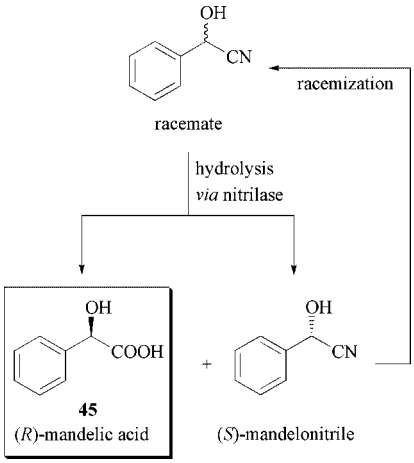


Fig. 97 Comparison of resolution of diastereomeric salt-pairs and enzymatic resolution for producing D,L-pantoyl lactone



Scheme 28. BASF process for the production of (*R*)–mandelic acid

6.1.4.3.5 Enzymatic Hydrolysis of Nitriles

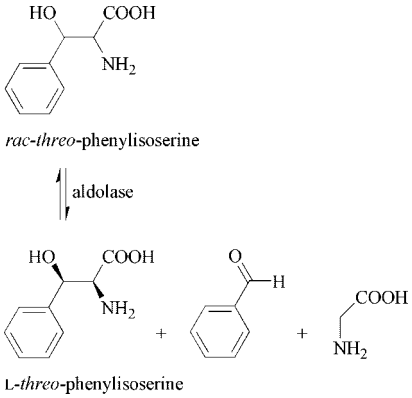
Production of (R)-Mandelic acid: The BASF Process That the conversion of nitriles into carboxylic acids also represent a suitable industrial resolution process has been reported by BASF [1096], [1294]. Based on racemic mandelonitrile, a stereoselective hydrolysis led to (*R*)-mandelic acid (**45**) in high yield and with high enantioselectivity (Scheme 28). It is noteworthy that during the process the cyanohydrin enantiomers are racemized in situ when the appropriate pH conditions are chosen. Thus, in spite of starting from a racemate a (theoretically) quantitative enzymatic conversion to the optically active (*R*)-mandelic acid can be achieved. The production of (*R*)-mandelic acid (**45**) is carried out on a scale of several tons. The hydrolysis of nitriles for the stereoselective manufacture of fine chemicals has also been applied by Celgene [1295].

6.1.4.3.6 Enzymatic Cleavage of *threo* Aldol Products

A chemoenzymatic process for the preparation of D- and L-*threo*-phenylisoserine and derivatives thereof has been developed by Celgene. This process integrates an Erlenmeyer condensation as an initial chemical step which produces racemic *threo* compounds, and a subsequent enzymatic cleavage of the undesired enantiomer with regeneration of the starting materials [1295]. As a biocatalyst, a novel D- or L-aldolase enzyme has been used (Scheme 29). Enantiomerically pure *threo*-phenylisoserines find a wide range of applications, e.g., for the preparation of the taxol side chain or a variety of phenicol antibiotics.

6.1.4.4 Biocatalytic Asymmetric Synthesis

Enzymatic reduction, oxidation, and ligase or lyase reactions, provide numerous examples in which prochiral precursor molecules are stereoselectively functionalized.



Scheme 29. Celgene-process for the production of L-threo–phenylisoserine

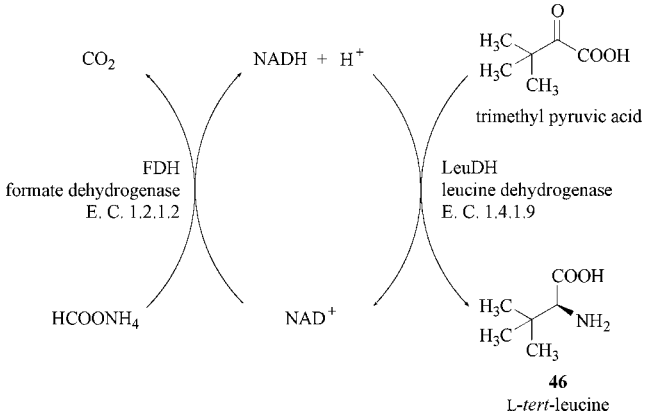
Ajinomoto's β-tyrosinase-catalyzed L-dopa process [1296], [1297], the formation of L-carnitine from butyro- or crotonobetaine, invented by Lonza [1298], and the IBIS naproxen route oxidizing an isopropylnaphthalene to an (*S*)-2-arylpropionic acid are representative, examples for many successful applications of enzymatic asymmetric synthesis on an industrial scale. A selection of recent industrial contributions in this field are summarized below.

6.1.4.4.1 Biocatalytic Reductive Amination of C=O Bonds

A very effective method for synthesizing α-amino acids with bulky side chains is the cofactor-dependent enzymatic reductive amination of α-keto acids, which is carried out by Degussa. For example, bulky-side-chain amino acids such as L-*tert*-leucine (L-Tle, **46**) and L-neopentylglycine (L-Npg) could be produced starting from the corresponding α-keto acid. In this reaction two enzymes, namely, leucine dehydrogenase (LeuDH, E.C. 1.4.1.9) and formate dehydrogenase (FDH, E.C. 1.2.1.2), and the cofactor NAD^+ act simultaneously in the transformation of the keto acid to the L-amino acid. The cofactor is used in catalytic amounts and is recycled during the reaction (Scheme 30). The use of LeuDH as amino acid dehydrogenase guarantees L stereospecificity, as no D enantiomer could be detected.

This type of reaction forming non-natural L-amino acids uses enzymes from the metabolism of proteinogenic amino acids, which additionally have an unexpectedly versatile substrate specificity by also accepting highly sterically hindered α-keto acids. L-Tle and L-Npg are of growing importance because of their extensive use as a building blocks in pharmaceuticals and as chiral auxiliaries in asymmetric synthesis [1299–1301].

The synthesis of chiral α-amino acids starting from α-keto acids by means of transamination has been reported by NSC Technologies [1302], [1303]. In this process, L-aspartate serves as the amino donor. The broad substrate specificity allows the conversion of numerous keto acid substrates.



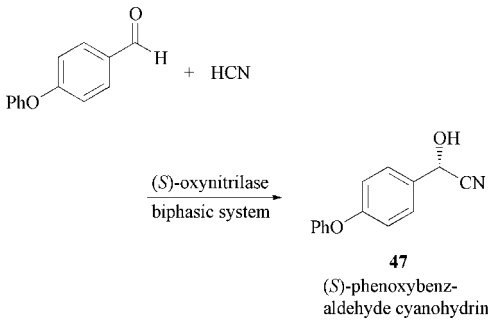
Scheme 30. L-Tle production by cofactor-dependent reductive amination

Another type of asymmetric reduction of a broad range of carbonyl compounds was developed by Celgene [1295]. Using ketones as substrate in the presence of a highly efficient transaminase as biocatalyst gave the corresponding amines in high yield and with high enantiomeric excess. It is noteworthy that transaminases have been found for the synthesis of (*R*)-amines as well as (*S*)-amines.

6.1.4.4.2 Biocatalytic Hydrocyanation of C=O Bonds

A further important asymmetric biocatalytic synthesis is the hydrocyanation of aldehydes [1304-1306] for the production of cyanohydrins, which are intermediates for a broad variety of pharmaceuticals and agrochemicals. For example, (*R*)-mandelonitrile is a versatile intermediate for the synthesis of (*R*)-mandelic acid, and (*S*)-*m*-phenoxybenzaldehyde cyanohydrin is a building block for the preparation of pyrethroids.

For the production of (*S*)-*m*-phenoxybenzaldehyde cyanohydrin (**47**) DSM established an enzymatic hydrocyanation process on an industrial scale (Scheme 31). An efficient (*S*)-oxynitrilase biocatalyst (E.C. 4.1.2.11) has been developed. This enzyme is derived from the plant *Hevea brasiliensis*, and has been cloned and overexpressed in a



Scheme 31. Asymmetric hydrocyanation using (*S*)-oxynitrilases

microbial host organism [1307]. In the presence of this biocatalyst the desired product (**47**) has been obtained with high enantioselectivity.

6.1.4.4.3 Biocatalytic Addition of Water to C=O Bonds

The enantioselective addition of water to fumaric acid gives malic acid [1308]. This highly efficient biocatalytic reaction is catalyzed by a fumarase (E.C. 4.2.1.2). Although substrate tolerance of this enzyme is narrow, high enantioselectivities are obtained.

6.1.4.4.4 Biocatalytic Amination of C=C Bonds

A biocatalytic enantioselective addition of ammonia to a C=C bond of an α,β-unsaturated compound, namely, fumaric acid, makes the manufacture of L-aspartic acid possible on industrial scale. This process, which is applied by, e.g., Kyowa Hakko Kogyo and Tanabe Seiyaku, is based on the use of an aspartate ammonia lyase (E.C. 4.3.1.1) as a biocatalyst [1309]. Another comparable reaction is the asymmetric biocatalytic addition of ammonia to *trans*-cinnamic acid, which is a technically feasible process for the production of L-phenylalanine [1310].

6.1.5 Outlook

The increasing use of biocatalysis in research and industrial production, especially in the developing field of chirotechnology is foreseeable. Advances in the following areas seem most promising:

1. Elucidation of the active site and the reaction mechanism of enzymes by a combination of X-ray analysis and computer-aided modeling may allow precise prediction of enzyme–substrate interaction.
2. Screening of microorganisms from exotic environments may lead to the discovery of promising new enzyme activities.
3. Using modern gene technology the catalytic properties of enzymes could be improved or custom-tailored for specific reactions. Even the design and engineering of synthetic catalysts may become feasible.
4. Considering the significant progress in the field of microbial pathway engineering, it is expected that the importance of this technology will also grow rapidly in the future. Microbial pathway engineering leads to biocatalysts which allow the production of desirable metabolites. Thus, similarly to fermentation, this method represents a biocatalytic one-pot synthesis in which several consecutive enzymatic steps are involved. Numerous important industrially relevant contributions in this field have been already made, for example, successful pathway engineering for carotenoids and polyketides [1311]. Further representative examples are the production of indigo, vitamin C, and 1,3-propandiol [1312].

There is a strong need for new biocatalytic methods producing enantiomerically pure compounds which are the basis for synthesis and production of biologically active compounds of agrochemical and pharmaceutical importance.

6.2
Therapeutic Enzymes

6.2.1
Requirements for the Use of Enzymes in Therapy

There is a long history of successful use of enzymes and other proteins as drugs [1313]. Although pharmaceutical research and development activities in the field of recombinant therapeutic proteins are mainly focused on antibodies, cytokines, and hormones rather than on enzymes, there are nevertheless three broad areas where enzymes are successfully employed in therapy: (1) to replace enzymes which are missing or defective as a consequence of inherited genetic disease; (2) to replace enzymes that are deficient or present only in inadequate quantity as a consequence of acquired disease in the organ(s) where they are normally synthesized; and (3) to provide a specific biological effect which is dependent on the catalytic activity of the enzyme [1314]. Furthermore, some enzymes are used in a supportive manner in combination with other therapies, or for the removal of toxic or otherwise undesirable substances from the bloodstream or a body tissue.

To be effective as therapeutic agents, the enzymes must meet a number of requirements:

- They must reach their site of action in the body and tissue compartment.
- They must be active under the conditions present at the intended site of action. This includes substrate and cosubstrate/coenzyme availability, appropriate redox potential, adequate pH value for activity, and absence (or saturation) of inhibitors.
- They must be sufficiently stable to ensure adequate pharmacokinetics, i.e., the required activity level for the necessary time period.
- They must be sufficiently soluble to allow application as a solution if administration is via the intravenous, intramuscular, or subcutaneous route.
- They must be sufficiently pure to avoid eliciting unwanted side reactions caused by contaminants, e.g., microbial endotoxins, pyrogens, or other harmful materials.
- There must be a therapeutic effect that depends on the particular activity of the enzyme applied.

Of course, enzymes as active pharmaceutical ingredients of drugs must fulfill a number of further requirements which are equally relevant for low molecular weight drug substances:

- Safety depending on the indication/disease. This means that the therapeutic benefit must surpass any adverse reactions, especially immunological complications.
- Efficacy, i.e., the therapeutic effect must be established in controlled clinical trials according to FDA/EMEA regulations.

– Convenience of the application form, again depending on the indication or disease: in the treatment of life-threatening diseases, convenience is clearly of less importance than for, e.g., digestion aids.

6.2.2
Coping with Peculiar Protein Properties

Enzymes have unsurpassed substrate and reaction specificity, as well as exceptional efficacy as biological catalysts. Furthermore, they have been optimized by evolution for action in living organisms. Especially in the case of genetically encoded enzyme defects, enzyme therapy will make rational therapy possible.

However, due to their specific molecular properties, enzymes (or, in general, proteins) would by no means be considered ''ideal'' therapeutic agents for several reasons:

– Instability: enzymes are polypeptides and therefore inherently labile with respect to heat, extreme pH values, denaturants, and biological degradation. This may lead to limited shelf life and short half-lives in the human body, e.g., due to proteolysis in the stomach and intestine or receptor-mediated clearance from the blood followed by proteolytic degradation in the liver.

– Presence of endogenous inhibitors, which are especially common in blood [1315] and may destroy the catalytic activity of the enzyme.

– Bioavailability: since the molecular surface of soluble enzymes is hydrophilic, proteins generally cannot pass through biological membranes. Thus enzyme drugs are not orally available, and, unless the enzyme is intended to act in the oral cavity or in the gastrointestinal compartment, an intravenous, intramuscular, or subcutaneous route of administration must be chosen. In some cases administration by inhalation using a nebulizer has proved possible for enzymes acting in the respiratory tract.

– Cell permeability: Since enzymes will not usually enter tissue through the intestinal wall or into human cells from the bloodstream, protein drugs are unsuitable for intracellular action. Therefore, at present many applications of enzymes as drugs are extracellular, such as purely topical uses, the removal of toxic substances, and the treatment of life-threatening disorders within the bloodstream.

– Immunogenicity: nonhuman proteins are foreign to the human body and therefore are expected to be immunogenic. When injected into the bloodstream, they may induce the formation of antibodies and cellular immune response [1316]. This might change pharmacokinetics and prevent repeated or prolonged application of the same protein drug (in contrast, immunogenicity is even desired when proteins are used as vaccines [1317]). However, even with

recombinant human enzymes, immunogenicity may be an issue, especially if muteins are applied or if the production process does not ensure the absence of misfolded or aggregated enzyme species.

- Allergic reactions may occur on inhalation of enzyme powders, especially with proteases.
- Manufacturing and characterization: Enzymes are complex macromolecules that require difficult multistep production processes, leading to high cost of the drug. Furthermore, in contrast to small molecules, only limited analytical characterization of the final enzyme product is possible, even with the highly sophisticated protein-analytical techniques available nowadays. This makes quality control at the end-product level difficult, so that process changes may require bioequivalence testing.

On the other hand, there are some important advantages in the use of enzymes in therapy:

- Generally, fewer adverse reactions (including teratogenic effects) for protein drugs than for chemically synthesized low molecular weight compounds, for which the safety profile of new chemical entities (NCEs) cannot be predicted.
- Shorter development times: since the advent of recombinant protein drugs in the 1980s, it has initially been found that the total time needed for research and development of a new drug from project initiation until marketing approval generally was significantly shorter, and the attrition rate (i.e., the fraction of development projects which must be terminated during preclinical or clinical development) is lower than for traditional low molecular weight compounds. However, the mean development times for biopharmaceuticals rose dramatically during the 1990s, and seem to be converging for biopharmaceuticals and NCEs since 1996 [1318], [1319].

Therefore, a number of challenges remain for the future development of enzymes for therapeutic use:

- Improve stability: Approaches to prolong the half-life in the circulation include coupling of enzymes to polyethylene glycol (PEGylation) [1320], [1321]; chemical cross-linking; the development of sustained-release forms and depot systems, e.g., by microencapsulation of the enzyme in polymers such as poly(lactide)–poly(glycolide) copolymers [1322], or by entrapment within artificial liposomes or red blood cell ghosts; as well as the removal of receptor binding sites by designing appropriate enzyme muteins.
- Diminish immunogenicity, e.g., by chemical (PEGylation) or genetic modification to cover or remove epitopes on the protein surface recognized by the immune system.
- Achieve specific targeting to tissues/cells [1323]. This may be possible by modification with sugar residues which are recognized by hepatocyte receptors,

or by genetic fusion to ''targeting devices'' such as recognition peptides or antibody fragments [1324]. Another use of enzymes in tumor therapy is antibody-directed enzyme prodrug therapy [1325], [1326]: an antibody with an attached enzyme that is not normally present in extracellular fluids or on cell membranes, is administered to achieve specific binding to the surface of cancer cells. This is followed by administration of a prodrug, which is a substrate of the enzyme and on enzymatic cleavage releases the physiologically active cytotoxic entity in a site-specific manner. This is expected to reduce the exposure of normal tissue to the active drug while maximizing concentrations in the tumor tissue.

– Improve tissue penetration by decreasing molecule size (muteins) or by fusion to sequences mediating membrane permeation, such as penetrating (a peptide derived from the third helix of the DNA-binding domain of the Antennapedia homeodomain protein) [1327], TAT protein from human immunodeficiency virus-1 [1328], VP22 protein from Herpes simplex virus [1329], or Pep-1, a designed 21-residue peptide carrier [1330]. However, these approaches are at present (2006) in the experimental stage and appear to be still far from being useful in clinical practice.

6.2.3 Sources of Enzymes and Production Systems

For many decades, enzymes obtained from natural nonhuman sources such as bacteria, fungi, animals, and snake venoms have been used for therapeutic purposes. Due to their immunogenicity, these enzymes usually are suitable only for external applications (e.g., digestive or topical). In these cases, purity, source, and dosage of these enzyme preparations are not considered critical.

The purification of enzymes from human sources in a form and quantity which would allow their administration to patients has been a major challenge [1331]. Nevertheless, a number of human enzymes obtained from blood, urine, placenta, or human cell culture have been introduced into clinical practice, such as blood fractions and coagulation factors, urokinase, alglucerase, and others. Besides the limited supply of starting material, a major problem with enzymes obtained from human sources is the reliable elimination of viral (or maybe viroid) contamination. Therefore, since the 1980s, human enzymes are preferentially obtained by recombinant DNA technology.

Whereas initially basic replacement protein products, i.e., recombinant proteins identical with the native human enzymes were developed, there is now a trend towards engineered ''second-generation'' protein products whose amino acid sequence has been deliberately altered to improve properties relevant for therapeutic efficacy or safety, or to improve the manufacturing process [1332].

An important aspect of the production of recombinant enzymes for therapy is the choice of the expression system. Most or perhaps all human enzymes in their native form are subject to posttranslational modifications such as glycosylation, phosphorylation, acetylation, myristoylation, or proteolytic processing. If the host cell used for

recombinant production is not identical to the cell in which the protein is produced naturally, posttranslational modifications may deviate from the authentic pattern. This may influence the pharmacokinetic, pharmacodynamic, and immunological properties of the enzyme. Whether this is of importance for use in therapy must be studied experimentally in each case.

At present, various expression systems are used to obtain therapeutic proteins [1333]:

- Production from human cell lines clearly will provide the best fit to the natural modification pattern, but may give rise to problems of viral contamination. However, with the advent of highly developed, immortalized human production cell lines such as the human retina-derived Per.C6 cell [1322], the use of human cells as hosts for recombinant production will increase.
- Production from mammalian cells [mostly Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cell lines] is often used, especially for glycosylated proteins in cases where glycosylation affects therapeutic efficacy [1334], [1335]. Glycosylation provided by mammalian host cells may be somewhat different in detail but is generally similar to the human glycosylation pattern.
- Production from yeast (*S. cerevisiae*) results in posttranslational modifications different from the human pattern; hence, it has been used until now primarily for fungal (nonhuman) proteins [1336]. Recently, the use of glycoengineered *Pichia pastoris* as the host cell has been described [1337], which allows human N-glycan structures to be obtained.
- Production from *E. coli* results in nonglycosylated products. In principle, bacterial production systems have significant advantages concerning lower development cost, improved process economics, and absence of viral contamination [1338], [1339]. However, since eukaryotic proteins are often not folded correctly in bacteria, refolding of the target protein from ''inclusion bodies'' may be necessary. This can be done on an industrial scale [1340] but may require extensive process optimization to obtain good product yield.
- Production from transgenic plants [1341], [1342] or the milk of transgenic animals [1343]: these production systems are under study and may provide interesting options, especially for proteins needed in very large amounts or for very complex proteins such as coagulation factor VIII. No therapeutic enzyme product produced in transgenic animals or plants has yet been approved for marketing [1344].

For several years it has been discussed whether gene therapy will be a future third generation of protein therapy following the first (therapy with naturally occurring proteins) and second (engineered protein muteins) generations. In this case, the proteins are no longer produced in a fermenter but by the patients themselves after application of the protein-coding gene by means of a suitable gene-transfer system. This approach appears especially attractive for long-term therapies, such as enzyme replacement therapy of hereditary defects (e.g. ADA deficiency or lysosomal storage diseases). Many preclinical and clinical trials have been performed in this field, but given the

progress of gene therapy to date and considering unresolved safety issues, this alternative apparently does not pose an immediate threat to the enzyme drug market.

6.2.4 Overview of Therapeutic Enzymes

Many of the requirements listed in Section 6.2.1 are not met by older enzyme products, i.e., enzymes from nonhuman sources approved prior to ca. 1980 which are still in use as generics in 2006 (some of them also in nutrition additives, oral hygiene products, or cosmetics; these applications are not treated here). On the other hand, in the field of modern innovative drugs, only a relatively small number of recombinant enzymes have been developed until now as the main active principles of therapeutics, in contrast to the increasing number of recombinant cytokines, hormones, and antibodies which have already been approved or are in clinical development. The following overview includes older enzyme preparations still in use, even if they would not meet the requirements of modern drugs, as well as newer enzyme drugs developed and approved according to present-day standards.

In the development of therapeutic enzymes, as with other biopharmaceuticals, special attention must be given to regulatory requirements, as well as to aspects of quality assurance, assay methods, and standardization issues. These topics are not treated here in detail.

Vaccines such as diphtheria toxin (an ADP ribosyltransferase) or tetanus toxin (an endopeptidase) are not discussed here. These are proteins whose toxic properties indeed rely on their enzymatic activity. However, they are used as immunogens for vaccination after chemical treatment with formaldehyde to convert them into enzymatically inactive toxoids. Thus, their medical use does not rely on their enzymatic activity but rather on their immunogenic properties.

The overview of enzymes (Table 39) used in therapy is ordered according to the E.C. numbering scheme of enzyme classes. The table includes enzymes presently marketed, as well as several enzymes which are at present in advanced clinical studies but have not yet been approved. For more information, the reader is referred to comprehensive books and reviews [1345–1348], [1349], [1350].

6.2.4.1 Oxidoreductases

Urate oxidase (E.C. 1.7.3.3), an enzyme which catalyzes the oxidation of uric acid to the more soluble allantoin, is present in many organisms (including mammals) but not in higher primates. A nonrecombinant urate oxidase product isolated from Aspergillus flavus has been used in some countries for many years (France, since 1975; Italy, since 1984) for prophylaxis or treatment of hyperuricemia in patients with hematological malignomas which are prone to the risk of tumor lysis syndrome (TLS). This is an oncologic emergency most commonly occurring spontaneously or after chemotherapy in patients with large tumor burdens such as acute leukemia, aggressive lymphoma, or bulky solid tumors. TLS is characterized by hyperuricemia leading to the precipitation of uric acid and to intraluminal tubular obstruction, hyperphosphatemia, and hypocalcemia. The consequences of TLS may be life-threatening and include acute renal failure and multiorgan failure.

Table 39. Enzymes used in therapy

E.C. number	Enzyme	Source	Trade name(s) (examples)	Therapeutic use (examples)
1.7.3.3	urate oxidase, recombinant	recombinant *Saccharomyces cerevisiae*	Elitek, Fasturtec	hyperuricemia in TLS patients
1.7.3.3	urate oxidase	*Aspergillus flavus*	Uricozyme	gout; hyperuricemia in TLS patients
1.15.1.1	superoxide dismutase	bovine liver or erythrocytes	Peroxinorm, Ontosein, Oxinorm	inflammatory arthrosis, polyarthritis, asthma, oxygen toxicity in premature infants
1.15.1.1	superoxide dismutase, recombinant	recombinant *E. coli* or yeast	OxSODrol	prevention of bronchopulmonary dysplasia
(1.15.1.1)	PEG-superoxide dismutase	bovine liver	Dismutec	closed head injury
(2.3.2.13)*	coagulation factor XIII	human plasma	fibrogammin-P	bleeding, factor XIII deficiency; fibrin glues
3.1.1.3	lipase	*Rhizopus arrhizus*		digestion aid
3.1.1.47	platelet-activating factor acetylhydrolase, recombinant	recombinant *E. coli*	Pafase	severe sepsis, ARDS, pancreatitis
3.1.4.12	sphingomyelin phosphodiesterase 1 (acid sphingomyelinase), recombinant	recombinant CHO cells		type B Niemann–Pick disease
3.1.6.12	*N*-acetylgalactosamine-4-sulfatase (arylsulfatase B), recombinant	recombinant CHO cells	Aryplase, Naglazyme	Maroteaux–Lang syndrome (mucopolysaccharidosis type VI)
3.1.6.13	iduronate-2-sulfatase, recombinant	recombinant CHO cells		Hunter syndrome (mucopolysaccharidosis type II)
3.1.21.1	desoxyribonuclease, recombinant	recombinant CHO cells	Pulmozyme	chronic obstructive pulmonary disease

3.1.21.1	desoxyribonuclease (streptodornase)	*Streptococcus haemolyticus*	Varidase	gastric ulcers (together with streptokinase)
3.1.21.-	T4 endonuclease V	bacteriophage T4-infected *E. coli*	Dimericine T	xeroderma pigmentosum
3.1.26.4	ribonuclease	*Rana pipiens*	Onconase	malignant mesothelioma, refractory breast cancer, renal cell cancer, antiviral
3.2.1.1	α-amylase	porcine pancreas		digestion aid
3.2.1.2	β-amylase	*Aspergillus oryzae*		digestion aid
3.2.1.3	acid α-1,4-glucosidase, recombinant	recombinant CHO cells		Pompe's disease
3.2.1.4	cellulase	*Trichoderma viride*		digestion aid
3.2.1.17	lysozyme	hen's egg white		infectious diseases (antibiotic)
3.2.1.22	α-galactosidase, recombinant	recombinant human cell line or CHO cells	Replagal, Fabrazyme	Fabry's disease
3.2.1.23	β-galactosidase (Lactase)	*Kluyveromyces fragilis*, *Aspergillus oryzae*, or *Aspergillus niger*	Lactaid, Lactrase, SureLac	lactose intolerance
3.2.1.35	hyaluronidase	bovine testes or recombinant CHO cells	Hylase, Vitrase , Hylenex	adsorption adjunct; heart attack
3.2.1.45	glucosylceramidase (β-glucocerebrosidase)	human placenta	Ceredase	Gaucher's disease
3.2.1.45	glucosylceramidase (β-glucocerebrosidase), recombinant	recombinant CHO cells	Cerezyme	Gaucher's disease
(3.2.1.45)	PEG-β-glucocerebrosidase	human placenta	Lysodase	Gaucher's disease
3.2.1.76	α-L-iduronidase, recombinant	recombinant CHO cells	Aldurazyme	mucopolysaccharidosis I
–	pankreatin (mixture of pancreatic enzymes, e.g. trypsin, chymotrypsin, lipase, α-Amylase)	porcine pancreas	Cotazym, Kreon, Mezym F, Nutrizym, Pancrex, Pangrol, Pankreatan, Pankreon, Panzytrat	digestion aid
3.4.21.1	chymotrypsin	bovine pancreas		wound healing, digestive aid

(continued)

Table 39. (continued)

E.C. number	Enzyme	Source	Trade name(s) (examples)	Therapeutic use (examples)
3.4.21.4	trypsin	bovine pancreas	Leukase, Granulex	debridement of wounds, digestion aid
3.4.21.5	thrombin	human plasma	Thrombinar, Velyn	superficial bleeding; fibrin glues
3.4.21.7	plasmin	bovine or human plasma	Fibrolan, Lysofibrin, Actase, Elase	debridement of wounds
(3.4.21.21)*	coagulation factor VII	human plasma	Factor VII S-TIM 4	bleeding, factor VII deficiency
3.4.21.21	coagulation factor VIIa, recombinant	recombinant BHK cells	NovoSeven	hemophilia A and B
–	coagulation factor VIII	porcine plasma	Hyate C	hemophilia A
–	coagulation factor VIII	human plasma	Autoplex, Factorate, Profilate HS, Monoclate-P, Hemate P, Hemofil M, Monarc-M, Beriate P	hemophilia A
–	coagulation factor VIII (containing von Willebrand factor)	human plasma	Alphanate, Humate-P, Koate DVI, Immunate STM	von Willebrand disease
–	coagulation factor VIII, recombinant	recombinant CHO cells	Recombinate, Bioclate	hemophilia A
–	coagulation factor VIII, recombinant	recombinant BHK cells	KoGENate, Helixate	hemophilia A
–	coagulation factor VIII, recombinant (B-domain deleted)	recombinant CHO cells	ReFacto	hemophilia A
(3.4.21.22)*	coagulation factor IX	human plasma	Mononine, AlphaNine, Berinin HS, Immunine, Prothromplex-T	hemophilia B
(3.4.21.22)*	coagulation factor IX, recombinant	recombinant CHO cells	BeneFIX	hemophilia B
3.4.21.34	kallikrein	porcine pancreas	Padutin, Bioactin, Prokrein, Onokrein P	peripheral vascular and coronary disease, fertility disturbances

3.4.21.63	oryzin (*Aspergillus* alkaline protease)	*Aspergillus sp.*		inflammation
3.4.21.68	tissue plasminogen activator, recombinant	recombinant CHO cells	Activase, Actilyse	acute myocardial infarction; acute pulmonary embolism; ischemic stroke
(3.4.21.68)	tissue plasminogen activator mutein, recombinant	recombinant eukaryotic cells	Cleactor	acute myocardial infarction
(3.4.21.68)	tissue plasminogen activator mutein, recombinant	recombinant *E. coli*	Rapilysin, Retavase, Ecokinase	acute myocardial infarction
(3.4.21.68)	tissue plasminogen activator mutein, recombinant	recombinant CHO cells	Metalyse	acute myocardial infarction
3.4.21.69	activated protein C, recombinant	recombinant CHO cells	Xigris, Zovant	severe sepsis
3.4.21.73	urokinase	human urine or human kidney cell culture	Abbokinase, Actosolv, Alphakinase, Rheothromb, Ukidan	acute myocardial infarction
3.4.21.73	urokinase, recombinant	recombinant *E. coli*	Saruplase	acute myocardial infarction
–	streptokinase	C_β hemolytic *Streptococci*	Kabikinase, Streptase, Kinalysin, Varidase	acute myocardial infarction; gastric ulcers (together with ribonuclease)
–	APSAC (*p*-anisoylated derivative of plasminogen-streptokinase complex)	human plasma (plasminogen)/C_β hemolytic *streptococci* (streptokinase)	Eminase	acute myocardial infarction
3.4.21.74	ancrod	*Agkistrodon rhodostoma* venom	Arwin, Venacil	improvement of blood rheology; lymphoblastic leukemia, lymphosarcoma
3.4.21.74	batroxobin	*Bothrops atrox* venom	Defibrase, Botropase, Reptilase, Vivostat	improvement of blood rheology; chirurgic tissue glues and hemostyptics
3.4.21.-	sfericase	*Bacillus sphaericus*		chronic bronchitis, acute pneumonia, chronic sinusitis
3.4.22.2	papain	*Carica papaya*	Panafil	digestion aid, debridement of wounds

(continued)

Table 39. (continued)

E.C. number	Enzyme	Source	Trade name(s) (examples)	Therapeutic use (examples)
3.4.22.6	chymopapain	papaya latex	Discase, Chymodiactin	chemonucleolysis of prolapsed invertebral disk
3.4.22.32	bromelain	*Ananas comosus* var.	Traumanase, Phlogenzym	digestion aid; inflammation
3.4.23.1	pepsin	porcine stomach	Enzymorn, Sentinel	support of gastic function; gastric ulcers
3.4.24.3	collagenase	*Clostridium histolyticum*	Iruxol, Biozyme C, Santyl	debridement of wounds; skin ulcers
3.4.24.28	protease	*Bacillus subtilis*	Travase	debridement of wounds
	extracts containing proteases, cellulases, RNases, α- and β-amylases	*Aspergillus oryzae*	NORTASE, COMBIASE	digestion aid
3.4.24.40	serrapeptase (serralysin)	*Serratia* E15	Danzen	inflammations
3.4.24.72	fibrolase, truncated form, recombinant	recombinant eukaryotic cells	Alfimeprase	peripheral arterial obstructive disease
3.5.1.1	asparaginase	*E. coli* or *Erwinia carotovora*	Crasnitin, Elspar, Kidrolase, Leunase, Erwinase	acute lymphocytic leukemia
(3.5.1.1)	PEG-asparaginase	*E. coli*	Oncaspar	acute lymphocytic leukemia
(3.5.4.4)	PEG-adenosine deaminase	bovine intestine	ADAGEN	SCID
4.2.2.4	chondroitinase ABC	*Proteus vulgaris*		treatment of lumbar disk herniation
4.3.1.8	uroporphyrinogen-1-synthase (porphobilinogen deaminase), recombinant	recombinant *E. coli*		acute intermittent porphyria

*E.C. number of activated form.

Several factors, including the complex manufacturing process, have limited the availability of A. flavus urate oxidase. Therefore, the cDNA coding for the enzyme was cloned and expressed in Saccharomyces cerevisiae. The purified recombinant enzyme (rasburicase) was approved for marketing in Europe in 2001, and in the USA in 2002 [1351]. Use of rasburicase in cancer treatment is under study [1352].

Detailed protein-chemical studies demonstrated a higher purity of rasburicase compared to the natural A. flavus enzyme; in particular, the fungal oxidase contained a cysteine adduct to Cys-103, likely attributable to the purification process, which was absent in the recombinant enzyme product [1353], [1354]. Because urate oxidase is a nonhuman protein, the drug is antigenic and potentially may induce hypersensitivity reactions. While nonrecombinant urate oxidase has a rate of acute hypersensitivity reactions of up to 5 %, the incidence appears to be much lower with the recombinant form.

A PEGylated version of recombinant porcine uricase is in phase II clinical development for the elimination of excess uric acid in patients with symptomatic gout for whom conventional therapy is contraindicated or has been ineffective. In 2001, PEG-rasburicase was granted orphan drug designation by the FDA for this indication [1355].

Superoxide ion (O_2^-) and hydrogen peroxide are crucial starting points in the formation of other reactive oxygen species (ROS) which may cause oxidative damage to cells and tissue destruction. This is specially evident in endothelial cells, damage of which appears to be involved in pathological situations such as myocardial infarction, lung injury and inflammation, ischemia-reperfusion damage, hypoxia, and hyperoxia. Superoxide dismutase (orgotein, E.C. 1.15.1.1), a copper–zinc protein identified in bovine liver in the 1960s, removes the superoxide radical by catalyzing its dismutation to molecular oxygen and hydrogen peroxide, and thus acts as part of the cellular ROS detoxification pathway. Bovine superoxide dismutase has been used since the early 1980s as an anti-inflammatory drug, proved to be therapeutically effective in the treatment of osteoarthritis, chronic polyarthritis, radiation cystitis, and interstitial cystitis, and still is used as a veterinary product (palosein). However, superoxide dismutase is susceptible to the known limits of native protein administration such as unsatisfactory pharmacokinetics and immunogenicity, although the incidence of allergic and anaphylactic reactions seems to be low. Recombinant human superoxide dismutase (sudismase) preparations obtained from recombinant *E. coli* or yeast are presently in clinical development for treatment of amylotrophic lateral sclerosis, bronchopulmonary dysplasia in premature neonates, asthma, and respiratory distress syndrome.

To improve the blood residence time and simultaneously reduce immunogenicity, bovine superoxide dismutase has been coupled to polyethylene glycol. This conjugate (pegorgotein) indeed was found to have greatly increased clearance time that depends on the molecular weight of the PEG used for modification [1356]. The therapeutic efficacy of pegorgotein has been studied in several indications, and the product is presently in phase III clinical development for treatment of stroke and closed head injury.

6.2.4.2 **Transferases**

The only enzyme of this class which is used in therapy is coagulation factor XIII (fibrin-stabilizing factor) obtained from human plasma. Factor XIII is a glycoprotein which circulates in the blood as a proenzyme. During the final stages of blood coagulation,

thrombin converts it to an active form called factor XIIIa (E.C. 2.3.2.13). This is a transglutaminase which, in the presence of Ca^{2+}, catalyzes the cross-linking of fibrin monomers through the formation of intermolecular ε-(γ-glutamyl)lysine bonds.

Factor XIII deficiency is a rare inherited disease characterized by poor wound healing and abnormal scar formation, with bleeding from the umbilical stump after birth as the most common clinical symptom. Deficiency of factor XIII can be corrected with infusions of fresh frozen plasma or human factor XIII concentrates. Although the cloning and recombinant expression of factor XIII has been reported in several publications since the 1980s, no recombinant factor XIIIa preparation has yet been approved for therapeutic use. Recombinant human factor XIII is presently in phase I clinical trials and has obtained orphan drug designation for the treatment of factor XIII deficiency in 2003 the EU and in 2004 in the USA [1357].

6.2.4.3 **Esterases**

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphatidyl choline) is a potent phospholipid mediator with pleiotropic effects, produced by a wide variety of cells. Serum levels of PAF are elevated in human sepsis, and when PAF is injected into animals, it produces physiological dysfunction similar to that seen in sepsis. These effects are mediated by various cytokines and other molecules [1358]. Although a statistically significant reduction in mortality associated with the use of the recombinant PAF acetyl hydrolase (E.C. 3.1.1.47, pafase) was reported as the result of two phase II trials in the sepsis indication, these results were not validated in a large phase III multicenter trial [1359], and further development was discontinued.

Lipase (E.C. 3.1.1.3) from porcine pancreas (see Section 5.1.1.6) or from *Rhizopus arrhizus* (rizolipase) is used as a digestion aid.

6.2.4.4 **Nucleases**

In lung diseases characterized by persistent airway infection such as cystic fibrosis (CF, mucoviscidosis) and chronic bronchitis, respiratory distress and progressive lung destruction are strongly associated with bacterial persistence and the accumulation of viscous purulent secretions in the airways. Infected-airway secretions contain mucus glycoproteins and abundant amounts of DNA which, as an extremely viscous polymer polyanion, is a major cause of the increased viscosity.

Recombinant human desoxyribonuclease I (dornase alfa, E.C. 3.1.21.1) reduces the viscosity of the sputum by hydrolyzing DNA. Dornase alfa produced from recombinant CHO cells is virtually identical to DNase I, an extracellular glycoprotein produced in human pancreas and salivary glands.

In trials on clinically stable CF patients, dornase alfa, administered through an inhalation device, was found to reduce CF-related morbidity, including the risk of respiratory tract infectious exacerbations requiring treatment with parenteral antibiotics, and to improve pulmonary function and quality of life. It was approved for treatment of CF in 1993.

Streptodornase, a desoxyribonuclease obtained from *Streptococcus* bacteria, is used together with streptokinase for the debridement of wounds, as well as to treat ulcers in order to liquefy blood clots and dead tissue so that these can be easily removed.

DNA damage plays an important role in the development of skin cancer, which is the most common cancer in white people in the USA and other countries. Patients with the rare genetic disease xeroderma pigmentosum have an autosomal recessive genetic defect in the pathway that repairs sun-induced damage of DNA. As a result, the rates of malignant disorders of the skin are 1000 times higher than in the general population. The DNA repair enzyme, T4 Endonuclease V (E.C. 3.1.21.-) is currently in phase III clinical development for the prevention of actinic keratosis in xeroderma pigmentosum patients and in phase II clinical trials for the prevention of skin cancer in renal transplant patients who have elevated rates of skin cancer due to the use of immunosuppressive drugs which inhibit DNA repair of UV damage, as well as in renal transplant recipients with sun-damaged skin and a history of nonmelanoma skin cancer. The compound is used as a liposomal lotion (T4N5 liposome lotion). It is a viral DNA glycosylase which specifically excises pyrimidine dimers in DNA. The enzyme binds to UV-irradiated DNA, progressively scans the DNA until a pyrimidine dimer is encountered, and then cleaves the glycosyl bond of the 5′-pyrimidine of the dimer as well as the 3′-phospho diester bond, resulting in breakage of the DNA strand. In a randomized study, it was shown that topical application of the endonuclease lowered the rate of development of new skin lesions (actinic keratoses and basal-cell carcinomas) during a year of treatment [1361].

Following studies which demonstrated inhibition of tumor growth after injection of bovine pancreatic ribonuclease (RNase) into tumor-bearing mice, the potential of ribonucleases (E.C. 3.1.26.4) to inhibit tumor growth has been investigated since 1955. However, at present only an amphibian RNase isolated from the leopard frog Rana pipiens (ranpirnase) is in phase III clinical trials in the indications malignant mesothelioma, refractory breast cancer, renal cell cancer, and prostate cancer [1362]. Ranpirnase, which is not inhibited by endogenous mammalian RNase inhibitors, is also being studied preclinically for use in antiviral therapy. Furthermore, preclinical studies are ongoing for RN321, an anti-CD22/RNase immunoconjugate that consists of a B-cell-specific monoclonal antibody conjugated with ranpirnase which is intended for treatment of B-cell lymphomas.

6.2.4.5 **Glycosidases**

α-Amylase (E.C. 3.2.1.1) from porcine pancreas is part of pancreatin preparations (cf. Section 5.1.1.6) and is used to support the digestion of dietary carbohydrate. This is also the case for extracts from Aspergillus oryzae containing cellulases (E.C. 3.2.1.4), proteases, and α- and β-amylase (E.C. 3.2.1.1/3.2.1.2). In fact, an enzyme preparation from *Aspergillus oryzae* cultures (Takadiastase) developed by Jokichi Takamine and used as a digestive aid in the 1890s, probably represented the first use of a microbial enzyme preparation for therapeutic purposes.

Lysozyme (E.C. 3.2.1.17) was discovered in 1922 by Alexander Fleming as a bacteriolytic substance present in many tissues and secretions. Its antibacterial effect is based on hydrolysis of the bacterial cell walls by cleaving the β-(1,4) glycosidic bonds between *N*-acetylmuraminic acid and *N*-acetylglucosamine residues. Lysozyme also has chitinolytic activity, which contributes to the cell-wall lysis of numerous gram-positive bacteria. The explanation for a reported antiviral action of lysozyme is elusive.

Lysozyme, obtained from hen egg white, is still used for treatment of infectious and inflammatory diseases, for the treatment of viral diseases, for eye therapy, and within the oral cavity and throat in anti-angina preparations. Chitinases (E.C. 3.2.1.14) are also under study for treatment of fungal infections.

Hyaluronidases (E.C. 3.2.1.35) randomly hydrolyze the β-*N*-acetyl hexosaminic bonds in hyaluronate, chondroitin 4- and 6-sulfate and their desulfated derivatives to yield even-numbered oligosaccharides with glucuronic acid residues at their nonreducing end. By hydrolysis of the connective tissue glycosaminoglycans, they facilitate the absorption of injected fluids and resorption of exudates. Therefore, hyaluronidase obtained from bovine testes (a mixture of several enzymes) is used to promote absorption and dispersion in the subcutaneous or intramuscular injection of relatively large volumes of fluids. Ophthalmic injectable formulations have also been developed for use in the treatment of vitrous hemorrhage in diseases such as diabetic retinopathy. A recombinant human PH20 hyaluronidase was approved in 2005 for use in removing the cumulus matrix surrounding oocytes in preparation for assisted reproductive technology (ART) procedures as well as for use as a spreading agent to facilitate the dispersion and absorption of other drugs. It is also under study for treatment of ischemic stroke.

Diseases which are caused by genetically encoded single-enzyme defects generally are candidates for rational therapy by enzyme replacement. Until now, this has been realized in severe combined immunodeficiency (see Section 6.2.4.7) and in glycolipid storage diseases such as Gaucher's disease and Fabry's disease. For some other indications, clinical development of enzyme replacement therapies is in progress [1363], e.g., recombinant acid sphingomyelinase (E.C. 3.1.4.12) for type B Niemann–Pick disease, recombinant lysosomal acid maltase (α-glucosidase, E.C. 3.2.1.3) for Pompe's disease, and recombinant human iduronate-2-sulfatase (E.C. 3.1.6.13) for mucopolysaccharidosis type II (Hunter syndrome).

Development of these recombinant enzyme-based drugs has been supported greatly by the U.S. and European ''orphan drug'' regulations allowing drugs directed to life-threatening diseases which affect less than 200 000 persons in the USA, or occur with a frequency of less than 5 in 10 000 persons in Europe, to obtain tax incentives and grant funding, as well as seven (USA) or ten (Europe) years market exclusivity. This gives the originator companies a good chance to pay off the development costs of these drugs in spite of the small market size.

Gaucher's disease is the most prevalent lysosomal storage disorder, characterized by the accumulation of glycolipid (glucosylceramide) primarily in peripheral tissues. It is inherited in an autosomal recessive manner, and the incidence is 1/600–1/2500 in ashkenazic jews and 1/50 000 in Caucasians. This disease is caused by deficiency in the enzyme β-glucocerebrosidase (glucosylceramidase, E.C. 3.2.1.45) which catalyzes the removal of glucose from glucosylceramide to form ceramide. In 1991, alglucerase (glucocerebrosidase derived from human placenta) was launched as the first enzyme replacement therapy for Type 1 Gaucher's disease. Meanwhile, the human enzyme has been replaced by recombinant glucocerebrosidase (imiglucerase; U.S. approval 1994, European approval 1997) produced in CHO cells. This was critical for overcoming the limitations of availability: 20 000 placentas were required to provide a one-year supply for a single patient. Imiglucerase differs from the native human enzyme by one amino

acid, Arg-495 being substituted by His, and has a modified oligosaccharide component. A PEGylated form of glucocerebrosidase is also in clinical studies. Among all therapeutically used enzymes, glucocerebrosidase was the best-selling with global sales of $\$ 0.88 \times 10$ in 2004.

Fabry's disease is an X-chromosomal, recessively inherited lysosomal glycosphingolipid storage disease which leads to the abnormal deposition of neutral glycosphingolipids (mainly globotriaosylceramide) in many tissues and cell types, such as kidney, heart, skin, and the cells lining the blood vessels. In the USA, it occurs with an incidence of 1 in 40 000, mostly in caucasians, but is also found in African Americans, Hispanics, and Asians. The disease is caused by a genetically encoded defect of the lysosomal α-galactosidase A (E.C. 3.2.1.22). Two recombinant human α-galactosidases (agalsidase alfa produced in a human cell line, and agalsidase beta obtained from recombinant CHO cells) have been shown to mobilize and clear the sphingolipid deposits and have been approved for long-term enzyme-replacement therapy of Fabry's disease [1365], [1366]. The phase III clinical trial preceding the approval of agalsidase beta enrolled 58 patients at eight centers and was the largest trial ever conducted for Fabry's disease.

Mucopolysaccharidosis type I (Hurler's syndrome, MPS1) is an inherited and progressive disorder that results from the body's inability to make lysosomal α-L-iduronate, an enzyme that helps breakdown mucopolysaccharides. Hurler's syndrome is inherited as an autosomal recessive trait. Approximately 1 in 115 000 individuals are affected. The symptoms of intermediate MPS1 usually develop between three and eight years of age. Infants with severe MPS1 appear normal at birth. Symptoms are progressive and include growth retardation, progressive mental retardation, deafness, joint disease including stiffness, heart valve problems, abnormal bones of the spine, and claw hand. In 2004, recombinant human α-L iduronidase (laridonase, E.C. 3.2.1.76) [1364], [1367], [1368] was approved in Europe and the USA for treatment of MPS1.

Mucopolysaccharidosis type VI (Maroteaux–Lamy syndrome, MPS VI) is an autosomal recessive lysosomal storage disease, caused by a deficiency of arylsulfatase B (*N*-acetylgalactosamine 4-sulfatase, E.C. 3.1.6.12). Patients suffer from growth retardation as well as from bone and joint changes, hepatosplenomegaly, enlargement of heart valves, and cardiomyopathy. A recombinant arylsulfatase B (galsulfase) [1369] was approved in 2005 for treatment of this inborn error of metabolism. Clinical trials have demonstrated that Naglazyme provides clinically important benefits for MPS VI patients, specifically improved endurance. Galsulfase reduces the excess carbohydrates (glycosaminoglycans) that are excreted in the urine of patients with MPS VI, an indication of enzymatic bioactivity.

Lactose is present in concentrations of about 4.7 vol % in milk and the whey left after the coagulation stage of cheesemaking. Its presence makes milk unsuitable for the majority of the world's adult population: the young of all mammals are able to digest milk but in most cases, this ability declines after weaning. Of the Thai, Chinese, and African American populations, 97, 90, and 73 %, respectively, are reported to be lactose intolerant, whereas 84 and 96 % of the U.S. white and Swedish populations, respectively, are tolerant. For people who have an impaired ability to digest lactose, lactase (β-galatosidase, tilactase, E.C. 3.2.1.23) is available as a nonprescription enzyme as a dietary supplement to alleviate the symptoms of lactose intolerance.

6.2.4.6 Proteases

6.2.4.6.1 Pancreatic and Gastric Proteases

Gastric and pancreatic insufficiency is frequently treated by substitution with crude enzyme preparations applied orally. These unspecific hydrolases are able to degrade plasma proteins and cell membrane constituents in vitro. Nevertheless, orally administered proteases appear to be nontoxic, which indicates that there is no significant absorption of the active enzymes from the gastrointestinal tract.

Pepsin (E.C. 3.4.23.1) from porcine stomach is used for support of gastric function to increase the digestive power of gastric juice. In combination with bismuth complexes it is also used for treatment of gastric ulcers.

Treatment of disturbances of the exokrine pancreas function leading to maldigestion, such as chronic pancreatitis, partial or total pancreatic ectomy, or gastrogenic maldigestion, requires not only replacement of proteolytic, but also of amylolytic and lipolytic activities. Therefore, pancreatin, a hog (or bovine) pancreatic powder containing trypsin (E.C. 3.4.21.4), chymotrypsin (E.C. 3.4.21.1), α-amylase (E.C. 3.2.1.1), and lipase (E.C. 3.1.1.3) activities, is frequently used. The digestion of neutral fats is usually promoted by the addition of bile acids. To protect the pancreatin enzymes against the acid pH of the stomach, they are administered preferentially as enterocoated formulations which release the enzymes at the alkaline pH of the duodenum.

6.2.4.6.2 Plasma Proteases

Kallikrein (kallidinogenase, E.C. 3.4.21.8) was discovered in 1926 as a substance in human urine exhibiting hypotensive properties. Serum kallikrein plays an important physiological role in maintaining low blood pressure due to its ability to cleave L-kininogen, a serum α-globulin protein, and release vasoactive kinin peptides (bradykinin or lysyl-bradykinin). Kinins exert several biological activities such as vasodilation, blood pressure reduction, smooth muscle relaxation and contraction, pain induction, and inflammation. Therapeutically, kallikrein is used in the treatment of peripheral vascular and coronary arterial diseases, as well as in delayed fracture and wound healing.

The removal of purulent exudate, necrotic tissue, or hard and resistant eschar is a prerequisite for the effective cleaning and sterilization of burns, ulcers, traumatic and surgical wounds, and for the initiation of the healing process. A variety of proteolytic enzymes such as bovine plasmin (fibrolysin, E.C. 3.4.21.7), trypsin (E.C. 3.4.21.4) from bovine pancreas, clostridial collagenase (E.C. 3.4.24.3), and a protease from *Bacillus subtilis*, which can remove fibrin layers from wounds, are used as debriding agents for the cleaning of necrotic wounds, ulcers, abcesses, empyemas, sinuses, and fistulas. The enzymes are usually combined with antibiotics and are topically applied as ointments or as a wet or dry dressing. Dry enzyme powder may also be insufflated into wounds or cavities, or inserted into fistulas by means of small gelatin capsules. Because of the presence of trypsin and plasmin inhibitors in serum, the proteases are expected not to attack living tissue. However, due to their heterologous source, the risk of allergenic reactions must be considered.

6.2.4.6.3 Coagulation Factors

α-Thrombin (Fibrinogenase, E.C. 3.4.21.5) is a trypsin-like serine proteinase which causes blood clotting through proteolytic cleavage of fibrinogen and protease-activated receptors. It also amplifies its own generation from the inactive precursor prothrombin by activating the essential clotting factors V and VIII. Thrombin from bovine plasma is used to treat superficial bleeding resulting from trauma or surgical intervention, and ultrasound-guided thrombin injection has recently emerged as a treatment of iatrogenic femoral artery pseudoaneurysms. Another source of exposure to exogenous thrombin is the use of commercial "fibrin glue" preparations which contain a human or bovine thrombin component together with a gelatin-based matrix containing fibrinogen, fibronectin, and coagulation factor XIII in surgical procedures, especially during cardiac surgery, and to support wound healing. Bovine thrombin preparations represent potential antigenic stimuli, and an immunologic response may occur in up to 30 % of patients [1370]. Thrombin cannot be used intravascularly because of the danger of inducing intravascular coagulation.

Thrombin can both promote and prevent blood clotting (thrombin paradox). Its anticoagulant effect is caused by its ability to complex with an endothelial cell membrane protein, thrombomodulin, and then process the endogenous circulating serine protease zymogen Protein C into activated Protein C [APC, drotregocin alfa (activated), E.C. 3.4.21.69]. APC cleaves and inactivates factor Va and factor VIII and thereby inhibits the generation of thrombin, which prevents clotting, and also inhibits plasminogen activator inhibitor-1 (PAI-1), leading to a profibrinolytic effect. Furthermore, APC was reported to exert an anti-inflammatory effect by inhibiting production of tumor necrosis factor by monocytes and by blocking leukocyte adhesion to selectins. A recombinant version of naturally occurring human activated Protein C produced in CHO cells was approved in 2001 for treatment of severe sepsis [1358]. This condition results from an out-of-control host response to invading microbes, which gives rise to a dramatic increase of cytokine levels as well as escalation of the clotting cascade throughout the body, leading to inflammation, septic shock, and multiple organ failure.

Hemophilia is the most common congenital disorder of coagulation and affects approximately 1 in 10 000 males around the world [1371]. Hemophilia A, the oldest recognized hereditary bleeding disorder, is due to a deficiency of the antihemophiliac factor (AHF, factor VIII) in the circulating blood, whilst hemophilia B (also known as Christmas disease), a clinically identical, but less common disorder, is caused by factor IX deficiency. Both factor VIII, which is not an enzyme but a protein cofactor required for the activation of factor X by factor IXa, and factor IX (a zymogen of the serine protease factor IXa) are essential glycoproteins in the clotting cascade. Patients with hemophilia A and B require life-long substitution therapy, which was introduced about a century ago using fresh blood. A major therapeutic advance was the preparation of bovine factor VIII in 1954, followed by the introduction of cryoprecipitates and then lyophilized concentrates from human plasma. However, the pooling of plasma donations introduced the risk of transmission of viral infections (e.g., hepatitis, HIV). Although pretesting of the plasma used in the manufacture of coagulation factor products, as well as physical and chemical process steps of viral inactivation, have been introduced, recombinant products are believed to offer the greatest margin of safety for

hemophiliac patients. Recombinant expression of factor VIII and IX was a great technical challenge, since both are glycoproteins which undergo several posttranslational modifications, and factor VIII comprises no less than 2332 amino acid residues. Nevertheless, various human coagulation factor preparations obtained from recombinant mammalian cells have been developed and approved in the 1990s: recombinant factor VIII (octocog alfa) from CHO or BHK (1992/93); a second-generation, B-domainless factor VIII (moroctocog alfa), which retains activity in clotting assays but can be formulated without the addition of human albumin, which further reduces the theoretical risk of transmission of human viruses (1999); and recombinant factor IX (nonacog alfa) obtained from CHO cells (1997).

However, highly purified factor VIII and factor IX preparations obtained from human plasma are still available. Furthermore, concentrates of human plasma-derived factor VIII containing von Willebrand factor are used to treat von Willebrand disease, which is the most common inherited disorder of hemostasis, with an incidence in the population of about 1 %.

One complication of replacement therapy, however, is the development of inhibitory antibodies directed against factor VIII or factor IX in a significant percentage of severe hemophilia patients. For this type of patients, porcine factor VIII isolated from porcine plasma as well as prothrombin complex concentrates are available. Furthermore, recombinant human factor VIIa (niastase, E.C. 3.4.21.21) manufactured from BHK cells is used for treatment of these patients. Factor VIIa possesses very little inherent proteolytic activity, but when bound to tissue factor on the subendothelial cell surface, is able to convert factor X into factor Xa (and factor IX into factor IXa) directly in the absence of factors VIII and IX, and therefore bypasses factors VIII and IX in the coagulation cascade. Recombinant factor VIIa has been approved (EU, 1996; USA, 1999) for treatment of hemophilia A patients with factor VIII inhibitory antibodies and hemophilia B patients with factor IX inhibitory antibodies. Factor VII concentrates obtained from human plasma are used to treat patients with factor VII deficiency, which is a rare disorder occurring in 1 in 500 000.

6.2.4.6.4 **Plasminogen Activators**

Acute myocardial infarction and ischemic stroke are two main causes of death and disability in the Western societies. Fibrinolytic therapy of these diseases is based on the premise that their proximal cause is thrombosis, i.e., occlusive thrombi prevent blood flow to vital organs, resulting in oxygen deprivation, cell necrosis, and loss of organ function [1372]. Early and sustained recanalization is expected to prevent cell death, reduce infarct size, preserve organ function, and reduce early and late mortality. Thrombolytic agents dissolve blood clots by activation of the inactive circulating zymogen plasminogen to the active protease plasmin, which then will degrade fibrin, the main constituent of blood clots.

The first (and, in Europe, still most widely used) used plasminogen activator, streptokinase (SK), produced by several strains of hemolytic Streptococci, is not an enzyme, but activates plasminogen by forming an equimolar complex with plasminogen, which then undergoes a conformational change resulting in the exposure of the active site of the plasminogen molecule. In a second step, this active site catalyzes the

activation of residual plasminogen to plasmin, which then again converts the plasminogen–SK complex to a plasmin–SK complex by proteolytic cleavage. Since streptokinase generates free circulating plasmin, its use is associated with a systemic lytic state. Furthermore, streptokinase acts as a strong antigen in humans, which is even more critical because preexisting antibodies from previous streptococcal infections are common. Nevertheless, prospective multicenter trials with streptokinase have demonstrated that lytic therapy significantly improves the one-year survival rate if acute myocardial infarction is treated within the first 3 h after the onset of symptoms.

To control the enzymatic activity of the plasmin(ogen)–SK complex, anistreplase (anisoylated plasminogen–streptokinase activator complex, APSAC) has been developed. In APSAC, the catalytic center of the plasminogen moiety is specifically and reversibly modified with an anisoyl residue. This should delay the formation of plasmin but has no influence on the lysine-binding sites involved in the (albeit weak) binding of the complex to fibrin, so that a certain degree of localization of protease activity at the clot would be expected. However, the deacylation of the complex occurs both in circulation and at the fibrin surface, and the additional fibrin specificity of thrombolysis by anistreplase appears to be marginal [1372].

Urokinase (urokinase-type plasminogen activator, u-PA, E.C. 3.4.21.73) is an enzyme of human origin, secreted by the kidney and eliminated in the urine. Urokinase is formed in vivo as a single-chain form (scu-PA, prourokinase, Mr 54 000) which is then processed at the Lys-158–Ile-159 bond into a two-chain derivative (tcu-PA or HMW urokinase). Further proteolytic cleavage result in a form with Mr = 33 000 (LMW urokinase). Whereas the two-chain forms HMW and LMW show similar activity in vitro towards chromogenic substrates, scu-PA displays very low activity towards low molecular weight substrates. In plasma, in the absence of fibrin, scu-PA does not activate plasminogen; in the presence of the fibrin clot, scu-PA (but not tcu-PA) induces fibrin-specific clot lysis due to enhanced activity versus fibrin-bound plasminogen. u-PA is a serine protease with a high substrate specificity for plasminogen, and since it is a human enzyme, does not cause any immunological complications. For several years, LMW-urokinase obtained from human urine or from human neonatal kidney cell culture has been used for thrombolytic therapy. However, in the past few years, some manufacturers have run into problems with raw material supply (human urine) and uncertainties about possible viral contamination (human cell culture), leading to temporary unavailability of urokinase in some countries. Recombinant versions of single-chain urokinase (saruplase), obtained from recombinant *E. coli*, have been in clinical studies for a number of years, but no recombinant urokinase products have yet been approved for marketing.

A major advance in thrombolytic therapy was made when recombinant tissue-type plasminogen activator (t-PA, alteplase, E.C. 3.4.21.68) became available. t-PA is a Mr = 70 000 serine protease which also exists in single- and two-chain forms, which are both proteolytically active, and comprises three N-glycosylation sites. t-PA has little plasminogen activator activity in the absence of fibrin, but its activity is strikingly enhanced in the presence of fibrin. Thus, when introduced into the systemic circulation, t-PA will initiate local fibrinolysis at the site of blood clots, but only limited systemic proteolysis. Recombinant t-PA obtained from CHO cells was approved for the treatment of acute myocardial infarction (AMI) in 1987, and for acute massive

pulmonary embolism in 1990. In large clinical trials, it was conclusively demonstrated that fibrin-selective recombinant t-PA combined with intravenous heparin is superior to nonselective streptokinase for early mortality reduction.

Like many plasma proteases, t-PA consists of several discrete structural modules, i.e., a ''finger'' domain, a ''growth-factor-related'' domain, two ''kringle'' domains, as well as a protease domain, structurally similar to trypsin, which contains the enzymatically active center. In the 1990s, many approaches were reported to design t-PA mutants with improved properties, e.g., higher fibrin specificity, greater zymogenicity (activity ratio of two-chain vs single-chain form), slower clearance from the circulation, and resistance to plasma proteinase inhibitors, but only three of these mutants have been clinically developed and approved for marketing: reteplase [1373] is a single-chain, unglycosylated deletion variant consisting only of the second kringle and the protease domains of human t-PA. The plasminogenolytic activities of alteplase and reteplase in the absence of a stimulator do not differ, but in the presence of stimulating fibrinogen fragments, reteplase activity is about four times lower as compared to t-PA, and its binding to fibrin is five times lower. The reteplase molecule no longer contains the structural elements responsible for the rapid clearance of t-PA from the bloodstream (finger domain and carbohydrate residues), and indeed shows significantly prolonged half-life in the circulation (14–18 min in humans, as compared to 3–4 min for wild-type t-PA). This allows application as a double bolus, instead of a bolus followed by a 90 min infusion, as is necessary with t-PA. Reteplase is produced in recombinant *E. coli* by a process involving protein refolding on an industrial scale. Several large clinical studies have demonstrated that a clinical benefit of reteplase over alteplace could not be shown, and the two agents can be considered equivalent [1374], [1375]. Reteplase was approved for treatment of AMI in 1996.

In the second t-PA mutant which is commercially available, tenecteplase (TNK-t-PA), the glycosylation site in kringle-1 was deleted by substitution of Asn-117 by Gln, whereas a new glycosylation site was introduced, at a different locus, by replacement of Thr-103 by Asn. Furthermore, the amino acids 296–299 were replaced by Ala residues, which confer resistance against inhibition by PAI-1. Tenecteplase, which is produced in recombinant CHO cells, has a plasma half-life of 17 min, similar to that of reteplase, and has a similar ability to wild-type t-PA to bind to fibrin. Large clinical trials with AMI patients have demonstrated that tenecteplase, given as a single bolus, was equivalent to alteplase in terms of 30-d mortality. Tenecteplase was approved for marketing in 2000.

Monteplase [1376], a modified human tissue plasminogen activator in which replacement of cysteine 84 in the epidermal growth factor domain by serine results in a prolonged half-life in vivo, was launched in Japan in 1998 for the treatment of myocardial infarction. The compound has also been approved for treatment of pulmonary embolism, and is in phase II trials for the treatment of cerebral embolism. The product is not available outside of Japan.

Other plasminogen activators, e.g., the plasminogen activator from the saliva of vampire bat *Desmodus rotundus* (desmoteplase, DSPAa-1), other t-PA muteins, a chimeric t-PA Kringle 2/u-PA plasminogen activator (amediplase, [1377]) and staphylokinase from *Staphylococcus aureus* (obtained from recombinant *E. coli*) have been intensively studied in preclinical and even clinical studies, but until now, none of them

has been approved for routine use [1378]. However, desmoteplase is presently in phase IIb/II studies for treatment of acute ischaemic stroke where this enzyme, due to its high fibrin selectivity, is expected to be useful.

6.2.4.6.5 Proteases from Snake Venoms

Snake venoms contain two types of thrombin-like proteinases, including thrombocytin and crotalocytin, which preferentially act on substrates other than fibrinogen and are used for research purposes only, as well as enzymes (E.C. 3.4.21.74) such as Ancrod, obtained from the Malayan pit viper *Calloselasma* (*Agkistrodon*) *rhodostoma*, and Batroxobin, obtained from *Bothrops atrox moojeni* or *Bothrops jararaca* [1379]. These latter enzymes act very specifically on the A-α chain of fibrinogen, which is transformed into fibrinopeptide A and fibrin I, but do not promote cross-linking of the resulting fibrin gel. Fibrin I stimulates release of plasminogen activators from the endothelium and is degraded by plasmin into noncoagulating degradation products. This leads to a decrease of fibrinogen plasma levels and to reduced viscosity and coagulability of the blood. Therefore, Ancrod and Batroxobin are used for the prevention of thromboses, improvement of blood rheology, and support of drug-induced fibrinolysis, as well as for anticoagulation in patients with heparin-induced thrombocytopenia (HIT syndrome). Loss of activity after prolonged therapy, resulting from antibody formation, is observed with both snake venom proteases. Batroxobin is also used in chirurgic tissue glues and hemostyptics in order to obtain fibrin I preoperatively from the patient's own blood, which is then used post-operatively for wound closure.

Alfimeprase is a truncated form of fibrolase, a fibrinolytic metalloproteinase (E.C. 3.4.24.72) first isolated from the venom of the Southern copperhead snake (*Agkistrodon contortrix contortrix*) in which the first three amino acid residues at the N-terminus are replaced by a single Ser residue. Both fibrolase and alfimeprase have been shown to have direct proteolytic activity against the fibrinogen A-α chain. In vivo pharmacology studies [1380] have shown that thrombolysis with alfimeprase is up to six times more rapid than with plasminogen activators. Alfimeprase can be bound and neutralized by serum α-(2)-macroglobulin, a prevalent mammalian protease inhibitor, which may reduce systemic bleeding complications due to the inhibitory effects of α-(2)-macroglobulin. Alfimeprase is presently in phase III clinical trials for treatment of peripheral arterial occlusive disease.

6.2.4.6.6 Plant and Microbial Proteases

In the same way as pancreatin, lytic enzymes from plants or microorganisms are used as digestive aids, e.g., papain (papayotin, E.C. 3.4.22.2), a cysteine endopeptidase obtained from the latex of *Carica papaya*, and bromelain (E.C. 3.4.22.4), obtained from the stem and fruit of pineapple (Ananas comosus var.).

In Europa and Japan (but not in the USA), enzyme preparations containing bromelain together with papain, trypsin, or chymotrypsin, as well as serrapeptase (E.C. 3.4.24.40), a protease from the nonpathogenic enterobacterium *Serratia* E15, have been widely used in rheumatic disorders [1381] and generally for anti-inflammatory treatment. A protease from Aspergillus sp (promelase, E.C. 3.4.21.63) has also been studied as an anti-inflammatory agent. However, enzyme use in this market segment is

declining versus other drugs such as salicylates, nonsteroid anti-inflammatory agents, and steroids.

Intravenous administration of brinase, a proteolytic enzyme preparation from *Aspergillus oryzae*, was found to be beneficial in the treatment of chronic arterial obstruction. Sfericase, the main intracellular protease of *Bacillus sphaericus* was found to reduce the viscosity of human pathological bronchial secretions while not modifying their elasticity, and has been studied for use in chronic bronchitis, acute pneumonia, and chronic sinusitis. Sutilain, a proteolytic enzyme isolated from *Bacillus subtilis*, has been used for the treatment of burns.

Chemonucleolysis is now an established treatment modality in the management of herniated or prolapsed lumbar intervertebral disk disease as an alternative to surgery. The enzyme chymopapain (E.C. 3.4.22.6), a proteolytic cysteine protease from papaya latex, is injected directly into the center of the affected disk (nucleus pulposus), where it digests the protein part of the insoluble mucopolysaccharide–protein complex. The fragmented proteoglycan diffuses from the disk, causing a reduction in hydrostatic pressure on the nerve root and thus an easing of pain and disability. This procedure has been in widespread use since its introduction in 1963, and its efficacy has been established in a placebo-controlled, double-blind study. However, the injection technique is complicated, and a relatively high complication rate is observed.

6.2.4.7 **Amidases**

Certain tumor cells may require unusual or specific nutrients derived from the bloodstream, or may require a nutrient in higher concentration than normal cells. Thus, continued growth of these tumors might be prevented by restricting their nutrient supply by administration of an appropriate enzyme. In this area, microbially derived L-asparaginase (E.C. 3.5.1.1), isolated from various microorganisms such as *E. coli* (colaspase) and *Erwinia carotovora* (crisantaspase), has been approved for the treatment of some forms of leukemia, and glutaminase is also under study for treatment of neoplastic diseases. Asparaginase has proved to be particularly promising for treatment of acute lymphocytic leukemia. Its action depends on the fact that tumor cells are deficient in aspartate ammonia ligase activity, which restricts their ability to synthesize the normally nonessential amino acid L-asparagine. Asparaginase deprives the circulating blood of asparagine. This does not affect the functioning of normal cells, which are able to synthesize enough for their own requirements, but induces a state of starvation in the susceptible tumor cells. The enzyme is administered intravenously and shows a half-life of about one day in dogs. This half-life can be increased 20-fold by use of PEG-modified asparaginase (Peg-asparaginase) which is likely to expand the scope of application and the therapeutic efficacy of this enzyme. However, the main problems of this therapy are the rapid development of resistance by the tumor cells, disturbances in the homeostatic system, as well as immunological complications.

Severe combined immunodeficiency (SCID) is a primary immune deficiency which usually results in the onset of one or more serious, and even life-threatening, infections within the first few months of life. There are several forms of SCID; one of them is linked to a chronic deficiency of the enzyme adenosine deaminase (ADA, E.C. 3.5.4.4). For these patients, the only available alternative to bone marrow transplant is enzyme

replacement with PEGylated bovine ADA (pegademase bovine) in infants from birth or children of any age at the time of diagnosis. Injection of unmodified ADA is not effective because of its short circulating life (less than 30 min) and the potential for immunogenic reactions to a bovine-sourced enzyme. The attachment of PEG to ADA increases its circulating life and masks epitopes on the surface of the ADA molecule to avoid immunogenic reactions.

6.2.4.8 Lyases

There are only two lyases presently under study for therapeutic application. Recombinant human porphobilinogen deaminase (rhPBGD, E.C. 4.3.1.8), an enzyme involved in the heme-synthesizing pathway, obtained orphan designation in 2002 for the treatment of acute intermittent porphyria, a clinical manifestation of an autosomal dominant genetic disorder caused by mutation in the PBDG gene and resulting in a 50 % reduction of enzymatic activity. This may lead to an increase in blood and urinary concentrations of heme precursors such as porphobilinogen, and a deficit in heme synthesis. The effects of recombinant human PBDG, until now, have only been demonstrated in experimental models [1382].

Administration of PBDG is expected to decrease plasma concentrations of heme precursors. Phase IIb studies are ongoing since 2004.

A chodroitinase ABC (E.C. 4.2.2.4), i.e., an enzyme which cleaves chondroitin sulfate into unsaturated disaccharides, has completed a phase I/II study in Japan for the treatment of lumbar disk herniation and may be useful in chemonucleolysis (cf. Section 6.2.4.6.6 [1383].

6.3 Analytical Applications of Enzymes

This section describes the principles of enzyme use in analytical measurements; the reader who wishes to get a deeper insight into this field is referred to special monographs, e.g., [1384]. Normally, enzymes are used as auxiliaries for determination of *substrate concentration, inhibitors,* or *enzyme activity* and as labels for enzyme *immunoassays.*

Reactions are monitored photometrically (absorption change, fluorescence, bioluminescence, nephelometry, turbidimetry), by electrochemical detection (amperometry, potentiometry), by calorimetry, and by radiochemical methods.

Enzymatic reactions may be used in clinical chemistry in complex matrices such as serum or urine, due to the high selectivity of enzymes for their natural substrates [1385].

Some enzymatic reactions can be followed directly (substrate depletion or product accumulation), for example, the electrochemical detection of H_2O_2 (at +600 mV vs Ag/AgCl) or the ferrocene mediator in the glucose oxidase reaction:

$$\beta\text{-D-glucose} + O_2 \rightarrow \delta\text{-D-gluconolactone} + H_2O_2$$

However, most enzymes catalyze reactions involving species that are not themselves readily measured [1385]. In these situations, products must be converted to species that are measurable, in a coupled or indicator reaction. The indicator reaction quantitatively

converts the product of the primary reaction into readily measurable species. It should be rapid and quantitative. To accomplish this in a ''linear manner'', normally a large excess of the indicator enzyme is employed and, if possible, saturating levels of cosubstrates or coenzymes of the indicator enzyme.

The most commonly used indicator enzymes are dehydrogenases and peroxidases.

Example of a dehydrogenase reaction:

$$\text{Reduced substrate} + \text{NAD(P)}^+ \rightarrow \text{oxidized substrate} + \text{NAD(P)H} + \text{H}^+$$

The formation of reduced coenzyme (NADH or NADPH) allows absorbance measurements at 340 nm, where the oxidized coenzyme shows almost no absorbance [R2].

Example of a peroxidase reaction:

$$\text{Reduced dye} + \text{H}_2\text{O}_2 \rightarrow \text{oxidized dye} + \text{H}_2\text{O}$$

Peroxidase indicator reactions can be used to follow any primary enzyme reaction that produces H_2O_2. These are mainly oxidases. Peroxidases are very specific for H_2O_2, but will react with a variety of organic dyes that are colorless in the reduced form but highly absorbing in the oxidized form. The coupled reaction of glucose oxidase and peroxidase is used in ''dry chemistry'', in so called enzyme dipsticks (see Section 6.3.4 for glucose measurement.

6.3.1
Determination of Substrate Concentration

Substrate concentrations are determined enzymatically in two general ways: (1) fixed-time (end-point) methods and (2) kinetic methods (measurement of reaction rate) [1387], [1388].

Fixed-Time (End-Point) Methods In the simplest case, the enzyme-catalyzed substrate conversion is practically complete, and either the decrease in substrate concentration or the increase in product concentration can be measured, e.g., by light absorption at a given wavelength.

The time required for completion (e.g., 99 % conversion) of the enzymatic reaction strongly depends on the maximum reaction rate (V) and the Michaelis constant (K_m) of the enzyme used. Fixed-time assays need a fixed, relatively long period of time and rely on quantitative or near-quantitative substrate conversion. They are used exclusively for substrate quantitation. They cannot be used for enzyme activity measurements, since saturating conditions must be maintained over the duration of the reaction.

Determination of Glucose with Glucose Oxidase Glucose oxidase (GOD, E.C. 1.1.3.4) is by far the most widely used analytical enzyme in the world. Millions of diabetics use it daily, mainly in hand-held biosensors.

To detect glucose with optical methods, in the first reaction, glucose is oxidized with glucose oxidase [9001-37-0] and hydrogen peroxide is formed:

$$\beta\text{-D-glucose} + \text{O}_2 \rightarrow \delta\text{-D-gluconolactone} + \text{H}_2\text{O}_2$$

In the second reaction, hydrogen peroxide is used to oxidize a chromogen to form a dye with the aid of horseradish peroxidase (E.C. 1.11.1.7) [9003-99-0]:

$$H_2O_2 + \text{chromogen} \rightarrow \text{dye} + H_2O$$

2,2′-Azino-bis(3-ethyl-2,3-dihydrobenzthiazolsulfonate) [30931-67-0] (ABTS) or tetramethylbenzidine (TMB) is used as chromogen [1392].

Determination of Urea Urea is hydrolyzed with urease (E.C. 3.5.1.5) [9002-13-5] and the ammonia formed is assayed by use of glutamate dehydrogenase (E.C. 1.4.1.3) [9029-12-3]

$$\text{Urea} + H_2O \rightarrow 2\,NH_3 + CO_2$$

$$\text{2-Ketoglutarate} + 2\,NH_4^+ + 2\,\text{NADH} \rightarrow 2\,\text{L-Glutamate} + 2\,NAD^+ + 2\,H_2O$$

The reaction is followed photometrically (decrease in NADH concentration at 340 nm).

Kinetic Assays The drawback of end-point methods is that a considerable amount of time may be required before the reaction comes to completion; this limits the sample frequency. In addition, many time- and reagent-consuming sample blanks must be determined, to eliminate interference from light-absorbing serum constituents. These problems can be overcome by assaying the substrate concentration via the rate of the enzyme-catalyzed reaction [1388].

These so-called kinetic assays monitor continuously concentrations as a function of time. As shown in Equation (6.1), up to around 10 % completion, the reaction obeys a pseudo-first order rate law which allows the initial reaction rate to be determined. At low initial substrate concentrations ($< 0.1\ K_m$), the initial rate will be directly proportional to the initial substrate concentration. A linear plot of initial rate versus the substrate concentration is used as for quantitation (calibration) [1385].

$$v = c_S \cdot V / K_m \tag{6.1}$$

Such assays are most conveniently run on automatic analyzers using the so-called kinetic fixed-time approach, in which the absorbance is read at two constant points of time, and the slope of the absorbance change is calculated. Only one calibration is required.

As said, these methods work only for substrate concentrations below the K_m value, which sets an upper limit on the dynamic range. This upper concentration limit is increased in the presence of competitive inhibitors that increase the apparent K_m of the enzyme. Kinetic substrate assays are described for many analyses.

Urea concentration is determined according to the reaction sequence mentioned previously.

Determination of Glucose

$$\beta\text{-D-glucose} + \text{ATP} \rightarrow \text{D-glucose 6-phosphate} + \text{ADP}$$

$$\text{D-Glucose 6-phosphate} + NADP^+ \rightarrow \text{D-Glucono-}\delta\text{-lactone 6-phosphate} + \text{NADPH} + H^+$$

In the first reaction, glucose is phosphorylated by hexokinase (E.C. 2.7.1.1) [9001-51-8]; then glucose 6-phosphate is dehydrogenated by the action of glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49) [9001-40-5]. The NADPH production is monitored photometrically at 340 nm.

Determination of Triglycerides Triglycerides (fats) are hydrolyzed by lipase (E.C. 3.1.1.3) [9001-62-1] and carboxyl esterase (E.C. 3.1.1.1) [9016-18-6]. Glycerol is then phosphorylated by glycerol kinase (E.C. 2.7.1.30) [9030-66-4]. The ADP formed in this reaction is rephosphorylated to ATP with phosphoenolpyruvate and pyruvate kinase (E.C. 2.7.1.40) [9001-59-6]. Finally, the pyruvate is hydrogenated by L-lactate dehydrogenase (E.C. 1.1.1.27) [9001-60-9] and the decrease in NADH concentration is followed:

$$\text{Triglyceride} + 3\,H_2O \rightarrow \text{Glycerol} + 3\,\text{Fatty acid}$$

$$\text{Glycerol} + \text{ATP} \rightarrow \text{Glycerol 3-phosphate} + \text{ADP}$$

$$\text{ADP} + \text{Phosphoenolpyruvate} \rightarrow \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \rightarrow \text{L-Lactate} + NAD^+$$

6.3.2 Determination of Enzyme Activity [1390]

Ideally, the substrate concentration must be so high for an enzyme assay that the value of K_m can be neglected. Then the reaction rate is equal to the maximum rate defined by the Michaelis–Menten equation. The reaction obeys pseudo zero-order kinetics. In many cases, the enzyme to be assayed cannot be determined directly but must be coupled to a second enzyme-catalyzed reaction that serves as the indicator reaction. In this case, the conditions chosen must make the enzyme reaction which is to be determined the rate-limiting step.

Activity of Alkaline Phosphatase (E.C. 3.1.3.1) [9001-78-9].

$$\text{4-Nitrophenylphosphate} + H_2O \rightarrow \text{Phosphate} + \text{4-Nitrophenolate}$$

This is a straightforward enzyme activity assay. Serum alkaline phosphatase is an important metabolic indicator. In vivo it catalyzes the dephosphorylation of $NADP^+$ and a variety of other substances, but in vitro a synthetic substrate can be used. The chromogenic substrate *p*-nitrophenyl phosphate is cleaved by the enzyme whose activity is to be determined. The rate of formation of the yellow product nitrophenol can be followed by the increase in light absorption at 405 nm.

Activity of Creatine Kinase (E.C. 2.7.3.2) [9001-15-4].

$$\text{Creatine phosphate} + \text{ADP} \rightarrow \text{Creatine} + \text{ATP}$$

The creatine kinase assay used to be the gold standard (the isoenzyme CK-MB) in heart attack diagnostics before being replaced by the troponins. This is an example of a

reaction coupled with an auxiliary enzyme. The ATP formed in the reaction catalyzed by creatine kinase is used to phosphorylate glucose to glucose 6-phosphate, which is then dehydrogenated, as described previously. The rate of NADPH formation at 340 nm is a measure of the rate of the reaction catalyzed by creatine kinase.

In this assay, diluted serum is preincubated with glucose, hexokinase, $NADP^+$, and G6P dehydrogenase to first allow any creatine phosphate and ADP (present in the serum) to be consumed. When a constant absorbance is reached at 340 nm, excess creatine phosphate and ADP is added. The increase is monitored with time. The slope of the initial linear section is directly proportional to the enzyme activity. By using standards, the assay can be calibrated.

6.3.3
Immunoassays

Enzymes are the most widely used labels for immunoassays, because a single enzyme label can by catalytic amplification provide multiple copies of detectable species. The detection limits therefore rival those of radioimmunoassays without the storage and disposal problems associated with radioisotopes [1391].

Enzyme immunoassays use enzymes as labels to determine the amount of immunocomplex (antigen–antibody) formed; for a review, see [1390], [1391], [1392]. The most popular immunoassay is the enzyme-linked immunosorbent assay (ELISA) technology using microtiter plates.

The ideal label for immunoassays is inexpensive, safe and has simple labeling procedures [1385]. It is covalently linked to the assay reagent at multiple sites for high sensitivity. The labeled species is stable and is easily detected using inexpensive instrumentation that is readily automated. The labeling should have minimal effect on the binding behavior.

Depending on the chemical reaction catalyzed by the respective enzyme, its activity can be measured photometrically, fluorometrically, or luminometrically. Enzymes can be bound to antibodies using such bifunctional coupling reagents as glutaraldehyde [111-30-8] [1392], 3-maleinimidobenzoyl-*N*-hydroxysuccinimide [15209-14-0] [1393], or bis(-maleido)-methyl ether [1394], depending on the nature of the reactive groups on the enzyme. Horseradish peroxidase is bound directly to the antibody by reaction of carbonyl groups in the carbohydrate part of the enzyme with amino groups of the antibody via Schiff base formation and subsequent reduction with sodium borohydride [1395].

Alkaline phosphatase (E.C. 3.1.3.1) [9001-78-9], used in enzyme immunoassays, should be homogeneous on electrophoresis. For conjugate synthesis, glutaraldehydeis recommended [1392]. Alkaline phosphatase activity can also be assayed optically by using 4-nitrophenylphosphate (see above) or fluorometrically by using 4-methylumbelliferylphosphate [3368-04-5] as substrate. A photometric amplification system for the immunoassay of alkaline phosphatase has been published [1396].

β-Galactosidase (E.C. 3.2.1.23) [9031-11-2] contains 12 mercapto groups; these are linked to antibodies by maleimide bifunctional reagents. The activity of β-galactosidase

can be measured photometrically with 4-methylumbelliferyl-β-galactoside [39940-54-0]; in histochemistry, the substrate of choice is 5-bromo-4-chloro-3-indolyl-β-galactoside [7240-90-6].

Horseradish peroxidase (E.C. 1.11.1.7) [9003-99-0] contains 12–14.5 wt % of carbohydrates, which can be used for coupling to antibodies. The enzyme activity can be assayed photometrically in different ways. The chromogen 2,2′-azino-bis[3-ethylbenzothiazoline-sulfonate] (ABTS) is especially suited as a substrate. 3,3′,5,5′-Tetramethylbenzidine [54827-17-7] is recommended as a substrate for use in immunohistochemistry.

Luminescence Reactions *Chemiluminescence* occurs when part of the free energy produced in a chemical reaction is emitted as light, because a reaction product is generated in its singlet excited state. *Bioluminescence* occurs in living organisms, where these reactions are catalyzed by enzymes. The corresponding enzymes are called *luciferases*, and the substrates being converted to light-emitting products are called *luciferins*; for reviews see [1397], [1398]; see also Section 2.2.5. Because light intensities can be measured with very high sensitivity, luminescence reactions are of increasing interest as indicator systems for immunoassays, giving detection limits comparable to those achieved with radioactive labels, e.g., see [1399], [1400].

Firefly Bioluminescence In the reaction catalyzed by luciferase (E.C. 1.13.12.7) [61970-00-1] of the American firefly *Photinus pyralis*, the light intensity is proportional to the reaction rate, and substrate assays using this reaction follow the rules of kinetic substrate determination. These considerations also apply to other enzyme-catalyzed chemiluminescence reactions.

Bioluminescence catalyzed by firefly luciferase [1401] has been used in immunoassays in two different ways: the luciferase can be used directly as a marker, e.g., [1402]; or pyruvate kinase can be used as a label, and the ATP formed in the rephosphorylation of ADP by phosphoenolpyruvate (see Phytase) is then assayed by the reaction catalyzed by firefly luciferase [1403].

Chemiluminescence Catalyzed by Horseradish Peroxidase The peroxidase from horseradish (E.C. 1.11.1.7) [9003-99-0] catalyzes the oxidation of luminol (5′-amino-2,3-dihydro-1,4-phthalazinedione [521-31-3] and luminol derivatives by hydrogen peroxide [1404], with concomitant light emission:

$$\text{Luminol} + H_2O_2 + 2\,OH^- \rightarrow \text{Aminophthalate} + N_2 + 2\,H_2O$$

Under normal conditions, this reaction is rather slow and, depending on the substrate concentrations, a lag phase of varying length is observed. Therefore, this reaction is not suitable for routine assay of peroxidase activity. However, several groups of compounds have been found recently that activate this reaction by eliminating the lag phase, which results in an increase in light intensity by a factor of 100–1000. The light signal is stable at a given set of concentrations. Firefly luciferin [2591-17-5] [1405], 6-hydroxybenzothiazoles [1406], and phenol derivatives such as 4-iodophenol [540-38-5] and 4-phenylphenol [92-69-3] are used as activators [1407]. At pH 6, the phenol

derivatives also act as activators of a photometric reaction catalyzed by peroxidase [1408].

Most primary enzyme reactions that produce hydrogen peroxide have been tested with a luminol-peroxidase indicator reaction [1385]. It has been used as an indicator reaction for glucose with glucose oxidase, and for cholesterol with cholesterol oxidase. Also, firefly luciferase can be employed as an indicator reaction for any primary enzymatic reaction that produces or consumes ATP (e.g., the hexokinase reaction). This assay is, however, not economic compared with the simple and cheap glucose assays.

6.3.4 Enzyme Dipsticks and Enzyme Sensors

Real-time and in situ quantitation of analytes is an old goal of analytical research. Biosensors and lateral-flow rapid tests are new tools aiming at this.

Enzyme lateral-flow rapid tests (dipsticks) are often also called ''dry chemistry'' [1409]. They first used immobilized reagents to detect analytes in urine, later also in whole blood.

The first dipsticks were developed for testing urine for pH, glucose, and protein (1964), nitrite (1967), urobilinogen (1972), ketone which ketone (1973), and bilirubin (1974) as yes/no or semiquantitative mode. Color comparators and later reading instruments were used for quantification. Thereafter, blood, serum, and plasma were targeted. Reflectometric readers were developed. Blood glucose was determined by using glucose oxidase and peroxidase in a coupled reaction on the dipstick.

Today, still dipsticks are mainly used for screening purpose in urine. They comprise several fields for different analytes, for example combi-tests for glucose, ascorbic acid, protein, pH, blood, and nitrite in urine.

Glucose measurement in whole blood, however, has shifted methodically mainly to enzyme biosensors.

Biosensors [1410, 1411] ideally are small and portable devices that allow the selective quantitation of analytes. They have two components: a transducer in intimate contact with a biochemical recognition element.

Transducers measure electrochemical, optical, or thermal processes that change in the presence of the analyte. Biorecognition is mainly achieved by enzymes, antibodies, chemoreceptors, and nucleic acids. In the presence of an analyte, these agents, immobilized at the surface of the transducer, cause a change in measurable property and convert the biochemical recognition event into a measurable electronic signal. The immobilized bio-recognition element can be reused.

The first enzyme sensor was introduced by Clarke and Lyons in 1962 [1412]. It consisted of glucose oxidase trapped on the surface of a platinum working electrode by a semipermeable dialysis membrane allowing substrates and products to freely diffuse to and from the enzyme layer. The conversion of glucose and molecular oxygen to hydrogen peroxide and gluconolactone is monitored electrochemically.

Different potentials allow different arrangements: At a potential of -0.6 V vs Ag/AgCl, the oxygen reduction current is measured. In this case, the working electrode is covered with a gas-permeable membrane (Teflon). This is an almost interference-free enzyme sensor.

At +0.6 V vs Ag/AgCl, the oxidation of hydrogen peroxide is monitored. This has the advantage that the oxidation current in the absence of glucose is almost zero. The measurement can be done within seconds. The glucose oxidase is mostly immobilized in a polymeric gel (enzyme membrane) and trapped between two dialysis membranes. It can be reused from 5000 to 10 000 times as it is a diffusion-controlled sensor having a large ''enzyme reserve''.

A disadvantage is the possibility of interferences caused by other substances in the sample, for example, ascorbic acid. Clinical benchtop analyzers are highly economical because they are reagentless, that is, they do not consume the enzyme, just buffer solution.

Both glucose sensors are dependent of the oxygen concentration in the solution and the enzyme membrane. The pocket device used by millions of diabetics uses a different principle: A mediator (1,1′-dimethylferricinium) replaces molecular oxygen. It uses an amperometric graphite foil as the transducer. The ferrocene species is co-adsorbed with glucose oxidase on the sensor chip in its reduced form.

For medical safety reasons, this enzyme sensor configuration is not reused; it is a one-use sensor. Thus, no efforts are needed to immobilize and to stabilize the enzyme. Only small amounts of glucose oxidase are used. The sensor operates at a very low potential, so electroactive interfering substances present in blood (e.g., ascorbic acid) are not detected. A drop of the patient's blood is placed onto the sensor chip containing dry ferrocene and glucose oxidase coadsorbed on the printed electrodes. The chip is inserted before into a portable, handheld potentiostat. Glucose is bound by active site of the enzyme and transfers two electrons to the FAD moiety. The reduced FAD in turn reduces two mediator molecules each of which transfers one electron to the electrode. The oxidation current measured by the device is then directly proportional to the blood glucose concentration.

A similar device is available for the determination of lactate, mainly for monitoring the fitness of athletes.

6.4
Enzymes for Food Analysis [1413], [1414]

Use of the catalytic activity of enzymes for analytical determination dates from the middle of the 19th century. Nevertheless, the use of enzymes for food analysis in its present form was established only about 30 years ago. This was made possible by the large-scale preparation of pure enzymes, the availability of suitable and inexpensive photometers, and the elaboration of both enzymatic procedures and methods for sample preparation.

In *food research*, the amounts of various food constituents are determined, and changes occurring during technological processing and subsequent storage are monitored. Because of the great specificity manifest by enzymes, enzymatic methods are now increasingly used for this purpose.

In *industry*, raw materials are checked, production controls are carried out, and both the end products and the competing products are analyzed. Enzymatic methods were accepted quickly by industry because of the obvious advantage of using easy, rapid, and highly specific methods of analysis.

Official inspections involve regular analysis of samples and checking that laws, regulations, and guidelines are being observed. Checks are also made to determine if the food industry is fulfilling its obligation to make complete declarations according to the law. Enzymatic methods were used very early for these purposes not only because of the accurate results obtained from enzymatic analysis but also because of the great flexibility of the methods and their applicability to different types of sample materials. Because of tariffs on food, *customs officials* routinely analyze food by enzymatic methods. *Military inspectors* also use enzymes in the general control of food as well as in stability and storage investigations.

The use of enzymes for analytical determinations is recommended today by many national and international commissions; in fact, standardized enzymatic methods are widely available.

6.4.1 Carbohydrates

Carbohydrates are determined routinely in food analysis. The sugar components are an important part of the overall analytical picture: they constitute a large part of the caloric value of food, and the authenticity of food samples can be deduced from the relative amounts of different sugars. The presence of lactose indicates the use of milk. Starch serves as a raising agent in meat products and as a thickening agent.

Glucose [50-99-7] is by far the most abundant sugar and is usually determined by using the hexokinase method [1413, vol. VI, pp. 163–172]:

$$\text{D}-\text{Glucose} + \text{ATP} \xrightarrow[\text{E.C.2.7.1.1}]{\text{Hexokinase}} \text{ADP} + \text{Glucose 6}-\text{phosphate} \tag{6.2}$$

$$\text{Glucose 6}-\text{phosphate} + \text{NADP}^{+} \xrightarrow[\text{E.C.1.1.1.49}]{\text{Glucose 6}-\text{phosphate dehydrogenase}} \text{D}-\text{Gluconate 6}-\text{phosphate} + \text{NADPH} + \text{H}^{+} \tag{6.3}$$

Other enzymatic methods can cause problems for a variety of reasons: e.g., the enzymes are nonspecific (glucose dehydrogenase), or reducing substances in the sample interfere with the reaction (glucose oxidase–peroxidase method). In addition, other methods do not allow the simultaneous determination of fructose in the same cuvette. Glucose is usually determined together with other carbohydrates such as fructose, sucrose, maltose, and starch.

Fructose [57-48-7]. The enzyme hexokinase also acts on fructose, which is determined after glucose (Reactions 6.4, 6.5, and 6.3) [1413, vol. VI, pp. 321–327]:

$$\text{D}-\text{Fructose} + \text{ATP} \xrightarrow[\text{E.C.2.7.1.1}]{\text{Hexokinase}} \text{ADP} + \text{Fructose 6}-\text{phosphate} \tag{6.4}$$

$$\text{Fructose 6}-\text{phosphate} \xrightarrow[\text{E.C.5.3.1.9}]{\text{Glucose phosphate isomerase}} \text{Glucose 6}-\text{phosphate} \tag{6.5}$$

Glucose and fructose are determined in wine and fruit juices, e.g., to detect the prohibited addition of sugar. Fructose and glucose are also determined together with sucrose.

Galactose [59-23-4] is easily determined by using the following reaction [1413, vol. VI, pp. 104–112]:

$$\text{D} - \text{Galactose} + \text{NAD}^+ \xrightarrow[\text{E.C.1.1.1.48}]{\text{Galactose dehydrogenase}} \text{D} - \text{Galactonic acid} + \text{NADH} + \text{H}^+ \tag{6.6}$$

This determination is of interest only for certain milk products (yogurt, soft cheese). The method can also be applied to acid hydrolysates of thickening agents like agar, guar, carrageenan, gum arabic, locust-bean gum, and tragacanth gum.

Mannose [3458-28-4]. The determination of free mannose is also of little interest. However, like galactose, it is an important component of thickening agents and can be estimated after subjecting these substances to acid hydrolysis according to Reactions (6.7), (6.8), (6.5), and (6.3) [1413, vol. VI, pp. 262–267]:

$$\text{D} - \text{Mannose} + \text{ATP} \xrightarrow[\text{E.C.2.7.1.1}]{\text{Hexokinase}} \text{ADP} + \text{Mannose 6}-\text{phosphate} \tag{6.7}$$

$$\text{Mannose 6}-\text{phosphate} \xrightarrow[\text{E.C.5.3.1.8}]{\text{Phosphomannose isomerase}} \text{Fructose 6}-\text{phosphate} \tag{6.8}$$

Sucrose [57-50-1]. The disaccharide sucrose does not occur in animals but occurs in many plants in varying amounts. On hydrolysis, sucrose yields an equimolar mixture of glucose and fructose (Reaction 6.9) and is estimated via glucose (Reactions 6.2 and 6.3) [1414, vol. 3, pp. 1176–1179]:

$$\text{Sucrose} + \text{H}_2\text{O} \xrightarrow[\text{E.C.3.2.1.26}]{\beta-\text{Fructosidase}} \text{D} - \text{Glucose} + \text{D} - \text{Fructose} \tag{6.9}$$

In addition to measurement of glucose, fructose produced in Reaction (6.9) can also be measured according to Reactions (6.4), (6.5), and (6.3) to ensure accurate results or increase the sensitivity of the determination.

Sucrose, glucose, and fructose are determined in fruit and vegetable products (e.g., juices, jams, tomato puree, or potatoes), bread and biscuits, honey, ice cream, confectionery, desserts, diet food, sugar refinery products, or beverages. A large excess of glucose interferes with the accuracy of sucrose and fructose analysis. Therefore, excess glucose should be removed as completely as possible with a mixture of glucose oxidase and catalase.

Maltose [69-79-4]. Enzymatic hydrolysis of maltose yields two glucose molecules which are determined as described above [1413, vol. VI, pp. 119–126]:

$$\text{Maltose} + \text{H}_2\text{O} \xrightarrow[\text{E.C.3.2.1.20}]{\alpha-\text{Glucosidase}} 2\,\text{D} - \text{Glucose} \tag{6.10}$$

Sucrose, which also contains an α-glucosidic bond, and maltotriose are hydrolyzed as well.

Maltose is determined in malt products, beer, baby food, and often together with partial hydrolysates of starch (glucose syrup, starch sugar; dextrins in beer).

Lactose [63-42-3]. The carbohydrate of milk, lactose, is an important constituent of infant diets. It is usually determined after hydrolysis (Reaction 6.11) via galactose and indicates the use of milk or milk products in the production of food [1413, vol. VI, pp. 104–112]:

$$\text{Lactose} + H_2O \xrightarrow[\text{E.C.3.2.1.23}]{\beta-\text{Galactosidase}} \text{D}-\text{Galactose} + \text{D}-\text{Glucose} \qquad (6.11)$$

Analysis via galactose gives accurate results because very few samples contain large amounts of galactose. Lactose can also be estimated via glucose (Reactions 6.11, 6.2, and 6.3), but excess free glucose impairs the precision of the results and must be removed with a mixture of glucose oxidase and catalase (see lactulose). The amount of lactose is determined in milk (decreases with mastitis), milk products, and other food as a measure of the milk content, e.g., baby food, bread, biscuits, ice cream, chocolate, desserts, and sausages.

Raffinose [512-69-6] occurs in relatively high concentrations in sugar beet. It accumulates in molasses during the production of sugar. Raffinose, which also occurs in soybean, is determined by using Reactions (6.12) and (6.6) [1413, vol. VI, pp. 90–96]:

$$\text{Raffinose} + H_2O \xrightarrow[\text{E.C.3.2.1.22}]{\alpha-\text{Galactosidase}} \text{D}-\text{Galactose} + \text{Sucrose} \qquad (6.12)$$

Raffinose is measured at different stages in the production of beet sugar. The addition of soybean protein to food is detected indirectly by measuring raffinose, because soybean flour can contain up to 10 % of this carbohydrate (addition of soybean is often subject to declaration).

Starch [9005-25-8]. The analysis of starch has played an important part in food analysis for over 100 years. It is determined via glucose after hydrolysis. The use of acid hydrolysis for this purpose is not advisable because other glucose polysaccharides and oligomers are also hydrolyzed by acids; in addition, undesired reactions can take place (e.g., conversion of glucose to fructose). Amylase is less suited for enzymatic hydrolysis because it does not break down starch to glucose completely. Amyloglucosidase is the enzyme of choice; however, in addition to starch, maltose and oligoglucosides are also hydrolyzed to glucose by this enzyme. Analysis is carried out by using Reactions (6.13), (6.2), and (6.3):

$$\text{Starch} + (n-1)H_2O \xrightarrow[\text{E.C.3.2.1.3}]{\text{Amyloglucosidase}} n\,\text{D}-\text{Glucose} \qquad (6.13)$$

Starch in the sample must be dissolved, which is achieved by heating in an autoclave or by treating with concentrated hydrochloric acid and dimethyl sulfoxide. All steps must be performed carefully according to instructions. Oligoglucosides (maltodextrins,

glucose syrup, starch sugar) are separated by extraction with ethanol–water mixtures which do not dissolve starch.

Starch is determined in products such as flour, bread, biscuits, and meat products, which contain starch as a raising agent. Oligoglucosides are estimated in fruit juices, beverages, confectionery, jam, and ice cream. Dextrins (low molecular mass starch) are determined in beer; they indicate the presence of fermentable carbohydrates.

6.4.2 Organic Acids

Organic acids and their salts frequently appear in metabolism and are of varying importance. Their presence in food has a considerable effect on taste and may indicate fermentation.

Acetic Acid [64-19-7]. Acetic acid, a component of the "volatile acids," is determined specifically by using Reactions (6.14)–(6.16) [1413, vol. VI, pp. 639–645]. The use of acetate kinase is not suitable because this enzyme also acts on propionate.

$$\text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow[\text{E.C.6.2.1.1}]{\text{Acetyl-CoA synthetase}} \text{Acetyl} - \text{CoA} + \text{AMP} + \text{Pyrophosphate} \tag{6.14}$$

$$\text{Acetyl} - \text{CoA} + \text{Oxaloacetate} + \text{H}_2\text{O} \xrightarrow[\text{E.C.4.1.3.7}]{\text{Citrate synthetase}} \text{Citrate} + \text{CoA} \tag{6.15}$$

$$\text{L} - \text{Malate} + \text{NAD}^+ \underset{\text{E.C.1.1.1.37}}{\overset{\text{L-MDH}}{\rightleftharpoons}} \text{Oxaloacetate} + \text{NADH} + \text{H}^+ \tag{6.16}$$

Reaction (6.16) is a preceding indicator reaction; the equilibrium must be considered when the calculations are being made.

Acetate is measured in wine, fruit and vegetable products, cheese, dressings, and vinegar as a check for fermentation.

Ascorbic Acid [50-81-7]. As a vitamin, ascorbic acid is of great biological importance in humans; it is also often used as a food additive in industry, e.g., for fruit and vegetable products. A chemical–enzymatic process (Reaction 6.17) is applied to the quantitative determination of ascorbic acid [1413, vol. VI, pp. 376–385]:

$$\text{L-Ascorbic acid } (\text{XH}_2) + \text{MTT} \xrightarrow{\text{PMS}} \text{Dehydroascorbic acid } (\text{X}) + \text{Formazan}^- + \text{H}^+ \tag{6.17}$$

where MTT = 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide; PMS = 5-methylphenazinium methyl sulfate.

Ascorbate is oxidatively removed in a sample blank reading in order to increase specificity:

$$\text{L} - \text{Ascorbic acid} + 1/2\text{O}_2 \xrightarrow[\text{E.C. 1.10.3.3}]{\text{Ascorbate oxidase}} \text{Dehydroascorbic acid} + \text{H}_2\text{O} \tag{6.18}$$

Ascorbic acid is determined in fruit and vegetable products, meat products, milk, beer, wine, and flour. The vitamin content is often declared.

Dehydroascorbic acid [490-83-5] can be determined by using Reactions (6.17) and (6.18), after it has been chemically oxidized to ascorbic acid [1413, vol. VI, pp. 376–385]:

$$\text{Dehydroascorbic acid} + \text{Dithiothreitol(reduced)} \rightarrow \text{L - Ascorbic acid} + \text{Dithiothreitol (oxidized)} \quad (6.19)$$

Aspartic Acid [56-84-8]. Aspartic acid is estimated primarily in apple juice [1413, vol. VIII, pp. 350–357]:

$$\text{L}-\text{Aspartate} + \alpha-\text{Oxoglutarate} \xrightarrow[\text{E.C. 2.6.1.1}]{\text{GOT}} \text{Oxaloacetate} + \text{L}-\text{Glutamate} \quad (6.20)$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow[\text{E.C. 1.1.1.37}]{\text{L}-\text{MDH}} \text{L}-\text{Malate} + \text{NAD}^+ \quad (6.21)$$

Citric Acid [77-92-9]. Citric acid is a key substance in metabolism. It occurs abundantly in plants and also in milk. It is determined by using Reactions (6.22) and (6.21) [1413, vol. VII, pp. 2–12]:

$$\text{Citrate} \xrightarrow[\text{E.C.4.1.3.6}]{\text{Citrate}(\textit{pro}-35)-\text{lyase}} \text{Oxaloacetate} + \text{Acetate} \quad (6.22)$$

The decarboxylation of oxaloacetic acid to pyruvic acid can occur either chemically or enzymatically (oxaloacetate decarboxylase present as an impurity in citrate lyase). However, loss of oxaloacetate does not lead to inaccurate results because the pyruvate formed is also estimated by using the L-lactate dehydrogenase reaction (6.38).

Fruit and vegetable products, bread, cheese, meat products, beverages, wine, tea, and confectionery are all analyzed for citric acid.

Formic Acid [64-18-6]. An end product of the metabolism of bacteria and fungi, formic acid is employed as a preservative in a variety of foods. However, the use of this additive must comply with the law. Formic acid is determined according to [1413, vol. VI, pp. 668–672]:

$$\text{Formate} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow[\text{E.C. 1.2.1.2}]{\text{Formate dehydrogenase}} \text{Hydrogen carbonate} + \text{NADH} + \text{H}^+ \quad (6.23)$$

Gluconic Acid [526-95-4]. Oxidation of glucose yields gluconic acid. δ-Gluconolactone is employed as a ripening agent in the production of sausages. The enzyme gluconate kinase is used to determine gluconic acid [1413, vol. VI, pp. 220–227]:

$$\text{D}-\text{Gluconate} + \text{ATP} \xrightarrow[\text{E.C. 2.7.1.12}]{\text{Gluconate kinase}} \text{D}-\text{Gluconate 6}-\text{phosohate} + \text{ADP} \quad (6.24)$$

$$\text{D}-\text{Gluconate 6}-\text{phosphate} + \text{NADP}^+ \xrightarrow[\text{E.C. 1.1.1.44}]{6-\text{PGDH}} \text{D}-\text{Ribulose 5}-\text{phosphate} + \text{NADPH} + \text{H}^+ + \text{CO}_2 \quad (6.25)$$

where 6-PGDH = 6-phosphogluconic acid dehydrogenase.

δ-Gluconolactone is also determined by using Reactions (6.24) and (6.25) after conversion to gluconic acid by alkaline hydrolysis (pH 10–11):

$$\text{D-Glucono-}\delta\text{-lactone} + \text{H}_2\text{O} \rightarrow \text{D-Gluconate} \quad (6.26)$$

Glutamic Acid [6899-05-4]. The enzyme glutamate dehydrogenase is applied to the quantitative determination of glutamic acid [1413, vol. VIII, pp. 369–376]:

$$\text{L}-\text{Glutamate} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow[\text{E.C. 1.4.1.3}]{\text{Glutamate dehydrogenase}} \alpha-\text{Oxoglutarate} + \text{NADH} + \text{NH}_4^+ \quad (6.27)$$

The equilibrium of this reaction strongly favors the reductive synthesis of glutamate. Originally, hydrazine was used to trap α-oxoglutarate to achieve quantitative conversion. This is more conveniently carried out today by using the enzyme diaphorase and iodonitrotetrazolium chloride (INT):

$$\text{NADH} + \text{H}^+ + \text{INT} \xrightarrow[\text{E.C. 1.8.1.4}]{\text{Diaphorase}} \text{NAD}^+ + \text{Formazan} \quad (6.28)$$

This color reaction is very sensitive; all reducing substances in the sample must be removed during its preparation to avoid interference.

3-Hydroxybutyric Acid [300-85-6]. The presence of hydroxybutyric acid in an egg indicates that fertilization has taken place and that the egg has been incubated for more than 6 d. Hydroxybutyric acid is estimated by using the following reaction:

$$\text{D-3-Hydroxybutyrate} + \text{NAD}^+ \xrightarrow[\text{E.C. 1.1.1.30}]{3-\text{HBDH}} \text{Acetoacetate} + \text{NADH} + \text{H}^+ \quad (6.29)$$

where 3-HBDH = 3-hydroxybutyrate dehydrogenase.

To ensure that this reaction is quantitative and to increase sensitivity, Reaction (6.29) is coupled to the color reaction (6.28).

Isocitric Acid [6061-97-8]. The determination of isocitric acid, an intermediate in the citric acid cycle, is of considerable importance for the analysis of fruit juice. The ratio of citrate to isocitrate is very constant; hence, analysis of isocitric acid can be used to show if citric acid has been added to the juice (adulteration). Isocitrate dehydrogenase is used to measure this acid quantitatively [1413 vol, VII, pp. 13–19]:

$$\text{D}-\text{Isocitrate} + \text{NADP}^+ \xrightarrow[\text{E.C. 1.1.1.42}]{\text{Isocitrate dehydrogenase}} \alpha-\text{Oxoglutarate} + \text{NADPH} + \text{CO}_2 + \text{H}^+ \quad (6.30)$$

The lactone or esters of isocitrate are first subjected to alkaline hydrolysis (Reaction 6.31) and then determined by using Reaction (6.30):

$$\text{D} - \text{Isocitric acid lactone (esters)} + \text{H}_2\text{O} \xrightarrow{\text{pH9–10}} \text{Isocitric acid (+alcohol)} \tag{6.31}$$

Isocitric acid is too expensive to be used to adulterate citrus fruit juices.

Lactic Acid [598-82-3]. One of the most important examples of the use of enzymes in food analysis is the enzymatic determination of the stereoisomeric forms of lactic acid. Only L-(+)-lactate is found in animals; it accumulates as the end product of glycolysis, e.g., in muscle. D-(−)-Lactate is formed by certain types of Lactobacilli. The analysis is carried out by using the following reactions [1413, vol. VI, pp. 582–592]:

$$\text{L}-\text{Lactate} + \text{NAD}^+ \xrightarrow[\text{E.C. 1.1.1.27}]{\text{L–LDH}} \text{Pyruvate} + \text{NADH} + \text{H}^+ \tag{6.32}$$

$$\text{D}-\text{Lactate} + \text{NAD}^+ \xrightarrow[\text{E.C. 1.1.1.28}]{\text{D–LDH}} \text{Pyruvate} + \text{NADH} + \text{H}^+ \tag{6.33}$$

The equilibria of the lactate dehydrogenase reactions favor formation of lactic acid. Originally, hydrazine was used to trap pyruvate in order to achieve quantitative conversion. Today, use of an enzymatic reaction, such as the following, is more convenient:

$$\text{Pyruvate} + \text{L}-\text{Glutamate} \xrightarrow[\text{E.C. 2.6.1.2}]{\text{GPT}} \text{L}-\text{Alanine} + \alpha-\text{Oxoglutarate} \tag{6.34}$$

L-Lactate is determined in fruit and vegetable products, meat additives, and baking agents. Both D- and L-lactate are measured in such milk products as yogurt and cheese as a check for microbial activity, and in wine and beer where the lactate content correlates with sensory results. L-Lactate can also be measured with an enzyme biosensor.

Malic Acid [6915-15-7]. L-Malic acid, an intermediate in the tricarboxylic acid (TCA) cycle, is found in fruit (e.g., grapes) and vegetables. In analytical determination, it is oxidized in the presence of malate dehydrogenase and NAD^+ to yield oxaloacetic acid: the generated oxaloacetic acid is no longer trapped with hydrazine, but with an enzymatic reaction [1413, vol. VII, pp. 39–47]:

$$\text{L}-\text{Malate} + \text{NAD}^+ \xrightarrow[\text{E.C.1.1.37}]{\text{L–MDH}} \text{Oxaloacetate} + \text{NADH} + \text{H}^+ \tag{6.35}$$

$$\text{Oxaloacetate} + \text{L}-\text{Glutamate} \xrightarrow[\text{E.C.2.6.1.1.1}]{\text{GOT}} \text{L}-\text{Aspartate} + \alpha-\text{Oxoglutarate} \tag{6.36}$$

L-Malic acid is analyzed in fruit products and wine. In comparison with chemical procedures which determine total malic acid content only, enzymatic methods can detect even small amounts of cheap D,L-malic acid added to the product. An enzymatic reaction for the determination of D-malate has been described in the literature, but the required enzyme is not available on the market.

Oxalic Acid [144-62-7]. More than half of all kidney stones are composed of calcium oxalate. Oxalic acid is also important because it influences the absorption of calcium in the intestine. It is determined by using Reactions (6.37) and (6.23) [1414, vol. 3, pp. 1551–1555]:

$$\text{Oxalate} \xrightarrow[\text{E.C.4.1.1.2}]{\text{Oxalate decarboxylase}} \text{Formate} + CO_2 \tag{6.37}$$

Oxalic acid is determined in beer (calcium oxalate is responsible for the Gushing effect), fruit, vegetables, and cocoa products. The oxalate oxidase method [1413, vol. VI, pp. 649–656] is not recommended for food analysis because too many factors interfere with the reaction.

Pyruvic Acid [127-17-3] is a key intermediate in metabolism. It is determined by using the following reaction [1413, vol. VI, pp. 570–577]:

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow[\text{E.C.1.1.1.27}]{\text{L-LDH}} \text{L-Lactate} + NAD^+ \tag{6.38}$$

Wine is analyzed for pyruvic acid, which is an SO_2-binding component. In evaluating the quality of raw milk (and pasteurized milk), the determination of pyruvate is used as an alternative to microbial counting. Another enzyme, pyruvate oxidase, is now available for detection of pyruvate by means of the hydrogen peroxide generated.

Succinic Acid [110-15-6]. Succinic acid is also an intermediate in the citric acid cycle; it is determined by using Reactions (6.39), (6.40), and (6.38) [1413, vol. VII, pp. 25–33]:

$$\text{Succinate} + \text{ITP} + \text{CoA} \xrightarrow[\text{E.C.6.2.1.4}]{\text{Succinyl-CoA synthetase}} \text{IDP} + \text{Succinyl} - \text{CoA} + p_1 \tag{6.39}$$

$$\text{IDP} + \text{PEP} \xrightarrow[\text{E.C.2.7.1.40}]{\text{Pyruvate kinase}} \text{ITP} + \text{Pyruvate} \tag{6.40}$$

Fruits and fruit products are analyzed for succinic acid, which indicates the degree of ripeness. The presence of this acid in whole eggs is a sign of microbial activity following contamination. Other items sampled are cheese, soybean products, and wine.

6.4.3 Alcohols

Enzymes are also used for the analytical determination of alcohol, such as ethanol, glycerol, the sugar alcohols sorbitol and xylitol, or cholesterol, in food.

Ethanol [64-17-5]. Anaerobic metabolism by many microorganisms, notably yeast, results in the formation of ethanol. Reaction (6.41), employed in the determination of ethanol, was one of the first routinely used enzymatic analyses. The acetaldehyde

generated was originally trapped with semicarbazide. However, its enzymatic oxidation (Reaction 6.50) is quicker and more efficient [1413, vol. VI, pp. 598–606]:

$$\text{Ethanol} + \text{NAD}^+ \xrightarrow[\text{E.C.1.1.1.1}]{\text{ADH}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+ \tag{6.41}$$

The ethanol concentration serves as an index of the quality of alcoholic drinks, such as wine, champagne, spirits, and beer. The ethanol content of products made from fruit indicates either the use of spoiled raw materials or the presence of yeast (e.g., in the case of kefir). The maximum ethanol concentration in drinks that are classified as ''low in alcohol'' or ''nonalcoholic'' has been established by law. The commercially available alcohol oxidase (E.C. 1.1.3.13) produces hydrogen peroxide but is rather unspecific for ethanol.

Glycerol [56-81-5] is widely distributed in nature, the bulk of it being bound as lipid. Glycerol is determined by using Reactions (6.42), (6.43), and (6.38) [1414, vol. IV, pp. 1825–1831]:

$$\text{Glycerol} + \text{ATP} \xrightarrow[\text{E.C.2.7.1.30}]{\text{Glycerol kinase}} \text{Glycerol 3}-\text{phosphate} + \text{ADP} \tag{6.42}$$

$$\text{ADP} + \text{PEP} \xrightarrow[\text{E.C.2.7.1.4.0}]{\text{Pyruvate kinase}} \text{ATP} + \text{Pyruvate} \tag{6.43}$$

The determination of glycerol in wine is of considerable importance because the ratios of ethanol to glycerol and of glycerol to gluconic acid indicate immediately whether the wine has been adulterated by adding glycerol. Beer, spirits, and marzipan are also analyzed for glycerol.

Sugar Alcohols *Sorbitol* [50-70-4] is obtained by reduction of fructose; it is of interest as a sugar substitute for people suffering from diabetes. The enzymatic determination of sorbitol can be carried out by using the following reaction, which is not specific, because the enzyme also acts on other polyols [1413, vol. VI, pp. 356–362]:

$$\text{D}-\text{Sorbitol} + \text{NAD}^+ \xrightarrow[\text{E.C.1.1.1.14}]{\text{Sorbitol dehydrogenase}} \text{D}-\text{Fructose} + \text{NADH} + \text{H}^+ \tag{6.44}$$

This analysis can be made specific for sorbitol by quantitatively measuring the fructose produced, via Reactions (6.4), (6.5), and (6.3). Another possibility is to couple Reaction (6.44) to Reaction (6.28). Under these conditions, *xylitol* [87-99-0], which also takes part in the sorbitol dehydrogenase reaction, can be measured as well [1413, vol. VI, pp. 484–490]:

$$\text{Xylitol} + \text{NAD}^+ \xrightarrow[\text{E.C.1.1.1.14}]{\text{Sorbitol dehydrogenase}} \text{Xylulose} + \text{NADH} + \text{H}^+ \tag{6.45}$$

The difference between the quantitative determination of sorbitol via fructose and via INT–diaphorase gives the amount of xylitol present in the sample. Diet foods, pomaceous fruit products, ice cream, confectionery, and biscuits are analyzed for sorbitol. Xylitol is estimated in diet foods, chewing gum, and confectionery.

6.4.4 Other Food Ingredients

Cholesterol [57-88-5] is an important steroid with diverse physiological functions; e.g., it is a component of the plasma membrane. At the same time, excessive deposition of cholesterol in vascular tissue leads to atherosclerosis. Cholesterol is determined according to Reaction (6.46) coupled to Reactions (6.47) and (6.48) [1413, vol. VIII, pp. 139–148]:

$$\text{Cholesterol} + O_2 \xrightarrow[\text{E.C.1.1.3.6}]{\text{Cholesterol oxidase}} \Delta^4\text{–Cholestenone} + H_2O_2 \tag{6.46}$$

$$H_2O_2 + \text{Methanol} \xrightarrow[\text{E.C.1.11.1.6}]{\text{Catalase}} \text{Formaldehyde} + 2H_2O \tag{6.47}$$

$$\text{Formaldehyde} + NH_4^+ + 2\,\text{Acetylacetone} \rightarrow \text{Lutidine dye} + 3H_2O \tag{6.48}$$

Cholesterol is used as a measure of the egg content of food, e.g., noodles and eggnog. In general, the cholesterol content of food made from animal fats is very important. When food containing plant materials is analyzed for cholesterol, phytosterols with a 3 β-hydroxyl group (other than lanosterol) interfere with the reaction.

Triglycerides an important group of lipids containing glycerol, are normally determined in clinical chemistry. Low-fat food is also analyzed enzymatically for triglycerides, which are hydrolyzed with esterase or lipase to yield fatty acids and glycerol (Reaction 6.49). The glycerol generated is determined by Reactions (6.42), (6.43), and (6.38) [1414, vol. IV, pp. 1831–1835]:

$$\text{Triglyceride} + 3H_2O \xrightarrow[\text{E.C.3.1.1.1/E.C.3.1.1.3}]{\text{Esterase and lipase}} \text{Glycerol} + 3\,\text{Fatty acids} \tag{6.49}$$

Acetaldehyde [75-07-0] is a flavor substance in beer, yogurt, and spirits. It is bound by SO_2 in wine. Acetaldehyde dehydrogenase linked to NAD is used to determine acetaldehyde [1413, vol. VI, pp. 606–613]:

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow[\text{E.C.1.2.1.5}]{\text{Aldehyde dehydrogenase}} \text{Acetic acid} + NADH + H^+ \tag{6.50}$$

Ammonia [7664-41-7]. The simplest compound containing nitrogen and hydrogen is ammonia. Fruit juice, milk, biscuits, cheese, diet food, and meat products are analyzed for ammonia [1413, vol. VIII, pp. 454–461]:

$$\alpha\text{-Oxoglutarate} + NADH + H^+ + NH_4^+ \xrightarrow[\text{E.C.1.4.1.3}]{\text{Glutamate dehydrogenase}} \text{L-Glutamate} + NAD^+ + H_2O \tag{6.51}$$

Nitrate The determination of nitrate by use of a stable enzyme was described for the first time in 1986:

$$\text{Nitrate} + \text{NADPH} + \text{H}^+ \xrightarrow[\text{E.C.1.6.6.2}]{\text{Nitrate reductase}} \text{Nitrite} + \text{NADP}^+ + \text{H}_2\text{O} \tag{6.52}$$

The determination of nitrate is of great importance. This compound is the precursor of nitrite and nitrosamines; hence, it is considered hazardous. Samples of water, beverages, meat, milk products, fruits and vegetables, and baby food are among the items analyzed for nitrate. Indeed, fertilizers, which are primarily responsible for the nitrate in food, are also subject to analysis.

Sulfite The enzymatic determination of sulfite was first described in 1983 [1413, vol. VII, pp. 585–591]:

$$\text{SO}_3^{2-} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow[\text{E.C.1.8.3.1}]{\text{Sulfite oxidase}} \text{SO}_4^{2-} + \text{H}_2\text{O}_2 \tag{6.53}$$

$$\text{H}_2\text{O}_2 + \text{NADH} + \text{H}^+ \xrightarrow[\text{E.C.1.11.1.1}]{\text{NADH peroxidase}} 2\text{H}_2\text{O} + \text{NAD}^+ \tag{6.54}$$

Sulfite is often used as a preservative in food technology and has diverse functions; e.g., it inactivates enzymes, prevents browning, and binds acetaldehyde in wine. Usually, too high concentrations are obtained when sulfur-containing samples, such as cabbage, leek, onion, garlic, and horseradish, are analyzed by either conventional distillation or the enzymatic procedure. However, use of the enzymatic method in wine analysis gives accurate results.

Creatine and Creatinine Both creatine [57-00-1] and creatinine [60-27-5] are found in muscle. The amount of meat contained in soup, sauces, and meat extracts is determined by analyzing these foods for creatine and creatinine (Reactions 6.55, 6.56, 6.43, and 6.38) [1413, vol. VIII, pp. 488–507]:

$$\text{Creatinine} + \text{H}_2\text{O} \xrightarrow[\text{E.C.3.5.2.10}]{\text{Creatininase}} \text{Creatine} \tag{6.55}$$

$$\text{Creatine} + \text{ATP} \xrightarrow[\text{E.C.2.7.3.2}]{\text{Creatine kinase}} \text{Creatine phosphate} + \text{ADP} \tag{6.56}$$

Lecithin [8002-43-5]. phosphatidylcholine) is the most important phospholipid occurring in plants and animals. The plural term lecithins refers to emulsifying agents, such as soybean preparations (soy lecithin contains 18–20 % phosphatidylcholine). Lecithin can be determined by using phospholipase D, which catalyzes its hydrolytic cleavage to form choline. The choline generated is subsequently measured as Reinecke salt. The method of choice, however, involves the use of phospholipase C from *Bacillus cereus* and of alkaline phosphatase to hydrolyze lecithin (Reactions 6.57 and 6.58), followed by heat

inactivation of alkaline phosphatase and determination of choline (Reactions 6.59, 6.43, and 6.38) [1413, vol. VIII, pp. 87–104]:

$$\text{Lecithin} + H_2O \xrightarrow[\text{E.C.3.1.4.3}]{\text{Phospholipase C}} 1,2-\text{Diglyceride} + \text{Phosphorylcholine} \tag{6.57}$$

$$\text{Phosphorylcholine} + H_2O \xrightarrow[\text{E.C.3.1.3.1}]{\text{Alkaline phosphatase}} \text{Choline} + p_1 \tag{6.58}$$

$$\text{Choline} + \text{ATP} \xrightarrow[\text{E.C.2.7.1.32}]{\text{Choline kis}} \text{Phosphorylcholine} + \text{ADP} \tag{6.59}$$

The sample can be prepared for analysis in various ways. An aqueous suspension can be made by ultrasonic treatment, or a sample solution can be prepared in *tert*-butyl alcohol and water (e.g., egg products, like eggnog). Alternatively, the sample can be dissolved in *tert*-butyl alcohol after extraction with organic solvents, or it can be prepared by alkaline hydrolysis with methanolic potassium hydroxide (e.g., cocoa products). Interpretation of the results can cause further problems, especially if acetone-soluble phosphorus compounds are used as comparison and if no difference is made between ''lecithin'' and ''lecithins''.

Urea [57-13-6] is the most important end product of protein metabolism, most of it being formed in the liver from ammonia. Urea is excreted via the kidneys. The enzyme urease is used for determination of urea (Reactions 6.60 and 6.51) [1413, vol. VIII, pp. 444–449]:

$$\text{Urea} + H_2O \xrightarrow[\text{E.C.3.5.1.5}]{\text{Urease}} 2NH_3 + CO_2 \tag{6.60}$$

Samples analyzed for urea are meat products and milk, where it serves as an index of protein in the diet of the cow. The urea content of wastewater and swimming pool water indicates the urine concentration in these waters.

6.5
Enzymes in Genetic Engineering [1418–1478]

Cloning techniques provide the basis for recombinant DNA technology and its applications in genetic engineering (Figs. 98 and 99). The term ''genetic engineering'' includes methods for the formation of new combinations of genetic material and for reintroducing and multiplying the recombinant nucleic acid molecules in a new and nonnatural environment.

The availability of a large variety of class II restriction endonucleases with different sequence specificities is a prerequisite for recombinant DNA technology (Section 6.5.1). *Analytical applications* of this enzyme class can be classified roughly as follows (see Fig. 99):

1. Molecular analysis of chromosome and genome structure (mapping, degree of methylation),
2. Characterization of phenotypically manifest or latent hereditary genetic defects on the DNA level (polymorphism analysis),

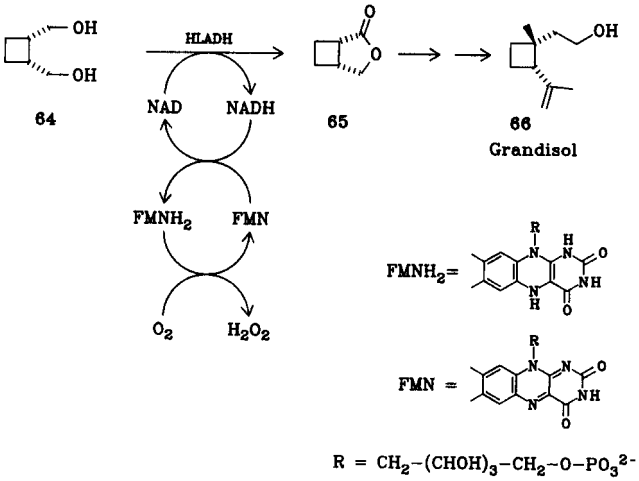


Fig. 98 Horse liver alcohol dehydrogenase (HLADH) catalyzed stereoselective oxidation of a meso diol with in situ NAD regeneration

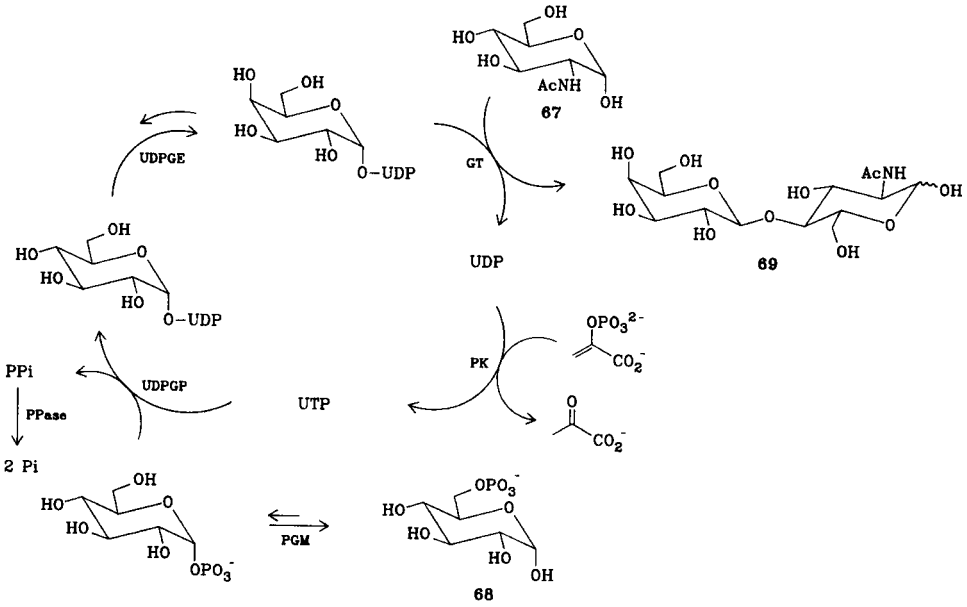


Fig. 99 Enzymatic synthesis of lactosamine.
GT = galactosyltransferase; PK = pyruvate kinase; PGM = phosphoglucomutase; UDPGP = UDP–glucose pyrophosphorylase; UDPGE = UDP–glucose epimerase; PPase = inorganic pyrophosphatase

3. Evidence of cell degeneration at an early stage by alteration of certain restriction sites in oncogene sequences,
4. Taxonomy of viruses or other disease-causing organisms by the correlation of characteristic fragment patterns,
5. Phylogenetic relationships by comparison of selected restriction sites in essential genes such as the hemoglobin gene.

In addition, DNA- and RNA-modifying enzymes are essential tools for *cloning technology*; these enzymes are discussed in Sections 6.5.2, Analytical Applications of Enzymes, Enzymes for Food Analysis, Enzymes in Genetic Engineering, Safe Handling of Enzymes.

Cloning technology allows the identical multiplication of specific genes and fragments thereof to high copy numbers. From these overproducing cells — the clones — the coding DNA sequences and, after expression, the corresponding proteins can subsequently be isolated in large quantities and high purity.

The starting materials for gene cloning are first of all complementary mRNA molecules which are being retro-transcribed by the retroviral enzyme reverse transcriptase (E.C. 2.7.7.49) into complementary double-stranded DNA molecules. The starting material may also be the structural gene itself which must first be isolated from the genome, i.e., the entire chromosomal DNA. Furthermore, in addition to the structural gene, essential *regulatory elements* must be available. Isolation of both specific gene fragments and regulatory sequences is achieved by fragmentation of the DNA with sequence-specific class II restriction endonucleases. This requires that the structural gene and the regulatory elements can be excised from the chromosome at exact nucleotide positions.

The cDNA or the fragments obtained by restriction endonucleases can then be modified further by DNA-modifying enzymes such as exonucleases, kinases, or phosphatases. The fragment ends can also be altered in their sequence specificity by attachment of synthetic oligonucleotides, i.e., *linkers* or *adapters*.

With the help of T 4 DNA ligase, the isolated fragments are inserted into special cloning vehicles, the *vectors*. These vectors may, for example, be extrachromosomal circular plasmids (e.g., pBR 322), appropriate derivatives of specific bacteriophages (e.g., λ, M 13), or eukaryotic viruses (e.g., SV 40).

The chimeric molecules obtained in this way, i.e., the recombinant DNA, are then inserted into new host cells, such as *Escherichia coli* bacteria which are easy to cultivate. All vectors carry the *origin element ori*, a DNA sequence for the autonomous replication of the DNA hybrid. The recombinant DNA is thus able to amplify to high copy numbers in transformed cells irrespective of the regular cell cycle. Growth of the host cells under selective conditions, e.g., on culture media containing specific antibiotics, ensures that only transformed bacteria replicate. Bacterial cells that do not take up recombinant DNA molecules die after a short period in such selective media. In this way, only transformed cells are able to grow, and the introduced vectors, and thus the inserted DNA fragments, are selectively amplified to high copy numbers. This is referred to as *cloning* because under selective growth conditions, all bacteria can be traced back to a homogeneous population of identically transformed cells, the cell clone.

Large quantities of identical recombinant DNA molecules can be purified easily from the cultivated bacteria. Subsequently, the inserted DNA fragment can again be excised from the vector with the help of the initially used class II restriction endonucleases. This DNA fragment that contains the gene to be cloned can be characterized, e.g., by physical mapping and sequencing.

In the final stage, the cloned DNA fragment is inserted into efficient expression vectors which have been provided with regulatory control elements. After renewed introduction into appropriate host cells, the regulatory elements are triggered by growth conditions in such a way that primarily the cloned genes are transcribed into complementary mRNA. In prokaryotes, the synthesized mRNA is directly translated into colinear proteins by the ribosomes. In eukaryotes, the mRNA is first synthesized as a precursor mRNA which is processed into mature mRNA by splicing. The structure and sequence of the various RNA species can be analyzed by using RNA-modifying enzymes. As in prokaryotes, the mature mRNA is then translated into colinear protein. Often the overproduced protein is synthesized in such high concentrations that it precipitates in the cell and can, therefore, be isolated from the cell mass in high yield and with high purity.

6.5.1
Restriction Endonucleases and Methylases [1479–1486]

6.5.1.1 Classification

In the search for enzymes that are the basis of restriction modification systems [1487], [1488], class I enzymes were discovered in 1968 [1489], [1490]. The first member of a class II restriction endonuclease, *Hin*dII, was found in 1970 [1491], [1492]. Later, enzymes of class III were also isolated [1493], which have properties between those of class I and class II. A selection of important class II restriction endonucleases and methylases is given in Table 43.

Class I enzymes are coded by the three gene segments *hsd*S, *hsd*R, and *hsd*M, expressing the functions for sequence specificity, restriction, and modification methylation. The corresponding endonucleases and DNA methyltransferases are enzyme complexes of high molecular mass. These enzyme complexes require magnesium ions, ATP, and SAM as essential cofactors. Class I enzymes bind to and methylate specific sequences, but cut unspecifically after translocation into the 3′-direction [1465], [1574–1576]. An example of a class I system is the *Eco*B restriction — modification system. The early steps of SAM-binding, i.e., enzyme activation and formation of the initial enzyme–DNA complex, are common to both restriction and modification; however, these reaction pathways diverge after the stable complex has been formed. In the case of recognition, ATP leads to the release of the enzyme from modified DNA; it stimulates translocation in unmodified recognition complexes. This reaction is coupled to ATP hydrolysis. Sequential cleavage of both DNA strands occurs in a limited sequence stretch some 100 to 1000 base pairs away from the binding site.

Class II enzymes are expressed as two single proteins from two different genes acting as separate enzymes. Class II endonucleases hydrolyze both DNA strands at specific

Table 43. Recognition sequences of important class II restriction endonucleases and methylases

Position no.	Recognition sequence	Enzyme	Number of recognition sites λ	Ad2	SV 40	ΦX 174	M 13mp7	pBR 322	pBR 328	Microorganism	References
A. Class II enzymes with palindromic recognition sequences											
A.1. Tetra-, hexa-, or octanucleotide recognition sequences											
A.1.1. Internal AT palindriomes											
1	AATT	–	188	0	39	25	65	8	13		–
2	AAATTT	–	16	13	4	5	3	0	0		–
3	$G/\overset{0}{A}\overset{+}{A}TT\overset{+}{C}$	*Eco*RI	5	5	1	0	2	1	1	*Escherichia coli* RY 13	[1441, 1534, 1554]
	$G\overset{M}{A}ATTC$	M · *Eco*RI	5	5	1	0	2	1	1	*Escherichia coli* RY 13	[1534, 1535]
4	CAATTG	–	8	4	4	1	0	0	0		–
5	TAATTA	–	8	4	2	1	3	0	0		–
6	$\overset{m}{G}A/TC$	*Dpn*I	116	87	8	0	8	22	27	*Diplococcus pneumoniae*	[1606, 1614]
	$/\overset{+}{G}A\overset{0}{T}C$	*Mbo*I	116	87	8	0	8	22	27	*Moraxella bovis*	[1644, 1645]
	$\overset{+}{G}ATC$	*Nde*II	116	87	8	0	8	22	27	*Neisseria denitrificans*	[1450, 1455]
	$/\overset{0}{G}A\overset{+}{T}C$	*Sau*3AI	116	87	8	0	8	22	27	*Staphylococcus aureus*	[1518, 1644]
	$\overset{M}{G}ATC$	M · *Eco dam*	116	87	8	0	8	22	27	*Escherichia coli* HB 101	[1514, 1585, 1646]
7	$A/\overset{0}{G}A\overset{+}{T}CT$	*Bgl*II	6	11	0	0	1	0	0	*Bacillus globigii*	[1532, 1536]
8	$G/\overset{0}{G}A\overset{+0}{T}CC$	*Bam*HI	5	3	1	0	2	1	1	*Bacillus amyloliquefaciens* H	[1460, 1512]
	$GGATC\overset{M}{C}$	M · *Bam*HI	5	3	1	0	2	1	1	*Bacillus amyloliquefaciens* RUB 500	[1551, 1543]
	$\binom{A}{G}/GATC\binom{T}{C}$	*Xho*II	21	22	3	0	4	8	7	*Xanthomonas holcicola*	[1530]

9	$\overset{0}{\text{CGAT}}/\overset{+}{\text{CG}}$	*Pvu*I	3	7	0	0	1	1	1	*Proteus vulgaris*	[1560]
10	$\overset{+}{\text{T}}/\text{GA}\overset{0}{\text{TCA}}$	*Bcl*I	8	5	1	0	0	0	1	*Bacillus caldolyticus*	[1612]
11	CATG/	*Nla*III	181	183	16	22	15	26	27	*Neisseria lactamica*	[1568]
12	ACATGT	–	2	9	0	0	3	1	0		–
13	$\text{GCATG}/\overset{0}{\text{C}}$	*Sph*I	6	8	2	0	0	1	1	*Streptomyces phaeochromogenes*	[1507, 1904]
14	$\overset{+}{\text{C}}/\text{CATGG}$	*Nco*I	4	20	3	0	0	0	1	*Nocardia corallina*	[1508]
	$\text{C/C}\binom{\text{A}}{\text{T}}\binom{\text{T}}{\text{A}}\text{GG}$	*Sty*I	10	44	8	0	0	1	2	*Escherichia coli* KM201 [pST27 *hsd*, S-a]	[1550]
15	TCATGA	–	8	3	2	3	1	4	4		–
16	TATA	–	113	65	17	11	16	8	13		–
17	ATATAT	–	11	3	0	0	4	1	3		–
18	GTATAC	*Sna*I	3	3	0	0	0	1	0	*Sphaerotilus natans* C	[1566]
19	CTATAG	–	0	4	2	1	0	0	0		–
20	TTATAA	–	12	4	3	1	2	0	0		–
A.1.2. Internal GC palindromes											
21	$\text{AG}/\overset{+}{\text{CT}}$	*Alu*I	143	158	34	24	24	16	14	*Arthrobacter luteus*	[1510]
	$\overset{M}{\text{AGCT}}$	M · *Alu*I	143	158	34	24	24	16	14	*Arthrobacter luteus*	[1570]
22	$\overset{+}{\text{A}}/\overset{+}{\text{AGCTT}}$	*Hin*dIII	6	12	6	0	0	1	1	*Haemophilus influenzae* R_d	[1529, 1555, 1674]
23	$\overset{0}{\text{GAGCT}}/\overset{+}{\text{C}}$	*Sac*I	2	16	0	0	0	0	0	*Streptomyces achromogenes*	[1565]
24	$\text{CAG}/\overset{+}{\text{CTG}}$	*Pvu*II	15	24	3	0	3	1	1	*Proteus vulgaris*	[1560]
25	TAGCTA	–	2	4	2	0	3	0	0		–
26	$\text{GG}/\overset{+0}{\text{CC}}$	*Hae*III	149	216	18	11	15	22	30	*Haemophilus aegyptius*	[1556, 1572]
	$\overset{M}{\text{GGCC}}$	M · *Hae*III	149	216	18	11	15	22	30	*Haemophilus aegyptius*	[1647]

(continued)

Table 43. (continued)

Position no.	Recognition sequence	Enzyme	Number of recognition sites λ	Ad2	SV 40	ΦX 174	M 13mp7	pBR 322	pBR 328	Microorganism	References
27	$AGG/\overset{0}{C}CT$	*Stu*I	6	11	7	1	0	0	0	*Streptomyces tubercidicus*	[1522]
28	$GGG\overset{+}{C}C/C$	*Apa*I	1	12	1	0	0	0	0	*Acetobacter pasteurianus* sub. *pateurianus*	[1503]
	$G\begin{pmatrix}A\\G\end{pmatrix}GC\begin{pmatrix}T\\C\end{pmatrix}/C$	*Ban*II	7	57	2	0	1	2	2	*Bacillus aneurinolyticus*	[1497]
29	$C/GG\overset{+}{C}CG$	*Xma*II	2	19	0	0	0	1	2	*Xanthomonas malvacearum*	[1630]
	$GC/GG\overset{+}{C}CGC\overset{0}{}$	*Not*I	0	7	0	0	0	0	0	*Nocardia otitidis-caviarum*	[1631]
	$\begin{pmatrix}T\\C\end{pmatrix}/GG\overset{+}{C}C\begin{pmatrix}A\\T\end{pmatrix}$	*Eae*I	39	70	0	2	3	6	7	*Enterobacter aerogenes*	[1467, 1558]
30	$TGG/\overset{+}{C}CA$	*Bal*I	18	17	0	0	1	1	1	*Brevibacterium albidum*	[1905]
31	CG/CG	*Fnu*DII	157	303	0	14	18	23	24	*Fusobacerium nucleatum* D	[1527]
32	$\overset{0}{A}/CGCGT$	*Mlu*I	7	5	0	2	0	0	0	*Micrococcus luteus*	[1507]
	$A/C\begin{pmatrix}A\\G\end{pmatrix}\begin{pmatrix}T\\C\end{pmatrix}GT$	*Afl*III	20	25	0	2	3	1	0	*Anabaena flosaquae*	[1458]
33	G/CGCGC	*Bss*HII	6	52	0	1	0	0	0	*Bacillus stearotheromphilus* H3	[1508]
34	$\overset{+}{C}CGC/GG$	*Sac*II	4	33	0	1	0	0	0	*Streptomyces achromogenes*	[1565]
35	$TCG/CG\overset{+}{A}$	*Nru*I	5	5	0	2	0	0	1	*Norcadia rubra*	[1665]
36	TGCA		272	206	36	18	15	21	19		
37	ATGCA/T	*Nsi*I	14	9	3	0	0	0	0	*Neisseria sicca*	[1620]
38	G/TGCAC	*Apa*LI	4	7	0	1	1	3	2	*Acetobacter pasteurianus*	[1462]
	$G\begin{pmatrix}A\\T\end{pmatrix}GC^{+}\begin{pmatrix}T\\A\end{pmatrix}/C$	*Hgi*AI	28	38	0	3	2	8	7	*Herpetosiphon giganteus* HP 1023	[1544]

	$G\binom{A}{G}GC^{+}\binom{T}{C}/C$	*Bsp*1286	38	105	4	3	4	10	10	*Bacillus sphaericus*	[1542]
39	$\overset{+}{C}TGC\overset{+}{A}/G$	*Pst*I	28	30	2	1	1	1	1	*Providencia stuartii* 164	[1496, 1545]
	CTGCAG	M · *Pst*I	28	30	2	1	1	1	1	*Providencia stuartii* 164	[1452]
40	TTGCAA	–	13	23	3	2	2	1	1		–
A.1.3. Internal CG palindromes											
41	$A/\overset{+}{C}GT$	*Mae*II	143	83	0	19	22	10	12	*Methanococcus aeolicus* PL-15/H	[1611]
42	AACGTT	–	7	3	0	3	2	4	5		–
43	GACGT/C	*Aat*II	10	3	0	1	0	1	1	*Acetobacter aceti*	[1675]
44	CACGTG	–	3	10	0	0	0	0	0		–
45	TAC/GTA	*Sna*BI	1	0	0	0	1	0	0	*Sphaerotilus natans*	[1553]
46	$\overset{+}{G}C\overset{+}{G}/C$	*Cfo*I	215	375	2	18	25	31	25	*Clostridium fromicoaceticum*	[1564]
	$\overset{+}{G}C\overset{+}{G}/C$	*Hha*I	215	375	2	18	25	31	25	*Haemophilus haemolyticus*	[1511]
	$\overset{M}{G}CGC$	M · *Hha*I	215	375	2	18	25	31	25	*Haemophilus haemolyticus*	[1601]
47	AGC/GCT	*Eco*47III	2	13	1	0	2	4	3	*Escherichia coli* RFL47	[1559]
48	$GG/CGC\overset{0}{C}$	*Nar*I	1	20	0	2	1	4	5	*Nocardia argentiensis*	[1549]
	$\binom{A}{G}G\overset{+}{C}GC/\binom{T}{C}$	*Hae*II	48	76	1	8	6	11	9	*Haemophilus aegyptius*	[1445, 1523]
	$G\binom{A}{G}/C^{+}G\binom{T}{C}C^{+}$	*Aha*II	40	44	0	7	1	6	7	*Aphanothece halophytica*	[1458]
	$G/G\binom{T}{C}\binom{A}{G}CC$	*Ban*I	25	57	1	3	6	9	12	*Bacillus aneurinolyticus*	[1497]
49	CGCGCG	–	1	48	0	0	1	1	0		–
50	TGCGCA	*Fsp*I	15	17	0	1	0	4	3	*Fischerella* species	[1519]
51	$\overset{+}{C}/\overset{+}{C}GG$	*Hpa*II	328	171	1	5	19	26	33	*Haemophilus parainfluenzae*	[1521, 1647]

(continued)

Table 43. (continued)

Position no.	Recognition sequence	Enzyme	Number of recognition sites λ	Ad2	SV 40	ΦX 174	M 13mp7	pBR 322	pBR 328	Microorganism	References
	$\overset{M}{C}CGG$	M · *Hpa*II	328	171	1	5	19	26	33	*Haemophilus parainfluenzae*	[1647]
	$\overset{+}{C}/\overset{0}{C}GC$	*Msp*I	328	171	1	5	19	26	33	*Moraxella* species	[1448]
	$\overset{M}{C}CGG$	M · *Msp*I	328	171	1	5	19	26	33	*Moraxella* species	[1504]
52	ACCGGT	–	13	5	0	0	0	0	1		–
53	$G\overset{+}{C}C/G\overset{+}{G}C$	*Nae*I	1	13	1	0	1	4	6	*Nocardia aerocolonigenes*	[1627]
	$\begin{pmatrix}A\\T\end{pmatrix}CCGG\begin{pmatrix}T\\C\end{pmatrix}$	*Cfr*10I	61	40	1	0	1	7	10	*Citrobacter freundii*	[1515]
54	$C\overset{+}{C}C/GGG$	*Sma*I	3	12	0	0	0	0	0	*Serratia marcescens* S_b	[1516]
	$C/\overset{+0}{C}CGGG$	*Xma*I	3	12	0	0	0	0	0	*Xanthomonas malvacearum*	[1516]
	$C/\begin{pmatrix}0\\T\\C\end{pmatrix}\overset{+}{C}G\begin{pmatrix}+\\A\\G\end{pmatrix}G$	*Ava*I	8	40	0	1	1	1	1	*Anabaena variabilis*	[1541, 1557]
55	T/CCGGA	*Bsp*MII	24	8	0	0	0	1	1	*Bacillus* species M	[1552]
56	$T/\overset{0}{C}G\overset{+}{A}$	*Taq*I	121	50	1	10	14	7	13	*Thermus aquaticus* YTI	[1522]
	$TCG\overset{M}{A}$	M · *Taq*I	121	50	1	10	14	7	13	*Thermus aquaticus* YTI	[1513]
57	$AT/CG\overset{+}{A}T$	*Cla*I	15	2	0	0	2	1	1	*Caryophanon latum* L	[1530]
	$ATCG\overset{M}{A}T$	M · *Cla*I	15	2	0	0	2	1	1	*Caryophanon latum* L	[1666]
58	$G/T\overset{+}{C}G\overset{+}{A}C$	*Sal*I	2	3	0	0	2	1	1	*Streptomyces albus* G	[1619]
	$GT\begin{pmatrix}T\\C\end{pmatrix}/\begin{pmatrix}A\\G\end{pmatrix}^{+}AC$	*Hin*cII	35	25	7	13	2	2	2	*Haemophilus influenzae* R_c	[1530]

	GT$\left(\begin{matrix}T\\C\end{matrix}\right)/\left(\begin{matrix}A\\G\end{matrix}\right)^{+0}$AC	*Hin*dII	35	25	7	13	2	2	2	*Haemophilus influenzae* R_d	[1491, 1492, 1505]
	GT/$\left(\begin{matrix}A\\C\end{matrix}\right)\left(\begin{matrix}T\\G\end{matrix}\right)^{+}$AC	*Acc*I	9	17	1	2	2	2	1	*Acinetobacter calcoaceticus*	[1467]
59	C/$^{+}$TCG^{+}AG	*Xho*I	1	6	0	1	0	0	0	*Xanthomonas holcicola*	[1562]
60	TT/CGAA	*Fsp*II	7	1	0	0	0	0	2	*Ficherella* species	[1519]
A.1.4. Internal TA palindromes											
61	ATAT	–	230	81	21	20	42	9	19		–
62	AAT/ATT	*Ssp*I	20	5	6	1	6	1	2	*Sphaerotilus* species	[1617]
63	$^{+}$GAT/ATC	*Eco*RV	21	9	1	0	0	1	1	*Escherichia coli* J62[pLG74]	[1498, 1509]
64	CA/TATG	*Nde*I	7	2	2	0	3	1	0	*Neisseria denitrificans*	[1454]
65	TATATA	–	4	5	0	0	1	1	1		–
66	GT/AC	*Rsa*I	113	83	12	11	18	3	4	*Rhodopseudomonas sphaeroides*	[1563]
67	AGT/ACT	*Sca*I	5	5	0	0	0	1	2	*Streptomyces caespitosus*	[1571, 1533, 1439]
68	G/GTACC^{+}	*Asp*718	2	8	1	0	0	0	0	*Achromobacter* species 718	[1650]
	GGTACC^{0}/C^{0}	*Kpn*I	2	8	1	0	0	0	0	*Klebsiella pneumoniae* OK8	[1444, 1496]
69	CGTACG	–	1	4	0	2	0	0	0		–
70	TGTACA	–	5	5	2	0	1	0	0		–
71	C/TAG	*Mae*I	13	54	12	3	4	5	4	*Methanococcus aeolicus* PL-15/H	[1611]
72	A/CTAGT	*Spe*I	0	3	0	0	0	0	0	*Sphaerotilus* species	[1548]
73	G/CTAGC	*Nhe*I	1	4	0	0	0	1	1	*Neisseria mucosa*	[1548]
74	C/CTAGG	*Avr*II	2	2	2	0	0	0	0	*Anabaena variabilis* uw	[1548]
75	$^{+}$T/CTAG^{+}A	*Xba*I	1	5	8	0	0	0	0	*Xanthomonas badrii*	[1468]
76	TTAA	–	195	115	47	35	61	15	17		–
77	ATTAAT	–	17	3	3	2	6	1	1		–

(continued)

Table 43. (continued)

Position no.	Recognition sequence	Enzyme	Number of recognition sites λ	Ad2	SV 40	ΦX 174	M 13mp7	pBR 322	pBR 328	Microorganism	References
78	$\overset{+\,0}{\mathrm{GTT/AAC}}$	*Hpa*I	14	6	4	3	0	0	0	*Haemophilus parainfluenzae*	[1521, 1524]
79	C/TTAAG	*Afl*II	3	4	1	2	0	0	0	*Anabaena flos-aquae*	[1458]
80	TTT/AAA	*Dra*I	13	12	12	2	5	3	5	*Deinococcus radiophilus*	[1567]
A.2. Class II enzymes with penta- or heptanucleotide recognition sequences											
81	$\mathrm{G/G}\begin{pmatrix}\mathrm{A}\\ \mathrm{T}\end{pmatrix}\mathrm{CC}^{+}$	*Ava*II	35	73	6	1	1	8	7	*Anabaena variabilis*	[1541, 1659]
	$\begin{pmatrix}\mathrm{A}\\ \mathrm{G}\end{pmatrix}\mathrm{G/G}\begin{pmatrix}\mathrm{A}\\ \mathrm{T}\end{pmatrix}\mathrm{CC}\begin{pmatrix}\mathrm{T}\\ \mathrm{C}\end{pmatrix}$	*Ppu*MI	3	25	1	0	0	2	0	*Pseudomonas putida* M	[1552]
	$\mathrm{CG/G}\begin{pmatrix}\mathrm{A}\\ \mathrm{T}\end{pmatrix}\mathrm{CCG}$	*Rsr*II	5	2	0	0	0	0	0	*Rhodopseudomonas sphaeroides* G30	[1528]
82	$\overset{+\mathrm{m}}{\mathrm{CC}}/\begin{pmatrix}\mathrm{A}\\ \mathrm{T}\end{pmatrix}\mathrm{GG}$	*Apy*I	71	136	17	2	7	6	10	*Arthrobacter pyridinolis*	[1662]
	$\overset{0\,0}{\mathrm{CC}}/\begin{pmatrix}\mathrm{A}\\ \mathrm{T}\end{pmatrix}\mathrm{GG}$	*Bst*NI	71	136	17	2	7	6	10	*Bacillus staerothermophilus*	[1499]
	$/\overset{0+}{\mathrm{CC}}\begin{pmatrix}\mathrm{A}\\ \mathrm{T}\end{pmatrix}\mathrm{GG}$	*Eco*RII	71	136	17	2	7	6	10	*Escherichia coli* R245	[1661, 1501, 1494]
83	$\overset{+}{\mathrm{CC}}/\begin{pmatrix}\mathrm{G}\\ \mathrm{C}\end{pmatrix}\mathrm{GG}$	*Nci*I	114	97	0	1	4	10	10	*Neisseria cinerea*	[1455]
84	$\mathrm{G}/\overset{+}{\mathrm{A}}\mathrm{NT}\overset{+}{\mathrm{C}}$	*Hinf*I	148	72	10	21	26	10	10	*Haemophilus influenzae* R_f	[1573]
85	$\mathrm{G/GN}\overset{++}{\mathrm{CC}}$	*Cfr*131	74	164	11	2	4	15	16	*Citrobacter freundii* RFL13	[1546]
	$\mathrm{G/GN}\overset{++}{\mathrm{CC}}$	*Sau*96I	74	164	11	2	4	15	16	*Staphylococcus aureus* PS96	[1546]
	$\begin{pmatrix}\mathrm{A}\\ \mathrm{G}\end{pmatrix}\mathrm{G/GNCC}^{+}\begin{pmatrix}\mathrm{T}\\ \mathrm{C}\end{pmatrix}$	*Dra*II	3	44	3	0	0	4	2	*Deinococcus radiophilus*	[1632, 1633]

86	$\overset{+}{\text{GC}}$/NGC	*Fnu*4HI	380	411	24	31	15	42	37	*Fusobacterium nucleatum* 4H	[1525]
87	$\overset{0+}{\text{CC}}$NGG	*Scr*FI	185	233	17	3	11	16	20	*Streptococcus cremonis* F	[1628]
88	/GTNAC	*Mae*II	156	118	14	17	25	17	18	*Methanococcus aeolicus* PL-15/H	[1611]
	G/GTNACC	*Bst*EII	13	10	0	0	0	0	0	*Bacillus stearothermophilus* ET	[1613]
89	$\overset{+}{\text{C}}$/TNAG	*Dde*I	104	97	20	14	29	8	9	*Desulfovibrio desulfuricans* Norway	[1564]
	CC/TNAG$\overset{0\,0}{\text{G}}$	*Sau*I	2	7	0	0	1	0	1	*Streptomyces aureofaciens* IKA18/4	[1443]
A.3. Class II enzymes with recognition sequences containing internal $(N)_x$sequences											
90	GGN/NCC	*Nla*IV	82	178	16	6	6	24	26	*Neisseria lactamica*	[1568]
91	CACNNN/GTG	*Dra*III	10	10	1	1	1	0	0	*Deinococcus radiophilus*	[1632, 1633]
92	GAANN/NNTTC	*Asp*700	24	5	0	3	2	2	1	*Achromobacter* species 700	[1506, 1650, 1677]
93	$\overset{+0}{\text{G}}$AANN/NNTTC	*Xmn*I	24	5	0	3	2	2	1	*Xnathomonas manihotis* 7AS1	[1526]
94	$\overset{+0}{\text{G}}$CC$(N)_4$/NGGC	*Bgl*I	29	20	1	0	1	3	5	*Bacillus globigii*	[1447, 1520, 1536]
95	$\overset{+\ +}{\text{CCA}}(N)_5$/NTGG	*Bst*XI	13	10	1	3	0	0	0	*Bacillus stearothermophilus* X1	[1634]
96	$\overset{0}{\text{G}}$GCC$(N)_4$/NGG$\overset{0\,0}{\text{C}}$C	*Sfi*I	0	3	1	0	0	0	0	*Streptomyces fimbriatus*	[1634]
B. Class II enzymes with nonpalindromic recognition sequences											
B.1. Class II enzymes											
97	GAATG-CN/NCTTAC/GN-N	*Bsm*I	46	10	4	3	1	1	3	*Bacillus stearothermophilus* NUB36	[1542]
98	TGACN/N-NGTCN ACTGN-N/NCAGN	*Tth*111I	1	6	0	0	0	2	0	*Thermus thermophilus* 111	[1495]

(continued)

Table 43. (continued)

Position no.	Recognition sequence	Enzyme	Number of recognition sites λ	Ad2	SV 40	ΦX 174	M 13mp7	pBR 322	pBR 328	Microorganism	References
B.2. Class IIS enzymes											
99	$\overset{+}{G}CAGC(N)_8CGTCG(N)_{12}$	*Bbv*I	199	179	22	14	8	21	11	*Bacillus brevis*	[1561]
100	$ACCTGC(N)_4TGGACG(N)_8$	*Bsp*MI	41	39	0	3	4	1	2	*Bacillus* species M	[1552]
101	$\overset{(+)}{G}\,\overset{(+)}{G}ATG(N)_9CCTAC(N)_{13}$	*Fok*I	150	78	11	8	4	12	11	*Flavobacterium okeanokoites*	[1507]
102	$GACG\overset{+}{C}(N)_5CTGCG(N)_{10}$	*Hga*I	102	87	0	14	7	11	12	*Haemophilus gallinarum*	[1677]
103	$GGT\,\overset{(+)}{G}\,\overset{+}{A}(N)_8CCACT(N)_7$	*Hph*I	168	99	4	9	18	12	18	*Haemophilus parahaemolyticus*	[1500]
104	$GAAG\overset{+}{A}(N)_8CTTCN(N)_7$	*Mbo*II	130	113	16	11	12	11	12	*Moraxella bovis*	[1569, 1639, 1645]
105	$CCTC(N)_7GGAG(N)_7$	*Mnl*I	262	397	51	34	61	26	30	*Moraxella nonliquefaciens*	[1466]
106	$GCATC\overset{0}{(}N)_5CGTAG(N)_9$	*Sfa*NI	169	84	6	12	7	22	17	*Streptococcus faecalis*	[1502]

phosphodiester bonds within, or very near, the recognition sequence. Their activity depends only on magnesium ions. The corresponding class II DNA methyltransferases methylate both DNA strands, independent of the presence of the respective nuclease in an SAM-dependent reaction. An example of a class II system is the *Eco*RI restriction and modification system. Class II restriction endonucleases are by far the most important tools for the synthesis of recombinant DNA because of their absolute sequence specificity for both binding and cleavage [1577], [1578], [1579], [1580], [1581], [1582], [1456].

Class III enzymes are also expressed from two genes. However, like class I enzymes, the corresponding endonucleases and DNA methyltransferases form enzyme complexes in which both activities act simultaneously. Class III enzymes recognize specific sequences, but cleave rather specifically 25 to 27 base pairs downstream from this region. The endonuclease activity depends on magnesium ions and ATP, whereas methylation occurs in the presence of SAM [1574], [1583], [1584], [1493], [1576].

Class II restriction endonucleases and methylases are found in all prokaryotic cells [1480], [1482], [1484]. In accordance with the proposals made by SMITH and NATHANS, the various enzymes are abbreviated by taking the first letters of the bacterial name [1585]. The various endonucleases and DNA-methyltransferases occur in almost all genera of the two kingdoms of eu- and archaebacteria. In particular, type II enzymes are known in both gram-positive and gram-negative eubacteria, in cyanobacteria, as well as in thermophilic, halophilic, methanogenic, and sulfur-dependent archaebacteria. In contrast, eukaryotic restriction–modification systems have not been described yet. Specifically cleaving eukaryotic endonucleases, such as the HO-nuclease in yeast or the retroviral endonucleases, have other functions and are characterized by more complex recognition sequences [1586], [1587].

In addition to class II restriction–modification systems of single specificity, a whole series of multiple component systems were discovered to contain several restriction–modification systems of different specificity. Particularly complex systems, which contain up to seven different specificities, are found in certain species of *Nostoc* (*Nsp* 7524I–V) and in *Neisseria gonorrhoeae* (*Ngo*I–VII) [1588], [1589].

Methods for separating the various enzyme species include size fractionation, column chromatography with anion and cation exchangers, and hydrophobic or affinity materials with DNA or DNA-like heparin as ligands [1590], [1591]. At the end of 1986 as many as 634 different class II restriction endonucleases with at least 111 different specificities were known, which have been isolated from 564 different bacterial strains (Table 43). The corresponding restriction methylases have been characterized only for the most important systems. Enzymes are described completely in [1480], [1482], [1484].

The active enzymes of the class II restriction endonucleases consist of protein dimers of identical subunits, whereas the corresponding DNA methyltransferases act as monomeric proteins. The molecular masses under denaturing conditions range from 20×10^3 to 70×10^3 dalton for both class II endonucleases and DNA methyltransferases. The only protein of higher molecular mass known so far is *Bst*I which is about 400×10^3 dalton [1578].

As an example, the *Eco*RI endonuclease monomer is a 277 amino acid protein with a molecular mass of 31 063 dalton, which is processed after translation by removing the terminal *N*-formylmethionine. The corresponding methylase protein consists of 326 amino acids with a molecular mass of 38 048 dalton. This protein is also processed by removing the N-terminal dipeptide fMet-Ala [1594], [1596].

Examples of cloned restriction modification systems are *Bsu*RI from *Bacillus subtilis* [1517], [1445]; *Eco*RI [1592], [1593], [1594], [1595], [1597], [1596]; *Eco*RII [1501]; and *Eco*RV [1598], [1599], [1600] from *Escherichia coli*; *Hha*II from *Haemophilus parainfluenzae* [1601], [1602]; *Pae*R7 from *Pseudomonas aeruginosa* [1603], [1440]; *Pst*I from *Providencia stuartii* [1604], [1453]; and *Pvu*II from *Proteus vulgaris* [1605]. According to gene mapping of the various systems, the proteins for both endonuclease and DNA methyltransferase may be plasmid or chromosomally coded. Further analysis of the two genes shows that they are either arranged in tandem to form transcription units or expressed as independent genes [1594], [1517], [1596], [1445]. When the known protein and genetic sequences of corresponding endonucleases and DNA methyltransferases were compared, no statistically significant sequence homology could be detected [1517], [1596]. Circular dichroism studies also indicate extensive differences in secondary structure [1596]. According to these observations, the two proteins have different evolutionary origins and interact with DNA in different ways.

6.5.1.2 Activity of Class II Restriction Endonucleases

An enzyme unit, U, is normally defined as the amount of protein that completely digests 1 μg of the DNA from bacteriophage λ in 1 h at 37 °C. To analyze the completeness of the reaction, the fragment mixture is separated in 0.5–2 % agarose gels by horizontal or vertical electrophoresis (see Section 2.2.15). The use of λ DNA as the standard test substrate has two advantages. This DNA has 48 502 base pairs and contains cleavage sites for almost all known restriction endonucleases, which allows a comparison of activities [1480], [1607], [1608], [1609]. Because the complete sequence of λ DNA is known, any cleavage pattern can be calculated in advance by computer.

6.5.1.2.1 Reaction Parameters

The activity of *Eco*RI and other class II restriction endonucleases not only depends on the presence of specific recognition sites and essential divalent cations, but also very strongly on such reaction parameters as temperature, ionic strength, and pH [1456]. The activity of class II restriction endonucleases is also frequently stimulated by the presence of reducing agents such as 2-mercaptoethanol or dithiothreitol.

On the plasmid pBR322, *Eco*RI has a temperature optimum of 40 °C. The apparent activation energy for the overall enzymatic reaction is 5.1 kJ/mol. The enzyme is inactivated irreversibly above 45–50 °C [1610]. The optimal pH for a specific *Eco*RI cleavage is 7.5; the optimal NaCl concentration, 150 mmol/L [1461].

The optimal temperature for enzymatic activity often corresponds to the optimal growth temperature of the organism from which the enzyme is isolated [1456]. Accordingly, enzymes such as *Bcl*I, *Bst*EII, *Mae*I–III, or *Taq*I with high temperature optima are isolated from thermophilic organisms [1612], [1613], [1615], [1611]. Enzymes are also known which require higher ionic strength (e.g., *Mae*III with 350 mmol/L NaCl) or higher pH values (e.g., *Bgl*I pH 9.5) for maximal activity [1616], [1611].

6.5.1.2.2 Additional Structural Requirements Influencing Activity

The structure of the substrate also strongly influences the activity of class II restriction endonucleases at individual cleavage sites. Important parameters are:

1. G–C content and base distribution in natural DNA;
2. Size of the DNA and cleavage frequencies;
3. Length and base composition of the flanking sequences;
4. Position of the cleavage sites with respect to each other;
5. DNA tertiary structure;
6. Modification of DNA and protein attachment.

Guanosine–Cytidine Content In natural DNA, the G–C content varies broadly. The DNA from *Micrococcus lysodeikticus* contains 72 % G−C residues, but the DNA from *Euglena gracilis* chloroplasts is composed of only 25 % G−C residues [1906], [1449]. Although this second DNA consists of about 130×10^3 base pairs, there is not a single cleavage site for *Sma*I recognizing only G−C base pairs [CCC/GGG]. Furthermore, the base distribution in natural DNA is nonstatistical. In eukaryotic SV40 DNA, the dinucleotide CpG occurs very rarely [1618]. As a result, the cleavage frequency for such class II restriction endonucleases as *Mae*II, *Cfo*I, *Hpa*I, *Taq*I, or *Fnu*DII, which contain the CpG dinucleotide in their tetranucleotide recognition sequences, is extremely low [1480], [1547]. By contrast, the number of cleavage sites on prokaryotic pBR322 DNA of similar size is about ten times higher, with values like those obtained from enzymes such as *Alu*I or *Hae*III, which have no CpG dinucleotides in their tetranucleotide recognition sequences [1480].

Length of DNA The influence of DNA length can be demonstrated by the *Sal*I cleavage of pBR322 DNA [1619]. The length of this plasmid is only about one-tenth the length of the bacteriophage λ DNA [1621], [1609], [1622]. The λ DNA has only two cleavage sites, but the much smaller pBR322 DNA still has one cleavage site. Because the number of cleavage sites per microgram of DNA increases fivefold with pBR322 DNA, digestion of the same amount of λ DNA with *Sal*I requires about five times more enzyme as compared with the plasmid DNA.

Flanking Sequences The influence of flanking sequences is demonstrated by the observation that short oligonucleotides consisting, for example, only of the hexanucleotide *Eco*RI recognition sequence GAATTC are not cleaved. Even if several hexanucleotides were ligated to longer DNA molecules to stabilize the double strand — which would be completely denatured in the short hexanucleotide at 37 °C — they could not be digested with *Eco*RI. Only if the recognition sequence is extended on both ends by one or more flanking nucleotides the enzyme can exert its activity. However, in these cases, higher quantities of enzyme and longer reaction times are required. Normal reaction rates can be achieved only by further increasing the length of the flanking sequences [1623].

Base Composition of Flanking Sequences The base composition within the flanking sequence distinctly influences the actual cleavage rate at the individual recognition site.

This effect was first observed for *Eco*RI which digests the various sites on λ or Ad2 DNA with rates differing by a factor of ten [1624], [1579]. A similar preferential digestion has also been observed with *Hin*dIII on λ, *Hga*I on Φ X 174, and *Pst*I on plasmid pSM1 DNA [1625], [1626]. In the latter case, significant resistance to cleavage was conferred on G–C-rich flanking regions.

More significant differences in individual cleavage rates were observed on pBR322 DNA with *Nae*I which recognizes the G–C-rich sequence GCC/GGC [1627]. The enzyme *Nae*I cuts pBR322 DNA at four different sites, two of which are digested rapidly and the third 5–10 times more slowly. Complete cleavage at the fourth site, however, proceeds 50 times slower [1628]. Similar slow cleavage sites were also observed for the enzymes *Nar*I [GG/CGCC], *Sac*II [CCG/CGG], and *Xma*III [C/GGCCG] which recognize sequences with alternating G and C residues [1480], [1629], [1630]. In all these examples, the slower reaction rates at particular sites are attributed to the sequences within the flanking regions. However, whether a particular sequence element or an altered DNA conformation is responsible for the strong decrease in cleavage rate is still unknown.

Relative Position of Cleavage Sites A similar decrease in cleavage rate is observed if two or more recognition sites that would be digested readily alone are only a few nucleotides apart from each other. After digestion at one of the two sites, the essential flanking sequences for efficient cleavage at the neighboring position are missing. Therefore, after the first cleavage, the DNA can only be hydrolyzed very slowly at the second site. This is the case for the multiple cloning sites of the M13mp derivatives that are used frequently for sequencing studies. In these polylinker regions, the recognition sequences for several restriction endonucleases are located directly next to each other [1463].

6.5.1.3 Specificity of Class II Restriction Endonucleases

The specificity of the presently known class II restriction endonucleases is defined by three criteria:

1. recognition sequence,
2. position of cleavage site, and
3. influence of methylation.

However, all three characteristics of enzyme specificity are known only for a limited number of enzymes. For many class II restriction endonucleases, only the recognition sequences have so far been determined [1480], [1484].

6.5.1.3.1 Palindromic Recognition Sequences

For the majority of class II enzymes, the recognition sequence on the double-stranded DNA is characterized by a two-fold axis of rotational symmetry. Such symmetric recognition sequences are called palindromes. In a palindromic sequence, a horizontal complementary arrangement exists in addition to the normal base pairing between A–T and G–C. For example, the *Eco*RI recognition sequence is

G/AATT-C
C-TTAA/G

Most palindromic recognition sequences are tetra-, penta-, hexanucleotides, but defined octanucleotides also exist [1631]. In addition, palindromic recognition sequences of extended length exist, e.g., for *Bst*XI, in which only the flanking nucleotides are specifically recognized. In most of these cases, the flanking trinucleotides are unambiguously defined. However, one case of nonequivocally defined flanking trinucleotides is known with *Dra*II [1632], [1633]:

$$\begin{pmatrix} A \\ G \end{pmatrix} G/GNCC \begin{pmatrix} T \\ C \end{pmatrix}$$

An example of an enzyme that recognizes flanking tetranucleotides is *Sfi*I [GGCCNNNN/NGGCC] [1634].

Palindromic recognition sequences can be arranged according to homologies within their recognition sequences (Table 44). In this approach, the sequences are ordered by starting from the shortest central palindromes AT, GC, CG, and TA. Tetranucleotide palindromes are created for each dinucleotide sequence by adding one nucleotide on the left side and its complementary nucleotide on the right side. Repeated application of this cycle generates hexa- and octanucleotide palindromes. Out of the 16 possible tetranucleotides, 11 sequences are covered by corresponding enzymes. For the 64 possible hexanucleotides, 49 enzyme specificities are known [1480].

Table 44. Palindromic recognition sequences of restriction endonucleases

	AT	**GC**	**CG**	**TA**	
A		*Alu*I	*Mae*II		T
G	*Sau*3AI	*Hae*III	*Cfo*I	*Rsa*I	C
C	*Nla*III	*Fnu*DII	*Hpa*II	*Mae*I	G
T			*Taq*I		A
AA		*Hin*dIII		*Ssp*I	TT
GA	*Eco*RI	*Sac*I	*Aat*II	*Eco*RV	TC
CA		*Pvu*II	*Pma*CI	*Nde*I	TG
TA			*Sna*BI		TA
AG	*Bgl*II	*Stu*I	*Eco*47III	*Sca*I	CT
GG	*Bam*HI	*Apa*I	*Nar*I	*Asp*718	CC
CG	*Pvu*I	*Xma*III		*Spl*I	CG
TG	*Bcl*I	*Bal*I	*Mst*I		CA
AC		*Mlu*I		*Spe*I	GT
GC	*Sph*I	*Bss*HII	*Nae*I	*Nhe*I	GC
CC	*Nco*I	*Sac*II	*Sma*I	*Avr*II	GG
TC	*Bsp*HI	*Nru*I	*Bsp*MII	*Xba*I	GA
AT		*Nsi*I	*Cla*I		AT
GT	*Sna*I	*Apa*LI	*Sal*I	*Hpa*I	AC
CT		*Pst*I	*Xho*I	*Afl*II	AG
TT			*Mla*I	*Dra*I	AA
	AT	GC	CG	TA	

Table 45. Cleavage positions of restriction endonucleases and generated fragment ends

Enzyme	Recognition sequence	End produced
*Hae*III	GG/CC CC/GG	blunt end
*Eco*RI	G/AATT-C C-TTAA/G	5′-protruding
*Pst*I	C-TGCA/G G/ACGT-C	3′-protruding
*Not*I	GC/GGCC-GC CG-CCGG/CG	5′-protruding
*Eco*RII	/CC(A/T)GG GG(A/T)CC/	5′-protruding
*Sau*I	CC/TNA-GG GG-ANT/CC	5′-protruding
*Xmn*I	GAANN/NNTTC CTTNN/NNAAG	blunt end
*Sfi*I	GGCCN-NNN/NGGCC CCGGN/NNN-NCCGG	3′-protruding
*Bst*XI	CCAN-NNNN/NTGG GGTN/NNNN-NACC	3′-protruding
*Mnl*I	CCTCNNNNNNN/N GGAGNNNNNNN/N	blunt end
*Hga*I	GACGCNNNNN/NNNNN-N CTGCGNNNNN-NNNNN/N	5′-protruding
*Mbo*II	GAAGANNNNNNN-N/N CTTCTNNNNNNN/N-N	3′-protruding

The cleavage sites for class II restriction endonucleases lie within or directly next to the recognition sequence; they are marked by a slash (/) in Table 45. As in recognition, the positions in the two complementary strands have a two-fold rotational symmetry. Either *blunt* double-stranded ends or *protruding* single-stranded 5′- or 3′-ends are formed. The lengths of the protruding terminal single-stranded regions depend on the position of the cleavage sites within the recognition sequence. Independent of cleavage positions, all class II restriction endonucleases form fragments that have 5′-phosphate and 3′-hydroxyl fragment ends. The only exception known so far is *Nci*I, which produces 3′-phosphorylated fragment termini [1635].

Some class II restriction endonucleases are able to digest DNA · RNA hybrids. The enzymes *Alu*I, *Hae*III, *Hha*I, and *Taq*I cut the DNA strand of the hybrid at the correct recognition sequences [1636].

Sequence-specific digestion of *single-stranded* DNA has also been shown for a number of enzymes. However, like double-stranded DNA, base-paired recognition sequences are necessary for cleavage of single-stranded DNA. Therefore, refolding effects between different recognition sites, which result in transiently formed secondary canonical structures, play a decisive role. The cleavage rates depend mainly on the stability of these double-stranded structures [1637], [1638], [1464].

6.5.1.3.2 Nonpalindromic Recognition Sequences

In addition to class II enzymes that act on palindromic sequences, class II S enzymes like *Fok*I, *Hga*I, *Mbo*II, or *Mnl*I have been found, which recognize nonsymmetric sequences. These enzymes digest the double-stranded DNA at precise distances downstream from their recognition sites [1639], [1640], [1641], [1466].

The spatial separation between recognition and cleavage has been utilized to digest any predetermined sequence in single-stranded DNAs like those of the various M13mp derivatives [1642], [1643]. Universal cleavage is achieved by applying a specifically

designed *adapter*. This synthetic DNA element is constructed from a constant double-stranded domain harboring (1) the recognition sequence of the class II S enzymes and (2) a variable single-stranded domain complementary to the sequence to be cleaved. The linearized single-stranded target DNA can be converted to double-stranded DNA by a Klenow-catalyzed polymerization reaction using the synthetic deoxyoligonucleotide as a primer. By this approach, double-stranded DNA molecules are created that can be cleaved precisely at any position. Thus, the new method permits the design of novel enzyme specificities by combining class II S restriction endonucleases with bivalent DNA adapter oligonucleotides mediating the novel sequence specificities.

6.5.1.3.3 Isoschizomers

Isoschizomers are class II restriction endonucleases that are isolated from various organisms but recognize identical sequences. Isoschizomeric enzymes, however, may have various cleavage sites within the recognition sequence or may show different sensitivity to particular modified bases. The great variability of isoschizomers is known with 36 different enzymes all recognizing the sequence GATC [1480].

In this collection of isoschizomers, well-known examples for enzymes with different methylation sensitivities are *Dpn*I, *Mbo*I, and *Sau*3AI [1644], [1645], [1606]. Whereas *Sau*3AI is insensitive to methylation of the internal adenine residue, *Mbo*I is completely inhibited by this methylation. In contrast, *Dpn*I is fully dependent on this modification because the enzyme does not cleave the unmethylated GATC sequence. The DNA from pBR322, isolated from wild-type *Escherichia coli* cells, is highly methylated at adenosine residues within GATC sequences by *E. coli dam* methylase that specifically recognizes GATC [1646], [1446]. Consequently, *Sau*3AI and *Dpn*I will cleave this DNA, whereas *Mbo*I is unable to digest this substrate.

Another important pair of isoschizomers, *Hpa*II and *Msp*I, shows a different sensitivity with respect to cytosine methylation. Modification of the central cytosine residue within their common tetranucleotide recognition sequence CCGG renders the cleavage site resistant to digestion with *Hpa*II but sensitive to cleavage with *Msp*I [1504], [1647]. This ability to discriminate between methylated and unmethylated cytosine residues is used to detect methylated CpG dinucleotides which are thought to be involved in eukaryotic gene regulation [1648], [1649], [1650].

An example of an isoschizomeric enzyme pair with different cleavage sites is *Asp*718 and *Kpn*I. Both enzymes recognize the sequence GGTACC but cleave between either 5′-terminal guanosine residues or 3′-terminal cytosine residues [1650], [1444]. The 5′-terminal protruding ends of *Asp*718 fragments can be efficiently labeled with T4 polynucleotide kinase, whereas the 3′-ends are suitable substrates for 3′-endlabeling reactions with Klenow enzyme. Labeling of the 3′-protruding ends of *Kpn*I fragments can be obtained via the tailing reaction with terminal transferase.

The most commonly used cloning vehicles often have no restriction sites for particular enzymes. In such cases, cloning can be achieved by the use of enzymes from an enzyme family. Members of an enzyme family all produce identical single-stranded fragment ends, whereas the complete recognition sequences of individual enzymes are different in the flanking nucleotides. The best known enzyme family is the

GATC family, whose members are *Sau*3AI, *Bgl*II, *Bam*HI, *Bcl*I, and *Xho*II [1480]. All these enzymes produce single-stranded ends of the sequence GATC. The respective hexanucleotide recognition sequences are lost after recombination of fragments produced by different enzymes of this family, but the common internal nucleotides remains the same in all possible combinations. In this way, for example, *Bgl*II fragments which are cloned into the *Bam*HI site of pBR322 DNA can be recovered from the vector by cutting with *Sau*3AI. Another enzyme family exists for the sequence CTAG with the enzymes *Mae*I, *Spe*I, *Nhe*I, *Avr*II, and *Xba*I [1480].

6.5.1.4 Changes in Sequence Specificity

Relaxation For many enzymes such as *Eco*RI, relaxation of the specificity occurs when the solvent is changed [1577], [1651], [1652], [1653], [1442], [1461]. This altered activity of *Eco*RI is known as *Eco*RI * activity. Relaxed specificity markedly increases the number of small fragments, whereas the large fragments disappear. According to sequencing data, the subcanonical sequences differ from the canonical recognition sites by at least one base pair [1654]. Relaxation of both flanking positions yields the tetranucleotide AATT as the predominant *Eco*RI * specificity. However, relaxation of internal positions has also been observed [1655].

Relaxation of the specificity of class II restriction endonucleases has been attributed generally to the effects of altered reaction environment on the nature of the complexes formed between the DNA helix and the *Eco*RI dimer. Because *Eco*RI * is an inherent enzyme property, it cannot be eliminated by more extensive purification. However, relaxation specificity can be suppressed by using suitable buffers. Relaxation can be prevented by the following conditions [1652], [1442], [1461]:

- high ionic strength,
- decreased pH,
- use of Mg^{2+} ions rather than Mn^{2+}, Co^{2+}, or Zn^{2+} ions,
- low enzyme concentration,
- low concentration of glycerol or other organic solvents such as dimethyl sulfoxide,
- which destabilize the double-stranded DNA helix, or
- short incubation periods.

In addition, incubation *temperature* strongly affects relaxation of specificity. As shown for *Pst*I, an increase in incubation temperature from 37 to 42 °C suppresses the relaxed specificity almost completely even at higher concentrations and longer incubation periods. In contrast, lowering the incubation temperature to 30 °C strongly favors *Pst*I * activity. When the incubation temperature is further lowered to 25 or 20 °C, the relaxed activity becomes predominant even under normal incubation conditions. Analogous observations have been made for *Bam*HI, *Bst*EII, *Eco*RI, *Sph*I, *Sal*I, and *Taq*I [1656].

Modification of DNA by Methylation Additional changes in the activity and specificity of class II restriction endonucleases are attributed to DNA modifications such as

methylation [1482]. Certain enzymes are inhibited not only by the corresponding methylase counterpart but also by DNA methylation mediated by *Escherichia coli dam* [GATC] or *dcm*I methylase [CC$\begin{pmatrix}A\\T\end{pmatrix}$GG] [1657], [1658], [1446]. These two methylases are not part of the restriction–modification systems but have other biological functions such as the regulatory role of *dam* methylase in mismatch repair and DNA replication [1659], [1660], [1451].

When the recognition sequences of *dam* or *dcm*I methylase and the particular class II restriction endonuclease overlap totally, the activity on DNA isolated from *E. coli* is completely inhibited. Well-known examples of different *dam* influences at N^6-methylated adenine residues in GATC sequences are the isoschizomers *Dpn*I, *Mbo*I, and *Sau*3AI mentioned previously [1644], [1645], [1642]. Similar considerations apply to the *dcm*I methylase that inhibits *Eco*RII but activates *Apy*I by 5-methylation of the internal cytosine residue [1661], [1662]; however, *Bst*NI is not affected by this particular methylation [1662], [1531]. The inhibitory effects of *dam* or *dcm*I can be eliminated by isolation of the DNA from *E. coli* cells in which both methylase genes have been inactivated by mutation [1657], [1646], [1658].

Partial overlapping with the *dam* recognition sequence is observed for a variety of enzymes and frequently inhibits their activity [1664], [1665]. As an example, after *dam* methylation, *Cla*I digestion is inhibited specifically at the heptanucleotide sequences **GATC**GAT or ATC**GATC**[1663]. All the other *Cla*I sites are unaffected. Therefore, sequential action of isolated *dam* methylase and *Cla*I endonuclease generates the following new sequence specificity:

$$\begin{pmatrix}A\\G\\T\end{pmatrix}\text{ATCGAT}\begin{pmatrix}T\\G\\A\end{pmatrix}$$

In contrast, the heptanucleotides mentioned above are cleaved specifically with endonuclease *Dpn*I after methylation of DNA with *Cla*I methylase [1666]. In this approach, the other *Dpn*I cleavage sites remain stable, and both enzyme pairs complement each other in creating new sequence specificities.

Permutation of Fragment Ends Another approach for creating novel cleavage sites applies the permutation of generated fragment ends with 5′-protruding termini [1667]. Sites generated by such enzymes as *Eco*RI, *Bam*HI, or *Sal*I can be rendered blunt-ended by filling in the protruding ends with deoxynucleoside triphosphates and Klenow enzyme. This ligation creates a new symmetry center which corresponds to the ligation point of the two filled-in ends. If, for example, a *Sal*I site [G/TCGAC] is treated in this way, a 10 base pair palindromic sequence GT**CGATCG**AC with a novel *Pvu*I cleavage site [(CGAT/CG)] is created. After *Pvu*I cleavage, a third restriction site may be generated by treatment with nuclease S1. After ligation, another novel cleavage site is created because the resulting octanucleotide G**TCGCGA**C contains a new *Nru*I cleavage site [TCG/CGA]. Analogous conversion of restriction sites can be accomplished for all the other enzymes that generate fragments with 5′-protruding ends. Interconversion of restriction sites could generate unique novel cloning sites without the need of synthetic linkers. This should improve the flexibility of genetic engineering experiments.

Modification by Bacteriophages Bacteriophage-induced DNA modification is also known to strongly influence the activity of class II restriction endonucleases. The respective phage genomes contain all or most of the cytosine or thymine residues substituted at the 5-position [1668], [1669]. Substitution of cytosine by 5-methylcytosine (*Xanthomonas oryzae* phage XP12) or glycosylated 5-hydroxymethylcytosine (*Escherichia coli* phage T4), or of thymine by phosphoglucuronated and glycosylated 5-(4′,5′-dihydroxypentyl)uracil (*Bacillus subtilis* phage SP15) renders the DNA resistant to almost all known class II restriction endonucleases. The only exception is *Taq*I, which fragments the modified DNAs extensively. Substitution of 5-hydroxyuracil or uracil for thymine (*B. subtilis* phages SPO1 or PBS1) results only in a decreased cleavage rate but not in complete inhibition of the various enzyme activities.

Endonuclease Inhibitors Other inhibitors of class II restriction endonucleases are DNA-binding agents such as ethidium bromide, actinomycin D, proflavine, distamycin A, or neotrypsin [1670], [1671], [1672]. Spermine or spermidine also have an inhibitory effect at high concentrations, whereas low concentrations of these substances have a stimulatory effect. This stimulation is similar to that observed after addition of proteins such as *E. coli* RNA polymerase or T4 gene 32 protein which bind tightly to the single-stranded termini generated by many class II restriction endonucleases [1676], [1675], [1673], [1674].

6.5.1.5 Novel Class II Restriction Endonucleases

New sequence specificities are also obtained by screening bacteria for novel class II restriction endonucleases [1485]. The first method for finding new enzymes consists of examining those bacterial strains that are resistant to certain bacteriophages. As an alternative procedure, representatives of previously unexamined families from the wide range of eu- and archaebacteria are screened systematically for the presence of new class II restriction endonucleases. For example, in a total of 348 microorganisms screened, 105 novel enzymes were found, predominantly in gram-negative bacteria [1677]. To detect the novel specificities, aliquots of bacterial extracts are incubated with a DNA of known sequence such as λ DNA for various periods under standard incubation conditions. The fragment mixtures are analyzed by electrophoresis on agarose gels.

The new sequence specificity is determined with computer assistance by correlating experimentally observed and predicted fragment patterns. In a following step, the corresponding fragments obtained with other DNAs, e.g., from pBR322 or SV40, are analyzed. In a third step, the precise cleavage positions with the recognition sequence are fixed in sequencing gels by accurate fragment length determination of both strands of a particular DNA fragment created with the novel enzyme.

The three steps in characterization of the specificity and cleavage positions of a new enzyme may be examplified by the class II restriction endonuclease *Mae*I. This new enzyme can be isolated from the archaebacterium *Methanococcus aeolicus* PL15/H, along with *Mae*II and *Mae*III which are also new enzymes with novel specificities [1611]. The enzyme *Mae*I recognizes the tetranucleotide sequence C/TAG and cleaves both DNA strands specifically between the 5′-flanking cytosine and thymine

nucleotides. The enzyme *Mae*II also recognizes a tetranucleotide sequence [A/CGT], whereas *Mae*III acts on the pentanucleotide sequence /GTNAC.

Novel class II restriction endonucleases with more complex recognition sequences have also been found. Examples are *Dra*II and *Dra*III which were isolated recently as minor species from *Deinococcus radiophilus* in addition to the already known main activity *Dra*I, an isoschizomer of *Aha*III [1457]. The enzyme *Dra*II recognizes a novel type of heptanucleotide with ambiguities in the flanking nucleotides:

$$\begin{pmatrix} G \\ A \end{pmatrix} G/GNCC \begin{pmatrix} G \\ T \end{pmatrix}$$

The enzyme *Dra*III is characterized by the nonanucleotide CACNNN/GTG with three internal undefined nucleotides [1632], [1633].

6.5.2 DNA Polymerases

Table 47 lists the properties of important DNA polymerases under A.

6.5.2.1 *Escherichia coli* DNA Polymerase I

The enzyme *E. coli* polymerase I, also called deoxynucleoside triphosphate: DNA deoxynucleotidyltransferase (DNA-directed) (E.C. 2.7.7.7) [9012-90-2], is obtained from *Escherichia coli*.

Properties *Escherichia coli* DNA poylmerase I consists of a single polypeptide with a molecular mass of 109×10^3 dalton [1679] and contains one Zn^{2+} ion per molecule [1678]. The enzyme consists of two domains joined by a protease-sensitive peptide linker [1680], [1681], although the complete protein is approximately spherical with a diameter of 6500 pm [1682], [1683]. *Escherichia coli* DNA polymerase I contains a single free mercapto group able to form a dissociable complex with Hg^{2+}. The protein can be carboxymethylated without loss of activity [1684].

One unit of *E. coli* DNA polymerase I is defined as the enzyme activity that incorporates 10 nmol of total nucleotide into acid-precipitable material in 30 min at 37 °C [1685].

Uses *Escherichia coli* DNA polymerase I is used predominantly for the in vitro labeling of DNA by *nick translation* [1476]. Several published procedures all follow a common principle (Fig. 100). Trace amounts of DNase I (see Section 6.5.4.1) introduce nicks into unlabeled DNA, thus exposing internal 3′-hydroxyl groups. These nicks move toward the 3′-terminus by the subsequent sequential action of *E. coli* DNA polymerase I. First, the enzyme removes the nucleotide on the 5′-side of the nick. In the coupled reaction that follows, the excised nucleotide is replaced with a labeled nucleotide at the 3′-terminus of the nick. Repeated action of *E. coli* polymerase I replaces all nucleotides with labeled nucleotides downstream from the nick. Below 22 °C, the reaction will not proceed further than one complete exchange of the existing unlabeled DNA strand for a

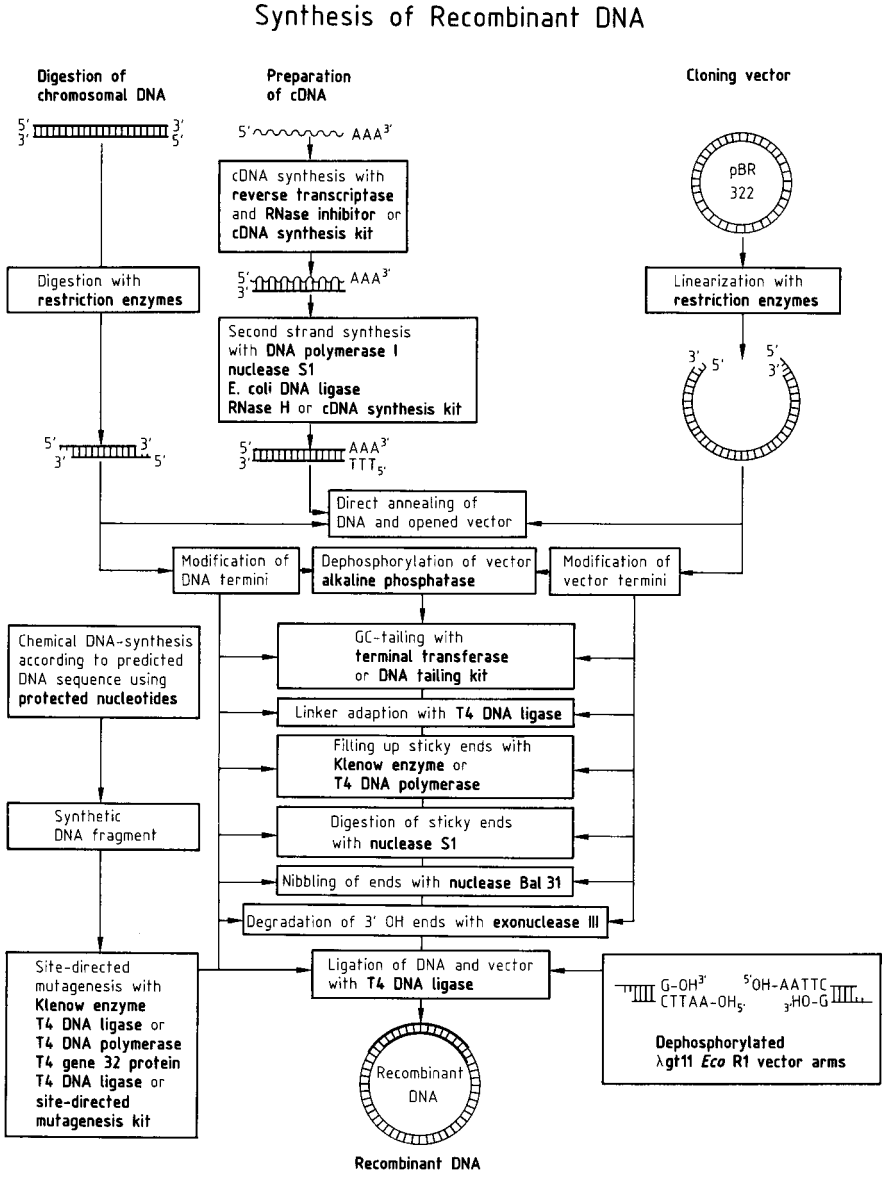


Figure 100. Use of DNA- and RNA-modifying enzymes in recombinant DNA synthesis For abbreviations, see text

labeled one, as shown in Figure 100. At the right ratio of DNA, DNase I, and *E. coli* DNA polymerase I, about 20–40 % of the labeled nucleotides are incorporated into the product, with a yield in label density of 10^8–10^9 dpm per microgram of DNA. Only 20–30 % of this incorporated label is commonly found in foldback DNA [1686]. The amount of foldback DNA is often higher if the unlabeled DNA fragment is relatively

small (<1000 nucleotides) [1687]. In this case, more 3′-ends are probably used for DNA synthesis than internal 3′-termini at nicks.

6.5.2.2 Klenow Enzyme

Klenow enzyme is the largest fragment obtained by proteolysis of *Escherichia coli* DNA polymerase I with subtilisin; it is also called deoxynucleoside triphosphate: DNA deoxynucleotidyltransferase (DNA-directed) (E.C. 2.7.7.7) [9012-90-2].

Properties Klenow enzyme has a molecular mass of 75×10^3 dalton. It can be obtained by subtilisin treatment, which digests the protease-sensitive peptide linker between the two functional domains of the native enzyme [1688]. The large fragment carries the 5′–3′ polymerase and the 3′–5′ exonuclease activities of intact DNA polymerase I, but lacks the 5′–3′ exonuclease activity located on the small fragment of 36×10^3 dalton. Klenow enzyme catalyzes the addition of deoxynucleotides to the 3′-hydroxyl terminus of a primer annealed to the template DNA.

One unit of Klenow enzyme is defined as the enzyme activity that incorporates 10 nmol of total nucleotide into an acid-precipitable fraction in 30 min at 37 °C [1685].

Uses Klenow enzyme is used in a wide variety of techniques, such as the following:

1. Conversion of 3′-recessed ends of restricted DNA fragments to blunt ends by the fill-in reaction [1689];
2. 3′-Endlabeling of DNA fragments by use of α-^{32}P-deoxynucleotides [1689];
3. Second-strand synthesis of cDNA [1690];
4. Homogeneous labeling of DNA fragments with random oligodeoxynucleotides as primers (oligolabeling) [1692];
5. Sequencing of DNA by the dideoxy chain termination method [1691] (Fig. 101);
6. Elongation of oligonucleotides mediating mismatch formation for site-directed mutagenesis [1693].

6.5.2.3 T4 DNA Polymerase

The enzyme T4 DNA polymerase, also called deoxynucleoside triphosphate: DNA deoxynucleotidyltransferase (DNA-directed) (E.C. 2.7.7.7) [9012-90-2], is obtained from phage T4-infected *Escherichia coli*.

Properties The enzyme T4 DNA polymerase consists of a single polypeptide with a molecular mass of 114×10^3 dalton [1694]. This polymerase displays a relatively broad pH optimum ranging from 8 to 9; at pH values of 7.5 and 9.7, ca. 50 % of the optimal activity is observed. Maximal polymerase activity requires 6 mmol/L of Mg^{2+}; this activity is decreased to one-fourth if Mg^{2+} is replaced by Mn^{2+} at its optimal concentration of 0.1 mmol/L.

One unit of T4 DNA polymerase is defined as the enzyme activity that incorporates 10 nmol of ^{3}H-dTTP into acid-precipitable DNA products in 30 min at 37 °C [1694].

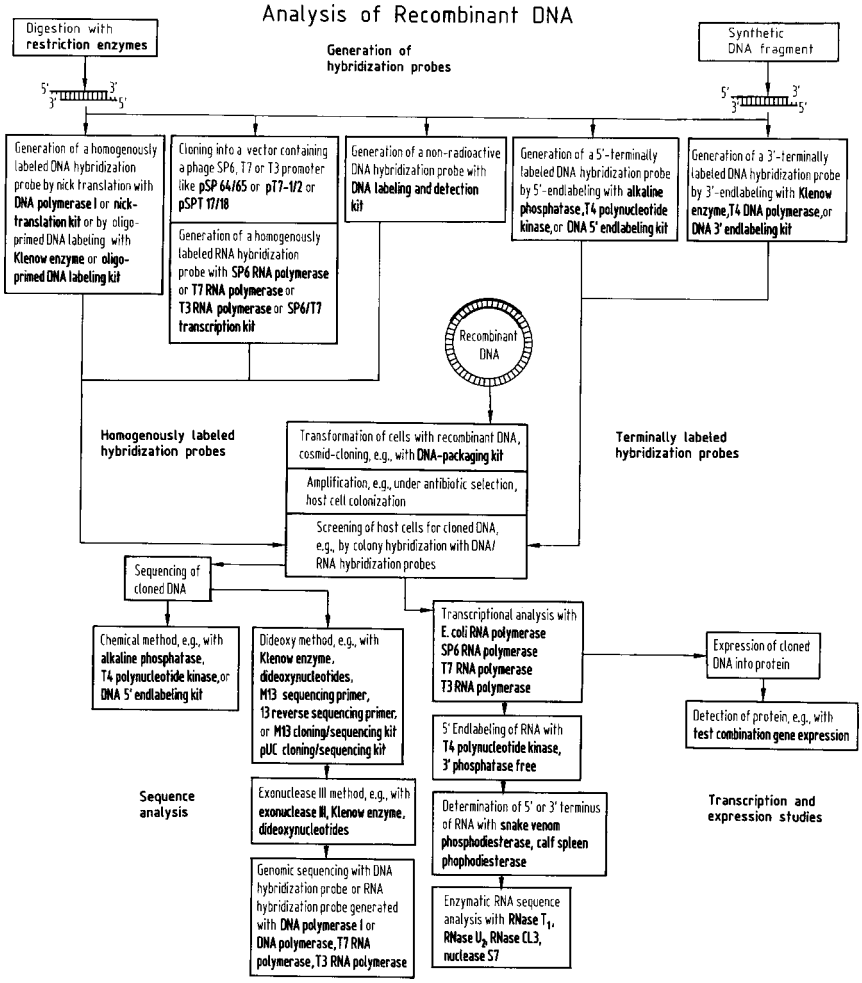


Figure 101. Use of RNA- and DNA-modifying enzymes in recombinant DNA analysis For abbreviations, see text

The T4 DNA polymerase catalyzes the addition of mononucleotides onto the 3′-hydroxyl terminus of a primer annealed to a single-stranded region of a DNA template. Because fully duplex DNA cannot serve as a template primer, DNA must first be made partially single-stranded by digestion of the 3′-termini with *E. coli* exonuclease III (see Section 6.5.4.2) [1685].

Single-stranded DNA can also serve as a template primer for T4 DNA polymerase. This polymerase is unable to use a DNA duplex that contains a nick as template primer. However, addition of the T4 gene 32 protein facilitates strand displacement, which allows the T4 DNA polymerase to replicate even the nicked duplex DNA in vivo and in vitro [1695], [1696]. Although T4 DNA polymerase lacks 5′–3′ exonuclease activity, it contains an extremely active 3′–5′ exonuclease that shows a strong specificity for

single-stranded DNA. Its turnover number is about 250 times greater than that of the 3′–5′ exonuclease associated with *E. coli* DNA polymerase I and about 3 times greater than the turnover number of its own 5′–3′ polymerase activity [1697].

Uses The enzyme T4 DNA polymerase can act as either a 5′–3′ polymerase or a 3′–5′ exonuclease. Polymerization occurs when all three of the following substrates are available to the enzyme: a polynucleotide template, a 3′-hydroxyl primer at least one residue shorter than the template, and the appropriate dNTPs or NTPs complementary to the template. In the absence of any one of these three components, the enzyme functions as an 3′–5′ exonuclease.

The T4 DNA polymerase can be used to label blunt or recessed 3′-termini of DNA by its 5′–3′ polymerase activity. In the case of blunt ends, the 3′–5′ exonuclease activity of the enzyme is responsible for digestion of duplex DNA to produce molecules with recessed 3′-termini. On subsequent addition of labeled dNTPs, the partially digested DNA molecules serve as primer–templates that are regenerated by the polymerase into intact, double-stranded DNA. Molecules labeled to high specific activity by this technique may be used as sensitive hybridization probes. They have two advantages over probes prepared by nick translation. First, they lack the artifactual hairpin structures that may be produced during nick translation. Second, they can be converted easily into strand-specific probes by cleavage with suitable restriction endonucleases in the unlabeled central region of the DNA molecule [1698]. In combination with T4 gene 32 protein, the T4 DNA polymerase is used for gap filling in site-directed mutagenesis experiments in which short oligonucleotides are employed for mismatch formation [1699].

6.5.2.4 Reverse Transcriptase

The enzyme AMV reverse transcriptase, also called deoxynucleoside triphosphate: DNA deoxynucleotidyltransferase (DNA-directed) (E.C. 2.7.7.49), is obtained from avian myeloblastosis virus.

Properties The enzyme AMV reverse transcriptase is one of the gene products of the RNA genome of avian myeloblastosis virus existing in two copies within the core structure of the retrovirus particle. Retroviruses which are capable of replicating without helper viruses have at least three genes arranged in the following order:

1. *gag* (encoding structural proteins of the inner coat and core),
2. *pol* (encoding reverse transcriptase and DNA endonuclease), and
3. *env* (encoding glycoproteins of the envelope).

A *gag–pol* precursor protein, $p180^{gag-pol}$, is one of the primary protein products; it is processed into a smaller protein, $p130^{gag-pol}$[1700], [1701]. Further proteolysis releases two polypeptides designated as α and β, the latter being phosphorylated [1702]. The enzymatically active forms of reverse transcriptase exist as α, ββ, and αβ. The molecular mass of the α-subunit is 68×10^3 dalton and of the β-subunit 92×10^3 dalton.

One unit of reverse transcriptase is defined as the enzyme activity that incorporates 1 nmol of ^{3}H-dTMP into acid-precipitable products in 10 min at 37 °C with poly(dA) · $p(T)_{15}$ as template primer [1703].

Because the amino acid sequence of α is a subset of the β-subunit, the mature form αβ is considered to be formed by proteolytic cleavage of the ββ dimer into αβ and p32, containing DNA endonuclease activity [1704]. Enzymatic activities associated with αβ are (1) RNA-dependent DNA polymerase, (2) DNA-dependent DNA polymerase, (3) RNase H, and (4) an unwinding activity normally attributed to single-strand binding proteins [1705]. The α-subunit contains both the polymerase and the RNase H activity which has the unique ability to degrade the RNA in DNA · RNA hybrids in an exonucleolytic manner [1706], [1707]. This exonucleolytic activity of RNase H can proceed from either the 5′- or the 3′-terminus. However, it cannot act on RNA linkages in covalently closed duplex circles. Reverse transcriptase was shown to synthesize poly(dT) transcripts in vitro in a progressive way with poly(rA)$_{1100}$ · oligo(dT)$_{12–18}$ as template and primer [1703]. In addition, the capacity of reverse transcriptase to use poly(rC) · oligo(dG)$_{12}$ as substrate is a useful feature for discrimination between the viral polymerase activity and the various host DNA polymerases [1708], [1709]. Effective priming is observed if the primer is more than eight nucleotides in length [1710].

Uses The enzyme AMV reserve transcriptase is widely used for synthesis of cDNA transcripts of specific RNA sequences in vitro (Fig. 102). Synthesis of the primary DNA strand is catalyzed by the RNA-dependent DNA polymerase activity. The DNA-dependent DNA polymerase activity is responsible for second-strand synthesis in cDNA formation when reverse transcriptase is used as polymerizing agent. The DNA-dependent DNA polymerase activity can be inhibited by addition of actinomycin D [1711]. Transcripts of cDNA are employed for analysis of structure, organization, and expression of eukaryotic genes. Comparison between cDNA and genomic DNA sequences elucidates genomic rearrangements, the existence of intervening sequences, and details of the splicing events [1712], [1713], [1714]. Synthesis of cDNA is also a powerful tool for the isolation of functional DNA sequences and the generation of corresponding hybridization probes. More recently, cloning of cDNA copies of the RNA virus genomes has opened another way to study details of their genomic structure [1715], [1716], [1717]. Several protocols for cDNA synthesis have been described [1718], and typical procedures have been reviewed recently [1719], [1721], [1722]. In addition, new methods have been developed for highly efficient cloning of full-length cDNA and sequencing of RNA [1723] by using AMV reverse transcriptase [1724].

6.5.2.5 Terminal Transferase

Terminal transferase, also called nucleoside triphosphate: DNA deoxynucleotidylexotransferase (E.C. 2.7.7.31) [9027-67-2], is obtained from calf thymus.

Properties Terminal transferase from calf thymus catalyzes the addition of deoxynucleotides to the 3′-hydroxyl terminus of a DNA primer [1725].

One unit of terminal transferase is defined as the enzyme activity that incorporates 1 nmol of dAMP into acid-insoluble products in 60 min at 37 °C with d(pT)$_6$ as primer [1726].

Terminal transferase from calf thymus has a molecular mass of 32×10^3 dalton. Under denaturing conditions, two nonidentical subunit structures are observed with molecular masses of 26.5×10^3 and 8×10^3 dalton. The isoelectric point is at pH 8.6, and

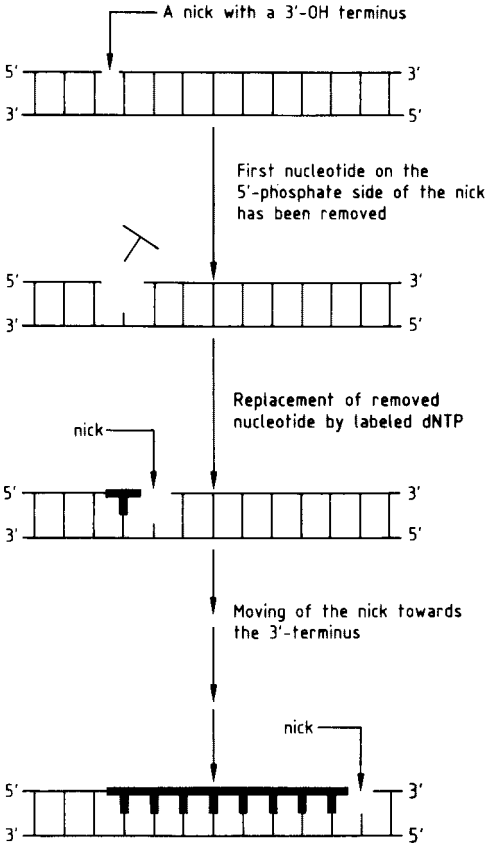


Fig. 102 Sequential action of DNase I and *Escherichia coli* DNA polymerase I during nick translation

the pH optimum at 7.2. At 35 °C, terminal transferase has an absolute requirement for an oligodeoxynucleotide primer containing at least three deoxynucleotides and a free 3′-hydroxyl end. However, the reaction is not template-dependent. Any dNTP, including substituted deoxynucleotides, can be polymerized by the enzyme [1727]. The highest polymerization rate for purine deoxyribonucleoside triphosphates is obtained when cacodylate buffer and Mg^{2+} are used, whereas the use of Co^{2+} instead of Mg^{2+} increases the polymerization of pyrimidine bases [1728].

Uses Because terminal transferase requires single-stranded DNA as primer, the incorporation efficiency is highest for dsDNA with 3′-protruding ends. However, blunt ends and 3′-recessive ends are also tailed by reverse transcriptase, with reduced efficiency [1729], [1717]. Terminal transferase is used mainly to add homopolymer tails to DNA fragments for the construction of recombinant DNA. By use of this tailing method, any double-stranded DNA fragment can be joined to a cloning vehicle. Optimal lengths for G–C tails are about 20–25 nucleotides, for A–T tails about 100 nucleotides [1476]. In

addition to the tailing reaction, terminal transferase is used for 3′-end-labeling of DNA fragments with α-^{32}P-labeled dNTPs or ddNTPs [1730], [1731] and for the addition of a single nucleotide to the 3′-ends of DNA for in vitro mutagenesis experiments [1729].

6.5.3 RNA Polymerases

Table 47 lists important RNA polymerases under B.

6.5.3.1 SP6 RNA Polymerase

The enzyme SP6 RNA polymerase, also called nucleoside triphosphate:RNA nucleotidyltransferase (DNA-directed) (E.C. 2.7.7.6) [9014-24-8], is obtained from phage SP6-infected *Salmonella typhimurium* LT2.

Properties The enzyme SP6 RNA polymerase consists of a single polypetide chain of 96×10^3 dalton [1732].

One unit of SP6 RNA polymerase is defined as the enzyme activity that catalyzes the incorporation of 1 nmol of GTP into acid-precipitable RNA products in 60 min at 37 °C [1732].

The SP6 RNA polymerase requires a DNA template and Mg^{2+} as a cofactor for RNA synthesis, and is strongly stimulated by bovine serum albumin or spermidine [1732]. The enzyme is not inhibited by the antibiotic rifampicin. This inhibitor affects bacterial RNA polymerases but not bacteriophage-coded RNA polymerases such as SP6 RNA polymerase [1732], [1733]. SP6 RNA polymerase possesses a very stringent promoter specificity which is distinct from that of analogous enzymes induced by bacteriophages T7, T3, gh1, or other T7-like bacteriophages [1734], [1735], [1736]. Thus SP6 RNA polymerase is able to transcribe only bacteriophage SP6 DNA [1737] or DNA cloned downstream from SP6 promoter as, for example, in the plasmids pSP64 or pSP65 [1738].

Uses The enzyme SP6 RNA polymerase is used for specific in vitro synthesis of RNA transcripts obtained from distinct DNA sequences cloned into the multiple cloning site of the vectors pSP64 or pSP65 [1738]. In these vectors, the SP6 promoter located in front of the multiple cloning site directs specific initiation of the transcription reaction. Restriction enzyme cleavage of the vector at a unique restriction site downstream from the inserted DNA sequences provides a discrete terminus for the run-off RNA transcript. Sense or anti-sense RNA transcripts of unique length can be generated, depending on the orientation of the integrated DNA (Fig. 103).

Splicing Analysis When this vector system is applied, SP6 RNA polymerase can be used to synthesize large amounts of precursor RNA for in vitro and in vivo studies of the splicing reaction [1739], [1740], [1738].

RNA Probes The enzyme SP6 RNA polymerase is also useful for the generation of homogeneously labeled, single-stranded RNA probes of highest specific radioactivity. These probes may be applied in Northern- and Southern-blot hybridization techniques [1741]. After DNase treatment and removal of the nonincorporated

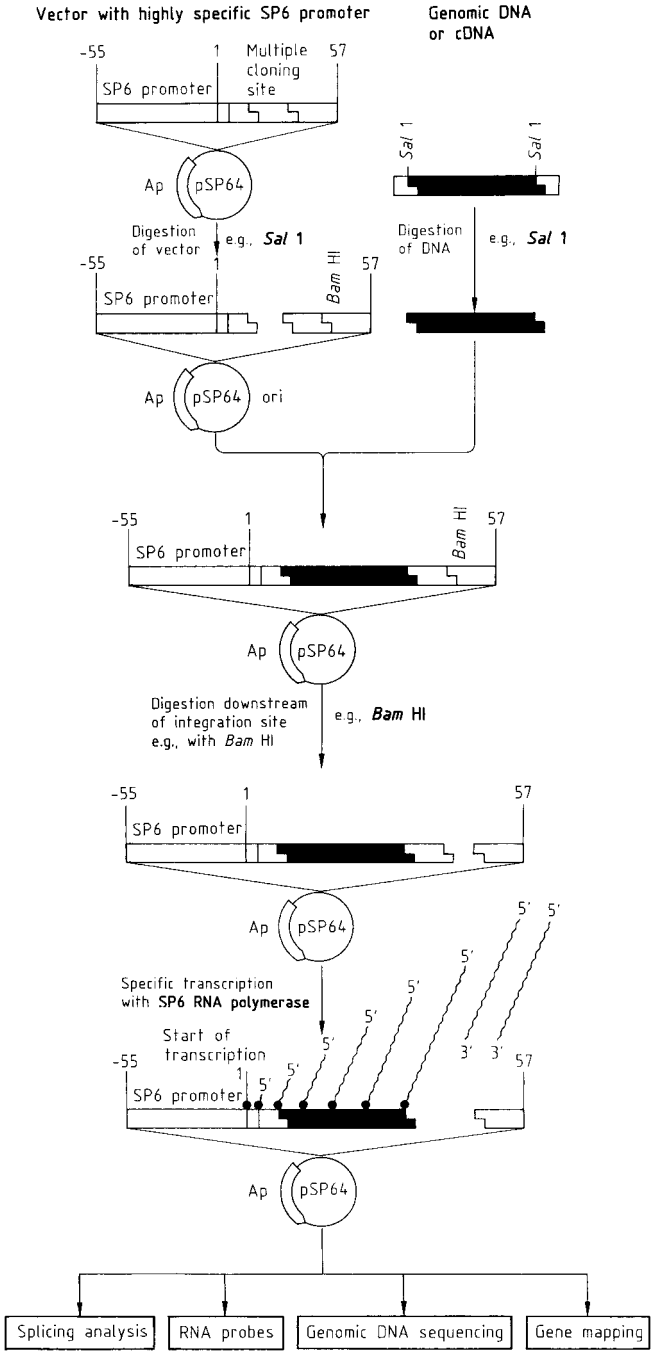


Fig. 4 Use of SP6 RNA polymerase for in vitro synthesis of mRNA

nucleotides by ethanol precipitation, the RNA probes generated with the pSP64/65 vectors are ready for hybridization without prior purification by gel electrophoresis. A tenfold increase in sensitivity may be observed when RNA probes are used instead of nick-translated DNA probes [1738].

Genomic DNA Sequencing Radioactively labeled RNA probes derived from SP6 RNA polymerase are also applied in genomic DNA sequencing [1742] and in situ hybridization experiments.

Gene Mapping M13 libraries may be screened with RNA probes synthesized by SP6 RNA polymerase in a one-step procedure that identifies the orientation of cloned DNA fragments.

Synthesis of Specific RNA An additional application of SP6 RNA polymerase is the synthesis of specific RNA strands for use in the RNase protection technique. After hybridization to total cell RNA, nonhomologous sequences are digested with a mixture of RNase A and RNase T_1. Only the homologous double-stranded regions will give stable hybrids of characteristic length [1741].

6.5.3.2 T7 RNA Polymerase

The enzyme T7 RNA polymerase, also called nucleoside triphosphate:RNA nucleotidyltransferase (DNA-directed) (E.C. 2.7.7.6) [9014-24-8], is obtained from phage T7-infected *Escherichia coli*.

Properties The enzyme T7 RNA polymerase consists of a single polypeptide chain of 98×10^3 dalton [1743].

One unit of T7 RNA polymerase is defined as the enzyme activity that incorporates 1 nmol of GMP into acid-precipitable RNA products in 60 min at 37 °C [1744].

The enzyme T7 RNA polymerase is highly specific for a T7 promoter (17 base pairs) including the transcription start site [1745]. The promoter sequence differs from that of bacteriophage SP6-coded RNA polymerase (Section 6.5.2.1) by only two nucleotides. Although the sequences are similar, T7 RNA polymerase specifically transcribes only T7 DNA or DNA cloned downstream of the T7 promoter. Appropriate cloning vectors are pT7–1 and pT7–2 containing the strong T7 promoter Φ10 in front of a polylinker region with eight unique restriction sites [1746]. In pT7–1 and pT7–2, the polylinker regions are integrated in opposite orientation. In addition to a DNA template with a T7 promoter, T7 RNA polymerase requires Mg^{2+} as cofactor and the four NTPs as substrates. Spermidine or bovine serum albumin stimulates the activity of the enzyme. In contrast to bacterial RNA polymerases, T7 RNA polymerase is not inhibited by rifampicin [1732], [1733].

Uses The T7 transcription system consisting of T7 RNA polymerase and vectors pT7–1 and pT7–2 is used for in vitro synthesis of specific RNA. For this purpose, the template DNAs are cloned into the polylinker regions of these vectors downstream from the T7 promoter. By cutting the cloned DNA at the 3′-end with an appropriate restriction

enzyme before the transcription reaction has started, run-off RNA transcripts of defined size are obtained. By using the T7 vectors pT7–1 and pT7–2 with oppositely oriented polylinker regions, DNA can be transcribed into sense and anti-sense mRNA.

The T7 system allows in vitro synthesis of either homogeneously labeled (in the presence of radioactive or biotinylated NTPs) or unlabeled RNA molecules. Since the ratio of the produced RNA to template DNA reaches 30 : 1, elimination of the DNA is usually not necessary but can be achieved by treatment with RNase-free DNase I. The RNA probes are highly specific for the complementary DNA strand; therefore, they are especially useful for different RNA or DNA blotting techniques [1747], [1748], in situ hybridization [1749], genomic sequencing [1742], or nuclease S1 studies. This property also allows the screening of M13 gene banks for clones containing DNA with a defined orientation. With unlabeled primary mRNA transcripts, splicing of RNA may be studied in vivo or in vitro. With the aid of in vitro synthesized anti-sense mRNA, expression of the corresponding genes can be suppressed after introduction of this RNA into eukaryotic cells [1750]. The T7 system also allows the synthesis of pure RNA transcripts for in vitro translation studies.

6.5.4 DNA Nucleases

Table 47 lists important DNA nucleases under C.

6.5.4.1 DNase I

The enzyme DNase I, also called deoxyribonuclease I (E.C. 3.1.21.1), is obtained from bovine pancreas.

Properties DNase I from bovine pancreas is a glycoprotein with a molecular mass of 31×10^3 dalton. The pH optimum of the enzyme is 7.0. One unit of DNase I is defined as the enzyme activity that produces after incubation of calf thymus DNA an absorbance increase of 0.001 in one minute at 25 °C [1751], [1752] .

DNase I is normally isolated as a mixture of four isoenzymes: A, B, C, and D. The complete amino acid sequence of isoenzyme A, which is the main component, has been determined [1753]. Isoenzymes A and B differ only in the composition of the carbohydrate side chain attached to asparagine 18, but isoenzymes C and D have the histidine at position 118 replaced by a proline [1754]. All DNase I isoenzymes contain two disulfide bridges, one of which can be reduced in the presence of Ca^{2+} without loss of activity [1755]. DNase I degrades double-stranded DNA in an endonucleolytic way to yield 5′-oligonucleotides [1756], [1757]. DNase I requires divalent metal ions for DNA hydrolysis, with maximal enzymatic activity in the presence of Ca^{2+} and Mg^{2+} or Ca^{2+} and Mn^{2+} [1758]. The mode of action and the specificity of the enzyme depend on the type of divalent cations present [1759], [1760]. DNase I appears to be primarily a digestive enzyme. However, DNase I-like activities have also been found in nondigestive tissues, which suggests that the enzyme might have other functions [1757]. It may play a role in the regulation of actin polymerization, because it forms a very stable complex with monomeric actin and causes depolymerization of filamentous actin [1539], [1761], [1762].

Uses DNase I is used for labeling dsDNA by nick translation [1476]. RNase-free DNase I is also applied to template digestion after in vitro synthesis of RNA with bacteriophage SP6-, T7-, or T3-coded RNA polymerases [1732].

6.5.4.2 Exonuclease III

Exonuclease III, also called exodeoxyribonuclease III (E.C. 3.1.11.2), is obtained from *Escherichia coli*.

Properties Exonuclease III is a monomeric protein with a molecular mass of 28×10^3 dalton [1763].

One unit of exonuclease III is defined as the enzyme activity that releases 1 nmol of acid-soluble nucleotides from calf thymus DNA treated with ultrasonic waves in 30 min at 37 °C [1764].

The enzyme is a multifunctional protein which catalyzes the hydrolysis of several types of phosphoester bonds in dsDNA. In addition, the following minor activities are found [1765], [1766], [1767]:

an endonuclease activity, specific for apurinic and apyrimidinic sites,

a 3′-phosphatase activity, and

an RNase H activity, which degrades the RNA strand in DNA · RNA hybrids.

The pH optima for the various endo- and exonucleolytic activities are between 7.6 and 8.5. A lower pH optimum between 6.8 and 7.4 has been reported for phosphatase activity. For optimal exonucleolytic activity, Mg^{2+} or Mn^{2+} is required, whereas Zn^{2+} inhibits exonuclease III [1685], [1767]. In addition, the activity depends strongly on temperature, salt concentration, and ratio of DNA to enzyme concentration [1768], [1769]. Also, the rate at which mononucleotides are released decreases in a base-dependent mode in the following order $C \gg A \sim T \gg G$ [1770].

Uses Exonuclease III is used mainly for the generation of single-stranded terminal regions from dsDNA by acting as a 3′–5′ exonuclease. The DNA modified in this way is used as a substrate for labeling DNA with Klenow enzyme or for sequencing studies [1771], [1768], [1769]. Another application of exonuclease III is to shorten progressively both strands of dsDNA by additional digestion of exonuclease III-generated single-stranded tails with nuclease S1 [1772]. Exonuclease is also used to determine stable lesions in defined sequences of DNA [1773].

6.5.4.3 Nuclease S1

Nuclease S1, also called *Aspergillus* nuclease S1 (E.C. 3.1.30.1), is obtained from *Aspergillus oryzae*.

Properties Nuclease S1 is a monomeric protein with a molecular mass of 38×10^3 dalton [1774], [1775]. The enzyme is a glycoprotein containing 18 % carbohydrate residues [1774]. Its isoelectric point is at pH 4.3–4.4 [1776].

One unit of nuclease S1 is defined as the enzyme activity that releases 1 μg of acid-soluble deoxynucleotides from denatured DNA in 1 min at 37 °C [1775].

The pH optimum is 4.0–4.3, with half-maximal rates at pH 3.3 and 4.9. Higher pH values (4.6–5.0) have been used to avoid possible nicking of DNA substrates by depurination. The enzyme requires Zn^{2+} for optimal activity. The ions Co^{2+} and Hg^{2+} can replace Zn^{2+}, but they are less effective [1775]. Nuclease S1 is optimally active at NaCl concentrations of 0.1 mol/L and is relatively unaffected by ionic strengths between 0.01 and 0.2 mol/L of NaCl [1777]. The enzyme is remarkably stable to such denaturing agents as urea, sodium dodecyl sulfate, or formamide [1778]. When the enzyme is used in high concentrations, the salt concentration must be high enough (>0.2 mol/L) to suppress nonspecific nicking [1779].

Uses Under optimal conditions, the rates of nuclease S1-catalyzed hydrolysis of single- and double-stranded nucleic acids have been estimated to differ by a factor of 75 000 [1780]. Thus, the main application of nuclease S1 is to trim single-stranded protruding ends of DNA or RNA without significant nibbling of the duplex ends [1781], [1782]. Mapping of transcripts is performed by analyzing S1-resistant RNA · DNA hybrids in alkaline agarose or denaturing polyacrylamide gels [1783], [1784]. High-resolution mapping is obtained after end-labeling the DNA with ^{32}P by analyzing the S1-resistant DNA fragments on sequencing gels [1786]. Use of this method allows mapping of the 5′- and 3′-ends of mRNA [1787]. In contrast to exonuclease VII, single-stranded loops in dsDNA or RNA · DNA hybrids are digested by nuclease S1 [1788], [1789]. Thus, mapping of introns in eukaryotic genes is possible by digesting the hybrid of mature mRNA and the ^{32}P-labeled codogenic DNA strand with nuclease S1 (Fig. 104).

A further application that takes advantage of this property is digestion of hairpin structures during cDNA synthesis [1719]. An S1 mapping based on analysis by two-dimensional gel electrophoresis is also described [1790]. The methodology of S1 mapping has been used successfully to study the regulation of cloned genes reintroduced into eukaryotic cells [1791].

6.5.4.4 Nuclease Bal 31

Nuclease Bal 31, also called exodeoxyribonuclease (E.C. 3.1.11), is obtained from *Alteromonas espejiani*.

Properties Nuclease Bal 31 progressively degrades both strands of dsDNA from the ends inward. The enzyme also has a ssDNA-specific endonuclease activity which cleaves supercoiled DNA to yield, for example, the linear form. No dsDNA-specific endonuclease activity is associated with the enzyme.

One unit of nuclease Bal 31 is defined as the enzyme activity that releases 600 base pairs in 10 min at 30 °C from 2 μg of linearized pUR222 DNA [1792].

Enzyme activity requires Ca^{2+} and Mg^{2+}, and is optimal at an NaCl concentration of 0.6 mol/L. Nuclease Bal 31 is not inhibited by sodium dodecyl sulfate or urea. Fragments of DNA shortened with nuclease Bal 31 can be ligated readily with T4 DNA ligase either directly or after filling in any single-stranded end with Klenow enzyme.

Uses In molecular cloning experiments, various specific manipulations of DNA sequences must be accomplished: for example, coding sequences must be joined to

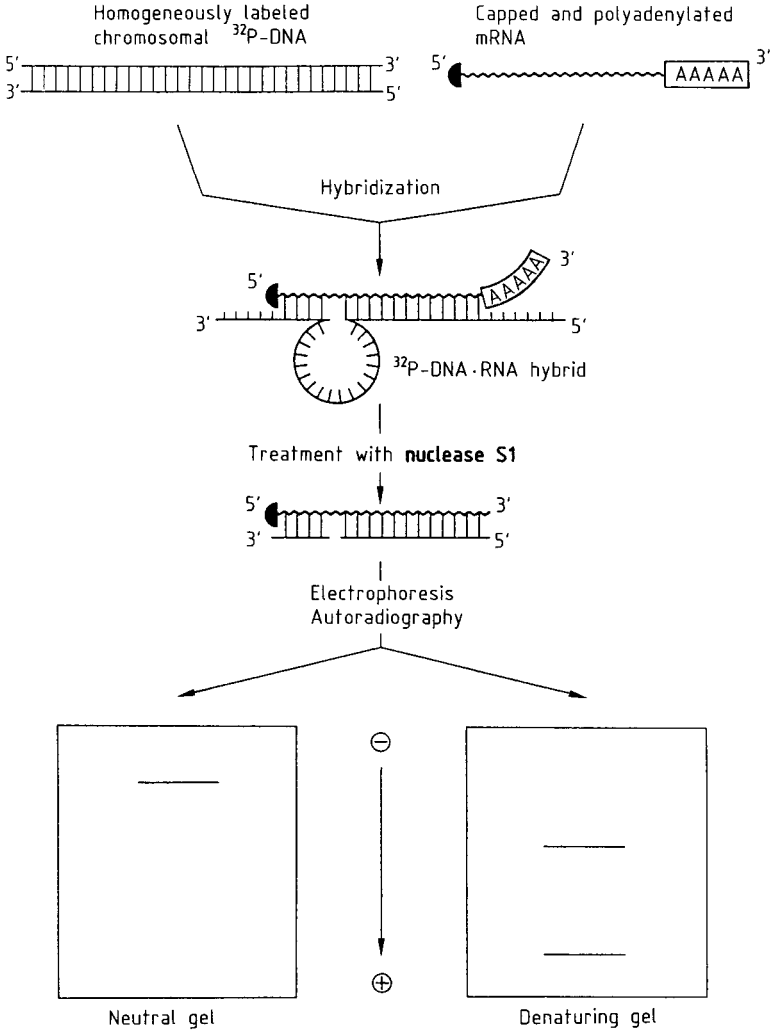


Fig. 104 Mapping of introns in eukaryotic genes by digesting a hybrid of mature mRNA and the coding strand of DNA with nuclease S1

a promoter in the proper reading frame, deletions of predetermined DNA segments are required to map functional regions, or transcription units must be localized by trimming to the minimum essential size. If suitable restriction sites do not exist at the proper location on the DNA sequence, nuclease Bal 31 can be applied.

Nuclease Bal 31 can also be used for mapping DNA with restriction endonucleases [1793]. The DNA segment to be mapped is shortened to various lengths with the nuclease. Each sample is then digested with the corresponding restriction endonuclease, and the resulting fragments are analyzed by gel electrophoresis. Along with shortening of the

original DNA segment, restriction fragments disappear from the gel in exactly the same order in which the restriction sites are arranged along the DNA strand.

Another use of nuclease Bal 31 is to transfer any restriction site located adjacent to a preselected position [1794]. A linearized plasmid containing a cloned DNA sequence is digested with nuclease Bal 31 to a predetermined length, cut with a single cutting restriction enzyme, circularized with T4 DNA ligase, and transformed into *Escherichia coli* cells. Clones are selected which are sensitive to digestion with the restriction enzyme used before.

The ssDNA-specific endonuclease activity of nuclease Bal 31 has also been exploited to investigate the secondary structure in supercoiled DNAs or an altered helical structure in nonsupercoiled DNA produced by carcinogenic or mutagenic agents [1538].

6.5.5 RNA Nucleases

Table 47 lists important RNA nucleases under D.

6.5.5.1 RNase H

The enzyme RNase H, also called endoribonuclease (E.C. 3.1.26.4), is obtained from *Escherichia coli*.

Properties The enzyme activity found in *E. coli* which hydrolyzes the RNA of RNA · DNA hybrids is designated RNase H, whereas the activity against the RNA in RNA · RNA duplexes is called RNase III [1795].

One unit of RNase H is the enzyme activity that produces 1 nmol of acid-soluble ribonucleotides from ^{3}H-poly(A) · poly(dT) in 20 min at 37 °C [1796].

RNase H acts as an endoribonuclease and degrades the RNA strand of RNA · DNA hybrids of natural origin, and of synthetic complexes like poly(A) · poly(dT). RNase H produces ribonucleotides with 5′-phosphate and 3′-hydroxyl termini. Almost no activity is detected with polyribonucleotides alone or polymers annealed to their complementary ribopolymer. For optimal activity, RNase H requires Mg^{2+} which can be replaced only partially by Mn^{2+}. The enzyme has its maximum activity in the presence of compounds containing mercapto groups and is inhibited by *N*-ethylmaleimide. The pH optimum is between 7.5 and 9.1. RNase H activity is relatively insensitive to salt; 50 % of its activity is retained in the presence of NaCl at 0.3 mol/L [1797].

Uses In addition to the use of *E. coli* RNase H for the study of in vivo functions such as RNA-primed initiation of DNA synthesis [1796], RNase H is applied in the synthesis of cDNA by combination of the classical first-strand synthesis with the novel second-strand synthesis mediated by *E. coli* DNA polymerase I, *E. coli* RNase H, and *E. coli* DNA ligase [1798], [1724]. Furthermore, RNase H is used to detect RNA · DNA regions in dsDNA of natural origin [1799], [1707]. A further application of the enzyme is the removal of poly(A) sequences of mRNA, which leads to increased electrophoretic homogeneity of mRNA in gel electrophoresis [1800]. RNase H may also be used for the site-specific enzymatic cleavage of RNA. With this method, a synthetic DNA oligomer

will hybridize only to complementary single-stranded regions of an RNA molecule, which are therefore digested by RNase H in a site-specific manner [1801].

6.5.5.2 Site-Specific RNases

6.5.5.2.1 RNase A

The enzyme RNase A, also called pancreatic ribonuclease, (E.C. 3.1.27.5), is obtained from bovine pancreas.

Properties RNase A is an endonuclease that cleaves RNA but not DNA. The enzyme specifically attacks pyrimidine nucleotides by cleaving the 3′-adjacent phosphodiester bond Py/pN. The molecular mass is 13.7×10^3 dalton and the pH optimum is 7.0–7.5 [1867], [1802]. Cyclic 2′,3′-pyrimidine nucleotides are obtained as intermediates. Pyrimidine 3′-phosphates and oligonucleotides with terminal pyrimidine 3′-phosphate groups are the final products.

One unit of RNase A is defined as the enzyme activity that produces total conversion of RNA as substrate in 1 min at 25 °C. The decrease in absorbance A_0 to A_1 corresponds to total conversion, A_1 being the final absorbance [1803]. RNase A is used as A- and G-specific RNase in RNA sequencing [1804], [1806].

6.5.5.2.2 RNase CL3

The enzyme RNase CL3, also called endoribonuclease (E.C. 3.1.27.1), is obtained from chicken liver.

RNase CL3 cleaves RNA predominantly at Cp/N bonds and produces fragments with 3′-terminal cytidine phosphate. Bonds of the type Ap/N and Gp/N are cleaved less frequently, and Up/N bonds are cleaved to a very small extent [1804]. The molecular mass is 16.85×10^3 dalton; the pH optimum is at 6.5–7.5 [1805].

One unit of RNase CL3 is defined as the enzyme activity that releases a sufficient quantity of acid-soluble oligonucleotides to produce an absorbance increase of 1.0 at 260 nm in 15 min at 37 °C [1805]. One unit corresponds to the decomposition of ca. 40 μg of poly(C).

RNase CL3 is used as minus-U-specific RNase in RNA sequencing [1804], [1806]. The Cp/N cleavage is more efficient in the presence of urea and at 50 °C, i.e., under conditions in which the secondary structure of RNA is destroyed [1806], [1807]. Under these denaturing conditions, approximately five to ten times as much enzyme must be used, but predominantly C-specific cleavage is obtained.

6.5.5.2.3 RNase T_1

Ribonuclease T_1 (E.C. 3.1.27.3), also called endoribonuclease, is obtained from *Aspergillus oryzae*.

RNase T_1 is an endonuclease that cleaves RNA but not DNA. The enzyme specifically attacks the 3′-adjacent phosphodiester bond Gp/N. RNase T_1 is therefore used as G-specific RNase in RNA sequencing [1804], [1806]. The molecular mass is 11.1×10^3 dalton, the pH optimum is at 7.4 [1808], [1809]. Cyclic 2′,3′-guanosine nucleotides are obtained as intermediates, and guanosine 3′-phosphates and oligonucleotides with guanosine 3′-phosphate terminal groups are the final products.

One unit of RNase T_1 is defined as the enzyme activity that releases a sufficient quantity of acid-soluble oligonucleotides to produce an absorbance increase of 1.0 at 260 nm with denatured DNA in 15 min at 37 °C [1808]. RNase T_1 is used especially for RNA sequencing and RNA fingerprinting.

6.5.5.2.4 RNase U_2

Ribonuclease U_2 (E.C. 3.1.27.4), also called endoribunuclease, is obtained from *Ustilago sphaerogena*.

RNase U_2 is an endonuclease that cleaves RNA but not DNA. The enzyme specifically attacks purine nucleotides by cleaving the 3′-adjacent phosphodiester bond Pu/pN [1829]. The molecular mass is 10×10^3 dalton, and the pH optimum is 3.5. Cyclic 2′,3′-purine nucleotides are obtained as intermediates. Purine 3′-phosphates and oligonucleotides with purine 3′-phosphate terminal groups are the final products. Reversal of the final step can be used for the synthesis of ApN or GpN [1810].

One unit of RNase U_2 is defined as the enzyme activity that releases a sufficient quantity of oligonucleotides to produce an absorbance increase of 1.0 at 260 nm with denatured DNA in 15 min at 37 °C [1810]. RNase U_2 is used as A- and G-specific RNAse in RNA sequencing [1804], [1806].

6.5.5.2.5 Nuclease S7

Nuclease S7, also called endonuclease (E.C. 3.1.31.1), is obtained from *Staphylococcus aureus*.

Nuclease S7 is an endonuclease that cleaves both DNA and RNA. The products obtained after cleavage of RNA or DNA with nuclease S7 are oligo- and mononucleotides with terminal 3′-phosphate groups. The enzyme manifests A or U specificity in RNA digestion at pH 7.5 [1811].

One unit of nuclease S7 is defined as the enzyme activity that releases a sufficient amount of acid-soluble oligonucleotides to produce an absorbance increase of 1.0 at 260 nm with denatured DNA in 30 min at 37 °C [1811].

The enzyme is used widely to optimize the standard rabbit reticulocyte lysate. The mRNA-dependent protein synthesis system with only low amounts of endogenous RNA is obtained by incubation of the lysate with the enzyme in the presence of $CaCl_2$[1812]. Because of the strict dependence of the nuclease on Ca^{2+} ions, it can easily be inactivated by a specific chelating agent such as ethylenediaminetetraacetic acid. During incubation at 50 °C and pH 7.5 in the presence of $CaCl_2$ and urea, NpA and NpU bonds are cleaved. Nuclease S7 is therefore also used as A- and U-specific RNase in RNA sequencing [1806]. This reaction generates RNA fragments with 3′-phosphate termini. Thus, in sequencing gels, the generated RNA fragments are shifted downstream by one nucleotide compared with RNase T_1 or RNase U_2 fragments of identical length.

6.5.5.2.6 Site-Specific RNases in RNA Sequence Analysis

Base-specific RNases and nucleases with RNA and DNA specificity can be used for enzymatic sequencing of RNA molecules [1829], [1830], [1831], [1832] (Fig. 107).

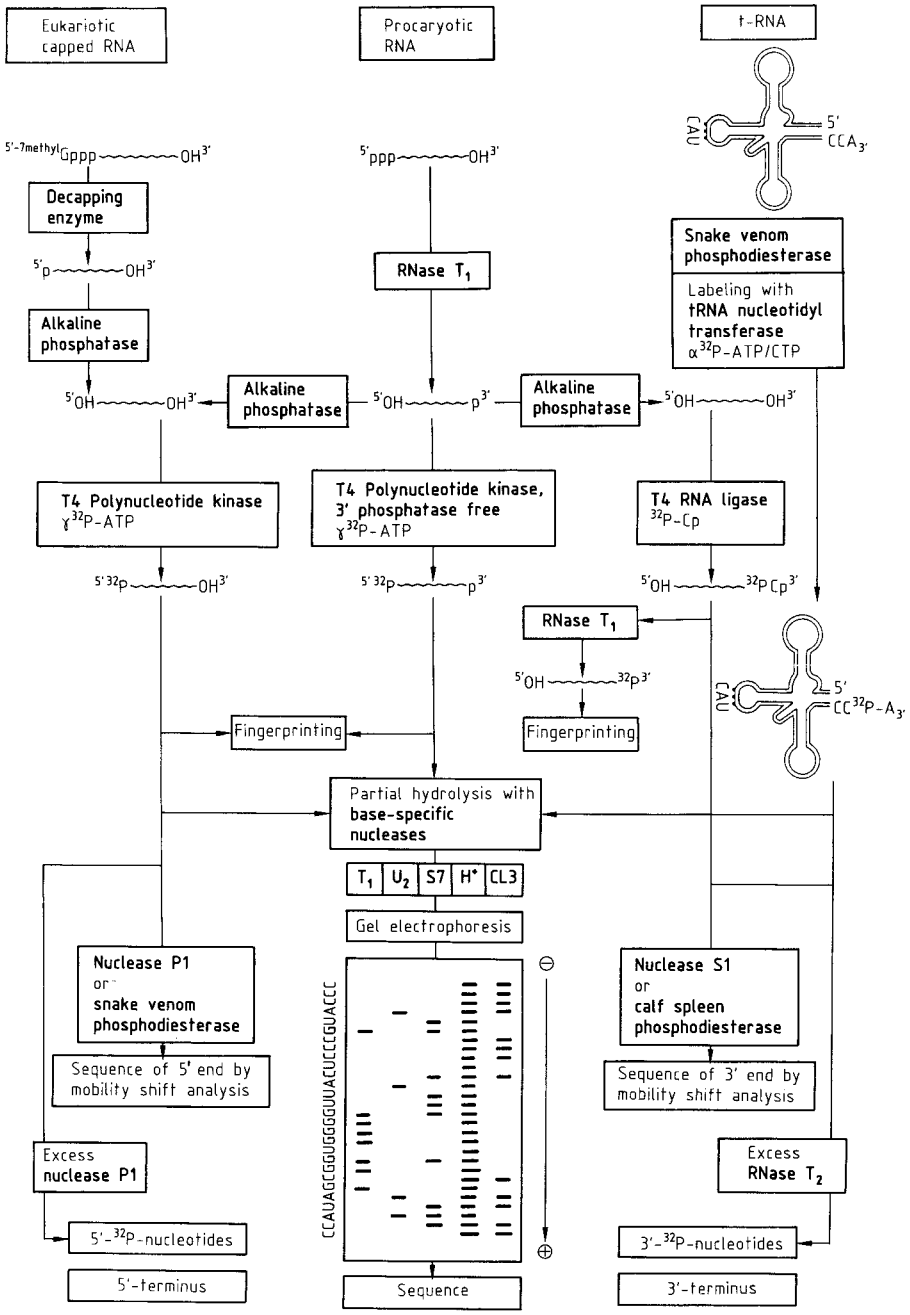


Fig. 6 Use of nucleases with RNA and RNA–DNA specility in sequencing RNA
H^+ = acid hydrolysis

Because the sequence of RNA molecules larger than some hundred nucleotides cannot be determined directly, two alternative approaches are possible:

1. Fingerprint analysis, for example, of RNase T_1-resistant fragments [1813], [1814].
2. Sequencing RNA after partial digestion of large RNA chains (e.g., with RNase T_1) to produce a set of fragments covering the whole molecule [1815].

In both approaches, the RNA fragments must be terminally labeled with ^{32}P in vitro prior to separation. End-labeling of RNA can be achieved in two ways:

1. Labeling the 5′-terminus by using T4 polynucleotide kinase and γ-^{32}P-ATP as substrate (left branch, Fig. 105); [1817], [1818], [1819].
2. Labeling the 3′-terminus by using T4 RNA ligase and 5′-^{32}P-Cp as substrate (right branch, Fig. 105) [1820].

In addition, 3′-end-labeling of tRNA may be obtained with tRNA nucleotidyltransferase. Any RNA molecules that fail to be polyadenylated can be labeled at the 3′-terminus with *E. coli* poly(A) polymerase [1821], [1822], [1823]. After isolation of the various RNase T_1 subfragments, RNA sequencing consists of three analytical steps, as shown in Figure 105, bottom part.

Determination of 5′- and 3′-Terminal Nucleotides Information about the nature of the 5′-terminally labeled nucleotide is obtained by complete digestion of the fragment with nuclease P1, resulting in 5′-^{32}P-nucleotides [1756], [1824], [1832]. Excessive digestion of 3′-terminally labeled fragments with RNase T_2 permits the identification of the 3′-termini by analysis of the liberated 3′-^{32}P-nucleotides.

Sequence Determination of the Terminal Nucleotides by Mobility-Shift Analysis Sequence information concerning limited regions of the 5′- and 3′-termini is obtained by partial enzymatic digestion of alternatively 5′- or 3′-labeled oligonucleotides with randomly acting endonuclease P1 or snake venom phosphodiesterase, acting as 3′-exonucleases; by randomly acting endonuclease S1 or calf spleen phosphodiesterase, acting as 5′-exonucleases; [1756], [1825], [1826], [1827], or by chemical treatment with $NaHCO_3$ or ethylenediaminetetraacetic acid [1823].

Complete Sequence Analysis by Partial Digestion with Base-Specific Nucleases In addition to chemical RNA sequencing [1828], RNA sequencing by reverse transcription in cDNA [1723], enzymatic RNA sequencing by partial digestion with the base-specific RNases mentioned in the previous sections (RNase T_1, RNase U_2, nuclease S7, and RNase CL3) is a well-established procedure [1804], [1806], [1829], [1832]. Reaction details are given in Table 46.

Identification of purine bases within the RNA chain is achieved by digestion of RNA with RNase T_1 and RNase U_2[1829], [1830], [1831], [1832]. Digestion with RNase T_1 (G-specific) results in fragments with 3′-terminal Gp residues. Digestion with RNase U_2 (A- and G-specific) under appropriate conditions results in fragments with mainly

Table 46. Partial digestion of RNA with base-specific RNases

Enzyme	Conditions	Specificity
RNase T_1	urea, 8 mol/L, pH 3.5; 50 °C	$^{5'}Gp/N^{3'}$
RNase U_2	urea, 8 mol/L, pH 3.5; 50 °C	$^{5'}Ap/N^{3'}$ and ($^{5'}Gp/N^{3'}$)
RNase S7	urea, 8 mol/L, pH 7.5; 50 °C	$^{5'}Np/A^{3'}$ and $^{5'}Np/U^{3'}$
RNase CL3	urea, 8 mol/L, pH 8.0; 50 °C	$^{5'}Cp/N^{3'}$ and ($^{5'}Ap/N^{3'}$ + $^{5'}Gp/N^{3'}$)

3′-terminal Ap residues (A-specific). Pyrimidine residues are localized by digestion of RNA with nuclease S7 at pH 7.5 and RNase CL3 at pH 8.0. Digestion with RNase CL3 results mainly in fragments with 3′-terminal Cp residues, less frequently in fragments with 3′-terminal Ap and Gp residues (minus-U-specific = C-, A-, and G-specific). Digestion with nuclease S7 at pH 7.5 results in fragments with 5′-terminal A and U residues (A- and U-specific) [1806].

Care must be taken in reading the sequence ladder, because — compared with T_1 or U_2 fragments of corresponding length — the electrophoretic mobility of 5′-endlabeled S7 fragments is shifted downward by exactly one nucleotide; 3′-endlabeled S7 fragments are shifted upward by one nucleotide. This difference in eletrophoretic mobility compared with T_1 and U_2 fragments is caused by the cleavage site of the enzyme upstream from A or U residues. Normal electrophoretic mobility is observed with RNA fragments obtained with RNase CL3 [1804], [1805]. Digestion with RNase CL3 results mainly in fragments with 3′-terminal Cp residues. The formation of fragments with 3′-terminal Ap and 3′-terminal Gp residues is much less frequent. 3′-Terminal Up residues are generated to only a very small extent. Thus the information obtained by digestion of RNA with nuclease S7 and RNase CL3 is useful for unambiguous discrimination between both pyrimidine residues.

6.5.6 Modifying Enzymes

Table 47 lists important DNA-modifying enzymes under E.

6.5.6.1 Alkaline Phosphatase

Alkaline phosphatase, also called orthophosphoric monoester phosphohydrolase (alkaline optimum) (E.C. 3.1.3.1) [9001-78-9], is obtained from calf intestine.

Properties The molecular mass of alkaline phosphatase from calf intestine is 140×10^3 dalton. The enzyme is a dimeric glycoprotein composed of two identical or similar subunits with a molecular mass of 69×10^3 dalton each. Alkaline phosphatase contains four Zn^{2+} ions per molecule [1817].

One unit of alkaline phosphatase is defined as the enzyme activity that hydrolyzes 1 μmol of 4-nitrophenyl phosphate in 1 min at 37 °C in a diethanolamine buffer (1 mol/L); 4-nitrophenyl phosphate concentration: 10 mmol/L; $MgCl_2$ concentration: 0.25 mmol/L; pH 9.8 [1833].

Table 47. Properties of important DNA- and RNA-modifying enzymes

Enzyme	Organism	M_n, 10^3 dalton	Sub-units	References (isolation)	Specificity[a]	Substrates[b]	pH optimum[c]	Cofactors	References (specificity)
A. DNA polymerases									
A.1. Prokaryotic DNA polymerases									
***E. coli* DNA polymerases**									
DNA polymerase I	*Escherichia coli*	109 [Zn^{2+}]	α	[1928], [1931], [1939]	3′–5′ polymerase	DNA matrix, 3′-OH DNA or RNA primer, dNTP	7–8	Mg^{2+}	[1955]
(Kornberg enzyme)					3′–5′ exonuclease	ssDNA			[1907], [1956]
					5′–3′ exonuclease	dsDNA, DNA/RNA hybrid			[1933], [2120]
					pyrophosphorylase	DNA matrix, 3′-OH DNA or RNA primer, pp_i			[1911]
					pyrophosphate exchange	DNA, dNTP, pp_i			[1911]
DNA polymerase I,	*Escherichia coli*	76 [Zn^{2+}]	α_C	[1680], [1681], [1996]	3′–5′ polymerase	DNA matrix, 3′-OH DNA or RNA primer, dNTP	7–8	Mg^{2+}	[1996], [2031]
large fragment (Klenow enzyme)					3′–5′ exonuclease	ssDNA			
					pyrophosphorylase	dsDNA			
					pyrophosphate exchange	DNA matrix, 3′-OH DNA or RNA primer, pp_i	7–8	Mg^{2+}	
DNA polymerase I, small fragment	*Escherichia coli*	36	α_N	[1680], [1681], [1816]	5′–3′ exonuclease	dsDNA	7–8	Mg^{2+}	[1688], [1816]
DNA polymerase II	*Escherichia coli*	120	α	[2068], [2143]	3′–5′ polymerase	DNA matrix, 3′-OH DNA or RNA primer,	7–8	Mg^{2+}	[1940], [1995], [1996]
					3′–5′ exonuclease	ssDNA			

(continued)

Table 47. (continued)

Enzyme	Organism	M_n 10^3 dalton	Sub-units	References (isolation)	Specificity[a]	Substrates[b]	pH optimum[c]	Cofactors	References (specificity)
DNA polymerase III	*Escherichia coli*	$[(175_{core})$ $379_{holo}]$	$[(\alpha,\varepsilon,\theta)_{core}$ $\tau,\gamma,\delta,\beta]_{holo}$	[1941], [1980], [1981]	3′–5′ polymerase	ssDNA matrix, 3′-OH RNA primer, dNTP, ATP	7.0	Mg^{2+}	[2069], [2070]
					3′–5′ exonuclease	ssDNA			[1969]
					5′–3′ exonuclease	ssDNA			[1969]
Phage-coded DNA polymerases									
T4 DNA polymerase	*Escherichia coli*, phage T4-infected	114	α	[1694], [1993]	3′–5′ polymerase	DNA matrix, 3′-OH DNA or RNA primer, dNTP	8–9	Mg^{2+}	[2022]
					3′–5′ exonuclease	ssDNA			[1694], [2000]
T5 DNA polymerase	*Escherichia coli*, phage T5-infected	96	α	[2003], [2139]	3′–5′ polymerase	DNA matrix, 3′-OH DNA primer, dNTP	8.5	Mg^{2+}	[2126], [2153]
					3′–5′ exonuclease	ss or dsDNA			[2134]
T7 DNA polymerase	*Escherichia coli*, phage T7-infected	96	α, β	[1917], [2083]	3′–5′ polymerase	DNA matrix, 3′-OH DNA primer, dNTP	7.6–7.8	Mg^{2+}	[1933], [2023]
					3′–5′ exonuclease	ss or dsDNA			[1917], [1927], [2083]

A.2. Eukaryotic DNA polymerases									
Type α DNA polymerases									
DNA polymerase α	*Drosophila melanogaster*	280	4 sub-units	[2092]					
	mouse myeloma cells	190	2 sub-units	[1977], [2116]					
	rat liver	155–250	5 sub-units	[1982], [2130]	3′–5′ polymerase	DNA (RNA) matrix, 3′-OH DNA primer, dNTP	7.5–8.5	Mg^{2+}	[1475], [1938]
	calf thymus	210–230	2 sub-units	[1919], [1922], [1926]	pyrophosphorylase				[2116]
	human HeLa cells	320 (α_1)		[1953]	pyrophosphate				
		600 (α_2)	5 sub-units	[1953]	exchange				[2116]
		220 (α_3)	2 sub-units	[1953]					
	human KB cells	150–160	2 sub-units	[1938], [2131]					
Type β DNA polymerases									
DNA polymerase β	calf thymus	44	α	[2111], [2113]					
	chicken embryo	40	α	[2077]	3′–5′ polymerase	DNA or RNA matrix,	8.4–9.2	Mg^{2+}	[2049], [2112],
	human KB cells	43	α	[2065]		3′-OH DNA primer, dNTP			[2065]
	Novikoff hepatoma cells	31	α	[2049]					
Type γ DNA polymerases									
DNA polymerase γ	chicken embryo	180	α_4	[2076]					
	mouse myeloma cells	140	α_4	[1978]					
	human HeLa cells	110		[1935], [2045]	3′–5′ polymerase	RNA (DNA) matrix, 3′-OH DNA primer, dNTP	8.5–9	Mn^{2+}	[1935], [1943], [1976]
	human lymphoblasts	120		[2024]					

(continued)

Table 47. (continued)

Enzyme	Organism	M_n, 10^3 dalton	Sub-units	References (isolation)	Specificity[a]	Substrates[b]	pH optimum[c]	Cofactors	References (specificity)
Type δ DNA polymerases									
DNA polymerase δ	calf thymus	140–200	α,β	[1540], [1954]	3′–5′ polymerase 3′–5′ exonuclease	RNA matrix, 3′-OH RNA primer, dNTP		Mg^{2+}	[1954], [2105]
Virus-coded DNA polymerases	rabbit bone marrow	122		[2105], [2106]	3′–5′ polymerase 3′–5′ exonuclease	RNA matrix, 3′-OH RNA primer, dNTP		Mg^{2+}	[1540], [2106]
Reverse transcriptase	avian myeloblastosis virus (AMV)	160 [Zn^{2+}]	α,β	[1705], [2146]	3′–5′ polymerase	RNA or DNA matrix, 3′-OH DNA or RNA primer, dNTP	8–8.5	Mg^{2+}	[1705], [1929], [2044]
					RNase H	RNA/DNA hybrid			[1991]
					DNA endonuclease	dsDNA			[2151]
					pyrophosphorylase				[2029], [2048]
					pyrophosphate exchange				[2029], [2048]

Reverse transcriptase	Moloney murine leukemia virus (Mo–MLV)	80 [Zn^{2+}]	α	[1990], [2062], [1705], [2145]	3′–5′ polymerase	RNA or DNA matrix, 3′-OH DNA or RNA primer, dNTP	8–8.5	Mn^{2+}	[1605], [1705], [1990], [2062]
					RNase H	RNA or DNA hybrid			
					DNA endonuclease pyrophosphorylase pyrophosphate exchange	dsDNA			
HSV-1 DNA polymerase	RC-37 cells, HSV-1-infected	144–150	α	[1934], [1957], [2101], [2128]	3′–5′ polymerase	DNA matrix, 3′-OH DNA primer	8–8.5	Mg^{2+}	[1934], [1957], [2101], [2128]
					3′–5′ exonuclease pyrophosphate exchange	ssDNA			
Vaccina virus DNA polymerase	human HeLa cells, vaccina virus-infected	110–115	α	[2107]	3′–5′ polymerase	DNA matrix, 3′-OH DNA primer, dNTP	8–9	Mg^{2+}	[2108]
					3′–5′ exonuclease	ssDNA			
Terminal transferases					pyrophosphate exchange				
Terminal transferase (Bollum enzyme)	calf thymus	62 [Co^{2+}]	α	[1726], [1908]	3′–5′ polymerase	3′-OH DNA primer, dNTP	7.2	Mg^{2+}, Co^{2+}	[1718]

(continued)

Table 47. (continued)

Enzyme	Organism	M_r 10^3 dalton	Sub-units	References (isolation)	Specificity[a]	Substrates[b]	pH optimum[c]	Cofactors	References (specificity)
B. RNA Polymerases									
B.1. Prokaryotic RNA polymerases									
Bacterial RNA polymerases									
RNA polymerase	*Escherichia coli*	454 [Zn^{2+}]	*	[1659], [2102], [2109], [2004], [2005], [2006], [2080]					
	Acetobacter vinelandii	400–500 [Zn^{2+}]	*	[1946], [1947]	3′–5′ RNA polymerase	DNA matrix, NTP	7–8	Mg^{2+}	[1947], [1973]
	Bacillus subtilis	400–500 [Zn^{2+}]	*	[2089], [2038]	de novo RNA synthesis	ATP and UTP or ITP and CTP	7–8	Mg^{2+}	[1944]
	Caulobacter crescentus	400–500 [Zn^{2+}]	*	[2095]	pyrophosphate exchange	poly[d(AT)], ATP, UTP, pp_i	7–8	Mg^{2+}	[1945], [2042]
	Lactobacillus curvatus	400–500 [Zn^{2+}]	*	[2054]					
	Micrococcus luteus	400–500 [Zn^{2+}]	*	[1923], [1961]					
Phage-coded RNA polymerases									
T7 RNA polymerase	*Escherichia coli*, phage T7-infected	98	α	[1735], [1743]			7–8	Mg^{2+}	[1735], [1745], [1743]
T3 RNA polymerase	*Escherichia coli*, phage T3-infected	105	α	[2094], [1734], [1979]			7–8	Mg^{2+}	[2094], [1979]
N4 RNA polymerase	*Escherichia coli*, phage N4-infected	350	α	[2007]			7–8	Mg^{2+}	[2110], [2007]
SP6 RNA polymerase	*Salmonella typhimurium*, phage SP6-infected	96	α	[1732], [1737]	3′–5′ RNA polymerase	DNA matrix, NTP	7–8	Mg^{2+}	[1732], [1737]
PBS2 RNA polymerase	*Bacillus subtilis*, phage PBS2-infected	260	α,β,γ,δ,ε	[2118]			7–8	Mg^{2+}	[2110], [2118]

Poly(A) polymerases									
Poly(A) polymerase	*Escherichia coli*	50	α	[1822]	terminal riboadenylate transferase	3′-OH RNA	7–9	ATP Mg^{2+} or Mn^{2+}	[1821], [1822]
Poly(A) polymerase	calf thymus	60	α	[2059]	terminal riboadenylate transferase	3′-OH RNA	7.4	ATP, Mn^{2+} or ATP, Mg^{2+}	[2059] [2073]
		120–140	α_2						
Poly(A) polymerase	human HeLa cells	63–75	α	[1999]	terminal riboadenylate transferase	3′-OH RNA	7.9	ATP, Mn^{2+} or ATP, Mg^{2+}	[1999] [1999]
		50–58	α						
Polynucleotide phosphorylases									
PNPase	*Escherichia coli*	252	α_3	[2010]	polyribonucleotide nucleotidyltransferase ADP-p_i exchange	NDP		Mg^{2+}	[2057] [2072]
PNPase	*Micrococcus luteus*	237		[2093]	polyribonucleotide nucleotidyltransferase ADP-p_i exchange	NDP		Mg^{2+}	[2093] [2057]

(continued)

Table 47. (continued)

Enzyme	Organism	M_n 10^3 dalton	Sub-units	References (isolation)	Specificity[a]	Substrates[b]	pH optimum[c]	Cofactors	References (specificity)
tRNA nucleotidyltransferases									
tRNA nucleotidyltransferase B	*Escherichia coli*	45–54	α	[1985]	tRNA nucleotidyltransferase	tRNA, ATP and CTP	9–9.4	Mg^{2+}	[2104]
tRNA nucleotidyltransferase	baker's yeast	70–71	α	[2051]	tRNA nucleotidyltransferase	tRNA, ATP and CTP	9.5	Mg^{2+}	[2052]
B.2. Eukaryotic RNA polymerases									
RNA polymerases									
RNA polymerase I	yeast	500–600 [Zn^{2+}]	11 sub-units	[2030], [2061]					
	wheat germ	400–500	7 sub-units	[2081], [2082]					
	Acanthamoeba castellanii	500–600	11 sub-units	[2085], [2086]					
RNA polymerase II	yeast	500–600 [Zn^{2+}]	9 sub-units	[2030], [2061]					
	wheat germ	500–600 [Zn^{2+}]	11 sub-units	[2081], [2082]	3′–5′ polymerase	ss (ds) DNA matrix, NTP	7–8	Mn^{2+}	[1920], [1960]
	Acanthamoeba castellanii	500–600	10 sub-units	[2085], [2086]					
RNA polymerase III	yeast	500–600 [Zn^{2+}]	10 sub-units	[2030], [2061]					
	wheat germ	500–600	14 sub-units	[2081], [2082]					
	Acanthamoeba castellanii	500–600	14 sub-units	[2085], [2086]					
Organelle-specific RNA polymerases									
RNA polymerase	maize chloroplasts	500	α,β,γ,δ	[1536], [1932]	3′–5′ polymerase	DNA matrix, NTP		Mg^{2+}	[2043], [1932]

RNA polymerase	yeast mitochondria	100–150	α_{2-3}	[1958]	3′–5′ polymerase	DNA matrix, NTP	Mg^{2+}	[1958]
2′,5′-Oligoadenylate synthetases								
2′,5′-Oligoadenylate synthetase	chicken embryonic cells	50–60		[2090]	2′,5′-oligoadenylate adenyltransferase	dsRNA, ATP	7.0–8.0	Mg^{2+}	[2090], [2091], [2026]
2′,5′-Oligoadenylate synthetase	Ehrlich ascites tumor cells	85		[2090], [1912]	2′,5′-oligoadenylate adenyltransferase	dsRNA, ATP	7.0–8.0	Mg^{2+}	[2091], [1987], [2026]
C. Nucleases								
C.1. Restriction endonucleases (see Table43)								
C.2. Endonucleases								
Double-strand specific endonuclease								
DNase I	bovine pancreas	31	α	[1751], [1753]	endonuclease	dsDNA	7.0	Ca^{2+}, Mg^{2+}	[1752], [1756], [1757]
Single-strand specific endonucleases								
Nuclease S1	*Aspergillus oryzae*	38 [Zn^{2+}]	α	[2087], [1775]	endonuclease	ssDNA (RNA), partially denatured dsDNA, DNA/RNA hybrid	4.0–4.3	Co^{2+}, Zn^{2+}	[2035], [1930], [1774]
P. citrinum nuclease	*Penicillium citrinum*			[2137]	endonuclease	ssRNA (DNA)	5.0	Zn^{2+}	[2138]
U. maydis nuclease	*Ustilago maydis*	42 [Zn^{2+}]	α	[1925]	endonuclease	ssDNA (RNA)	8.0	Mg^{2+}, Ca^{2+}, Co^{2+} or Zn^{2+}	[1924]

(continued)

Table 47. (continued)

Enzyme	Organism	M_n 10^3 dalton	Sub-units	References (isolation)	Specificity[a]	Substrates[b]	pH optimum[c]	Cofactors	References (specificity)
Mung bean nuclease	mung beans	39 [Zn^{2+}]	α	[2087]	endonuclease	ssDNA or ssRNA	5.0	Zn^{2+}	[1775]
WS nuclease	wheat germ	43 [Zn^{2+}]	α	[1948]	endonuclease	ssDNA (RNA)	4.8–5.5	Zn^{2+}	[1948]
AP endonucleases									
Endonuclease III	*Escherichia coli*	27	α	[2142]	AP endonuclease DNA glycosylase	dsAP DNA thymine dimers	7	EDTA resistant	[1909] [2013]
Endonuclease IV	*Escherichia coli*	33	α	[1970]	AP endonuclease	dsAP DNA	8.0–8.5	EDTA resistant	[2136], [1970]
Endonuclease V	*Escherichia coli*	20	α	[2141]	AP endonuclease	dsAP DNA	9.2–9.5	Mg^{2+}	[2136], [2141]
Endonuclease VII	*Escherichia coli*	45	α	[2135]	AP endonuclease	ssAP DNA	7.0	Mg^{2+} or Ca^{2+}	[2135], [2136]
Mlu AP endoA	*Micrococcus luteus*	35	α	[2009]	AP endonuclease	dsAP DNA	7.5	(Mg^{2+})	[2009]
Mlu AP endoB	*Micrococcus luteus*	35	α	[2009]	AP endonuclease	dsAP DNA	6.5–8.0	(Mg^{2+})	[2009]
Hin AP endo	*Haemophilus influenzae*	30	α	[2119]	AP endonuclease 3′-exonuclease 3′-phosphatase	dsAP DNA	7–8	Mg^{2+} or Mn^{2+}	[2119]
Bsu AP endo	*Bacillus subtilis*	56	α	[2098]	AP endonuclease			(Mg^{2+})	[2098]
CL AP endo	calf liver	28	α	[1949]	AP endonuclease		9.5	Mg^{2+}	[1949]

HP AP endo	human placenta	27–31	α	[1964]	AP endonuclease			(Mg^{2+})	[1964]
C.3. Exonucleases									
***E. coli*exonucleases**									
Exonuclease I	*Escherichia coli*	70–72	α	[1972], [2014]	3′–5′ exonuclease	ssDNA	9.5	Mg^{2+}	[2020], [2075]
Exonuclease III	*Escherichia coli*	28	α	[1928], [2066]	3′–5′ exonuclease	dsDNA	7.6–8.5	Mg^{2+}	[1707]
					DNA 3′-phosphatase	3′-P DNA			[1983]
					AP endonuclease	apurinic–apyrimidinic DNA			[1764]
					RNase H	RNA/DNA hybrid			[1785]
Exonuclease IV	*Escherichia coli*			[2099]	3′–5′ exonuclease	ssDNA	8.0–9.5	Mg^{2+}	[2099], [2067]
Exonuclease V	*Escherichia coli*	270	α,β	[2149]	3′–5′ exonuclease 5′–3′ exonuclease	dsDNA	9	ATP, Mg^{2+}	[2074]
Exonuclease VII	*Escherichia coli*	88	α	[1788]	3′–5′ exonuclease 5′–3′ exonuclease	ssDNA	7.9	EDTA resistant	[2114]
Exonuclease VIII	*Escherichia coli*	140	α	[2148]	5′–3′ exonuclease	dsDNA	8.0–9.0	Mg^{2+}	[1952]

(continued)

Table 47. (continued)

Enzyme	Organism	M_n, 10^3 dalton	Sub-units	References (isolation)	Specificity[a]	Substrates[b]	pH optimum[c]	Cofactors	References (specificity)
Exonuclease V-analogous enzymes									
*Hin*d exonuclease V	*Haemophilus influenzae*	290	α,β,γ	[2117], [1774], [2071]	3′–5′ exonuclease 5′–3′ exonuclease	dsDNA	9	ATP, Mg^{2+}	[2117], [2071]
Bsu exonuclease V	*Bacillus subtilis*	300	α,β	[1997], [2033]	3′–5′ exonuclease 5′–3′ exonuclease	dsDNA	9	ATP, Mg^{2+}	[2033]
Pae exonuclease V	*Pseudomonas aeruginosa*	300		[1984], [1997]	3′–5′ exonuclease 5′–3′ exonuclease	dsDNA	9	ATP, Mg^{2+}	[1984]
Sin exonuclease V	sea urchin intermedius	450		[2140], [1997]	3′–5′ exonuclease 5′–3′ exonuclease	dsDNA	9	ATP, Mg^{2+}	[2140]
Other exonucleases									
Nuclease Bal 31	*Altermonas espejiani*	73	α	[2152], [1916]	3′–5′ exonuclease 5′–3′ exonuclease	ss or dsDNA	8.0	Mg^{2+}, Ca^{2+}	[2152], [1792], [1916], [1538]
N. crassa nuclease	*Neurospora crassa*	55 [Zn^{2+}]	α	[1962], [1963]	exonuclease endonuclease	linear ss or dsDNA circular ssDNA or ssRNa	7.5–8.5	Mg^{2+}	[2132], [2133]

Phage-coded exonucleases									
λ exonuclease	*Escherichia coli*, phage λ-infected	52	α	[2150], [2012]	5′–3′ exonuclease	dsDNA, 5′-P terminus	8–9	Mg^{2+}	[1965], [2047]
D. RNA nucleases									
D.1.*E. coli*ribonucleases									
RNase I	*Escherichia coli*	low	α	[2046]	endonuclease	ssRNA, (A/U)p/N	8.1		[2046]
RNase II	*Escherichia coli*	68–85	α	[1918]	3′–5′ exonuclease	ssRNA, 3′-OH terminus	7–8	Mg^{2+}, K^{+}	[2001]
RNase III	*Escherichia coli*	50	α_2	[2125], [1914]	endonuclease	ss or dsRNA, secondary structures	7–8	Mg^{2+} or Mn^{2+}; NH_4^{+}, Na^{+}, or K^{+}	[1915], [2025]
RNase IV	*Escherichia coli*			[1881]	endonuclease	ssRNA secondary structures			[2034]
RNase H	*Escherichia coli*	40	α	[2122], [1796], [1986]	endonuclease	DNA/RNA hybrid	7.5–9.1	Mg^{2+} or Mn^{2+}	[2123], [1795]
D.2. Other ribonucleases									
RNase A	bovine pancreas	13.7	α	[1951]	endonuclease	ssRNA, (C/U)/pN	7.0–7.5		[2097], [1806]
RNase CL3	chicken liver	16.85	α	[1804], [1805]	endonuclease	ssRNA, C(A/G)p/N	8.0		[1806]
RNase T_1	*Aspergillus oryzae*	11.1	α	[2127], [1809]	endonuclease	ssRNA, Gp/N	3.5		[2127], [1806]

(continued)

Table 47. (continued)

Enzyme	Organism	M_n, 10^3 dalton	Sub-units	References (isolation)	Specificity[a]	Substrates[b]	pH optimum[c]	Cofactors	References (specificity)
RNase U_2	*Ustilago spherogena*	10.0	α	[1810]	endonuclease	ssRNA, Ap/N	3.5		[1829], [2127], [1806]
Nuclease S7	*Staphylococcus aureus*	16.8	α	[1812], [2058]	endonuclease	ssRNA, Np/(A/U)	7.5	Ca^{2+}	[1806]
D.3. tRNA-processing enzymes									
RNase P	*Escherichia coli*	17.5 + 120	α, Ml RNA	[1936]	endonuclease RNA	tRNA or rRNA precursor	8	Mg^{2+}; NH_4^+, Na^+, or K^+	[1936], [1937], [2050]
RNase D	*Escherichia coli*	40		[2124]	exonuclease	tRNA precursor, 3′-terminus	9–10	Mg^{2+}	[2147], [1937]
RNase P_2	*Escherichia coli*			[1676]	endonuclease	tRNA precursor			[2027]
RNase O	*Escherichia coli*			[2036]	endonuclease	tRNA precursor	7.5–10	Mg^{2+} or Mn^{2+}	[2036]
E. DNA-modifying enzymes									
E.1. Restriction methylases (see Table43)									
E.2. Phosphatases									
Alkaline phosphatase	calf intestine	140 [Zn^{2+}]	α_2	[1817]	phosphatase	5′-P or 3′-P DNA or RNA	7.5–9.5	Zn^{2+}	[1694], [1992]

Alkaline phosphatase	*Escherichia coli*	80 [Zn^{2+}]	α_2	[1720]	phosphatase	5′-P or 3′-P DNA or RNA	7.5–9.5	Zn^{2+}	[2016]
Phosphodiesterase	calf spleen			[2100]	phosphodiesterase	5′-OH DNA or RNA	5–7		[2100]
Phosphodiesterase	*Crotalus durissus*			[2018]	phosphodiesterase	3′-OH DNA or RNA	5–7		[1537]
E.3. DNA ligases									
Bacterial DNA ligases									
DNA ligase	*Escherichia coli*	77	α	[1989], [1856]	polydeoxyribonucleotide synthase	5′-P DNA or RNA, 3′-OH DNA or RNA (overlapping single-stranded ends of dsDNA or dsRNA)	7.5–8	Mg^{2+}, NAD	[1998], [2032], [2055]
Phage-coded DNA ligases									
T4 DNA ligase	*Escherichia coli*, phage T4-infected	68	α	[1845], [1847]	polydeoxyribonucleotide synthase	5′-P DNA or RNA, 3′-OH DNA or RNA (overlapping or blunt, single-stranded or double-stranded ends of ds DNA or dsRNA)	7.2–7.8	Mg^{2+}, ATP	[1624], [2129], [1998], [2032], [2055]
					pyrophosphate exchange	ATP, pp_i		Mg^{2+}	[1850]
E.4. Kinases									
T4 polynucleotide kinase	*Escherichia coli*, phage T4-infected	140	α_4	[1861], [1847]	5′-DNA kinase	5′-OH DNA or RNA, NTP or dATP	7.4–8.0	Mg^{2+}	[2002], [2019], [2021]
					3′-phosphatase	3′-P DNA or RNA	5.5–6.0	Mg^{2+}	[1864]

(continued)

Table 47. (continued)

Enzyme	Organism	M_r 10^3 dalton	Sub-units	References (isolation)	Specificity[a]	Substrates[b]	pH optimum[c]	Cofactors	References (specificity)
T4 polynucleotide kinase, 3′-phosphatase-free	*Escherichia coli*, phage T4*pse*T1-infected	140	α_4	[2103], [1882]	5′-DNA kinase	5′-OH DNA or RNA, NTP or dATP	7.4–8.0	Mg^{2+}	[2103], [2021], [1882]
DNA kinase	rat liver	80		[1959]	5′-DNA kinase	5′-OH DNA, ATP	5.5	Mg^{2+}	[1959], [2008], [1741]
DNA kinase	calf thymus	70		[2088]	5′-DNA kinase	5′-OH DNA, ATP	5.5	Mg^{2+}	[2088], [1741]
DNA kinase	human HeLa cells			[2039]	5′-RNA kinase	5′-OH RNA, ATP	5.5	Mg^{2+}	[2039], [1741]
E.5. DNA topoisomerases									
Type I DNA topoisomerases									
ω protein	*Escherichia coli*	100–120	α	[1910]	transient ssDNA breakage:		7–8	Mg^{2+}	[2064]
M. luteus topoisomerase I	*Micrococcus luteus*	100–120	α	[1950]	ccDNA relaxation DNA–catenane	dsDNA dsDNA			
HeLa topoisomerase I	human HeLa cells	100	α	[1966]	knot formation				
Type II DNA topoisomerases									
E. coli DNA gyrase	*Escherichia coli*		α,β	[1988]	transient dsDNA breakage: cccDNA formation	dsDNA, ATP	7–8	Mg^{2+}	[2144]
M. luteus DNA gyrase	*Micrococcus luteus*	420–430	$(\alpha\beta)_2$	[1967]	DNA-dependent ATPase cccDNA relaxation	dsDNA, ATP dsDNA			

T4 topoisomerase I	*Escherichia coli*, phage T4-infected		α,β	[1968], [2053]	DNA–catenane knot formation	dsDNA dsDNA		
E.6. DNA helix unwinding enzymes								
rep protein	*Escherichia coli*, phage ΦX174-infected	65	α	[2028]	DNA unwinding DNA–dependent ATPase	dsDNA, *E. coli* ssb protein ssDNA, ATP	Mg^{2+}	[2028], [2079]
Helicase III	*Escherichia coli*	40	α_2	[1913]	5′–3′ DNA unwinding DNA–dependent ATPase	dsDNA, *E. coli* ssb protein ssDNA, ATP	Mg^{2+}	[2078]
E.7. Single-strand DNA-binding proteins								
E. coli ssb protein	*Escherichia coli*		α_4	[2115]	ssDNA binding	ssDNA, 8 nucleotides per monomer	6.0 (IP)	[1942]
T4 gene 32 protein	*Escherichia coli*, phage T4-infected		$\alpha_{1-2/n}$	[2096]	ssDNA binding	ssDNA, 7 nucleotides per monomer	5.5 (IP)	[1942]
T7 DNA-binding protein	*Escherichia coli*, phage T7-infected		α	[2017]	ssDNA binding	ssDNA	7 (IP)	[1942]
fd gene 5 protein	*Escherichia coli*, phage fd-infected		α_{1-2}	[2084]	ssDNA binding	ssDNA, 4 nucleotides per monomer	8.0 (IP)	[1942]

(continued)

Table 47. (continued)

Enzyme	Organism	M_n, 10^3 dalton	Sub-units	References (isolation)	Specificity[a]	Substrates[b]	pH optimum[c]	Cofactors	References (specificity)
CT HDP-I protein	calf thymus		α	[1921]	ssDNA binding	ssDNA, 7 nucleotides per monomer	7.8 (IP)		[1942]
F. RNA-modifying enzymes									
F.1. RNA ligases									
T4 RNA ligase	*Escherichia coli*, phage T4-infected	43	α	[2121], [1878]	polyribonucleotide synthase	5′-P RNA (DNA), 3′-OH RNA (DNA)	7.2–8.4	Mg^{2+}	[1994], [2055], [2056], [2060], [2063]
					pyrophosphate exchange	ATP, pp_i		Mg^{2+}	[2121]
F.2. CAP-forming enzymes[c]									
Capping enzyme	smallpox virus	127	α,β	[1975], [2041]	RNA triphosphatase	pppRNA	7.8	Mg^{2+}	[1974], [2040]
					RNA guanyltransferase	GTP + ppRNA			
					RNA (guanine-7) methyltransferase	GpppRNA + SAM			

					GTP–pp_i exchange nucleoside	GTP + pp_i		
					NTP triphosphate phosphohydrolase			
F.3. CAP-splitting enzymes[d]								
Decapping enzyme (TAP)[e]	*Nicotiana tabacum* var. Wisconsin 38	280	α_4	[2037]	acid pyrophosphatase	7-methyl-GpppN	6.0	[1971], [2037]

[a] AP = apurinic.
[b] cc = closed circular; ccc = covalent closed circular.
[c] IP = isoelectric point.
[d] CAP = 7-methyl-GpppN.
[e] TAP = tobacco acid pyrophosphatase.
* $[(\beta'\beta\alpha_2)_{core}{}^{a}]_{holo}$

The purified enzyme is most stable in the pH range 7.5–9.5 and is inactivated rapidly at lower pH. Protection against acidic denaturation is observed in the presence of inorganic phosphate [1834]. Alkaline phosphatase can also be inactivated either by treatment with NaOH or by heating for 45 min at 65 °C in the presence of nitrilotriacetic acid or ethylenediaminetetraacetic acid to chelate the essential Zn^{2+} ions [1835]. Alternatively, alkaline phosphatase can be inhibited by inorganic phosphate. In the first two methods, RNA or DNA may be damaged. However, treatment with the chelating agents completely inactivates alkaline phosphatase without damage to the polynucleotide.

Uses Alkaline phosphatase catalyzes the hydrolysis of numerous phosphate esters, such as esters of primary and secondary alcohols, sugar alcohols, cyclic alcohols, phenols, and aminoalcohols. Phosphodiesters do not react. The enzyme is used preferentially to selectively cleave terminal phosphate groups from oligonucleotides and monophosphate esters. In molecular biology, alkaline phosphatase is used primarily for dephosphorylation of 5′-phosphorylated DNA or RNA ends. These 5′-hydroxylated substrates can be effectively 5′-endlabeled with T4 polynucleotide kinase and γ-^{32}P-ATP as substrate [1817], [1719], [1836], [1837], [1818], [1838], [1839]. 3′-Phosphorylated ends of RNA are also dephosphorylated with alkaline phosphatase [1838], [1831]. Either DNA or RNA that is ^{32}P-labeled at the 5′-terminus is most frequently used in chemical DNA sequencing [1837], [1818] or in RNA sequencing by degradation of end-labeled RNA with base-specific RNases [1838], [1831]. However, alkaline phosphatase is also involved in the labeling of DNA and RNA fragments used for mapping and fingerprinting studies [1840], [1841]. The enzyme is further used in the construction of recombinant DNA molecules. Self-annealing of the vector DNA can be suppressed considerably by dephosphorylation of the linearized vector molecule prior to insertion of the DNA fragment to be cloned [1842], [1843], [1844].

6.5.6.2 T4 DNA Ligase

The enzyme T4 DNA ligase, also called poly(deoxyribonucleotide) : poly(deoxyribonucleo-tide) ligase (AMP-forming) (E.C. 6.5.1.1) [9015-85-4], is obtained from bacteriophage T4-infected *Escherichia coli*.

Properties The molecular mass of T4 DNA ligase is 65×10^3 dalton [1845], [1846]. The enzyme is composed of a single subunit [1847]. The T4 DNA ligase depends on ATP as a cofactor in the joining reaction [1848]. The energy of ATP hydrolysis to yield AMP and pyrophosphate is used to form a phosphodiester linkage between polynucleotide chains. The enzyme also catalyzes an exchange reaction between pyrophosphate and ATP [1849].

One unit of T4 DNA ligase is defined as the enzyme activity that converts 1 nmol of ^{32}P from pyrophosphate into material that can be absorbed by Norit in 20 min at 37 °C [1849]. The optimal pH range for T4 DNA ligase is 7.2–7.8; at pH 6.9 and 8.0, the enzyme has 46 and 56 %, respectively, of its activity at pH 7.6 [1850]. The enzyme requires Mg^{2+} for activity; Mn^{2+} is only 25 % as effective as Mg^{2+}. Low concentrations of NH_4^+ ions have no effect on the T4 DNA ligase reaction. Higher levels of monovalent cations inhibit the enzyme completely [1851].

Uses The T4 DNA ligase is used to ligate DNA fragments with either 5′- or 3′-protruding or blunt ends. This enzyme is the only DNA ligase known that can catalyze blunt-end joining [1852]. An important use of T4 DNA ligase is the preparation of recombinant DNA molecules for cloning experiments. Hydrogen-bonded recombinant DNA molecules can be generated by annealing two DNA fragments containing cohesive ends; T4 DNA ligase is the enzyme of choice for joining such cohesive ends since it requires a smaller overlapping sequence than *E. coli* DNA ligase.

Cohesive ends can be generated by cleavage of DNA with class II restriction endonucleases. An alternative approach is the addition of complementary homopolymer tails to the appropriate fragments with terminal transferase (G—C tailing). Cohesive ends can also be generated by blunt-end ligation with a synthetic DNA linker that contains the recognition sequence for a restriction endonuclease producing cohesive termini.

After labeling internal 5′-ends with T4 polynucleotide kinase, T4 DNA ligase can also be used to identify 3′- and 5′-end groups at single-stranded interruptions by nearest neighbor analysis [1850]. Further, T4 DNA ligase can be applied to determine the ability of other enzymes to act at nicks and gaps in duplex DNA molecules. In addition, T4 DNA ligase can be used to study the primary and secondary structure of DNA molecules [1853] and may be applied to the chemical synthesis of double-stranded DNAs with specific nucleotide sequences [1854].

6.5.6.3 *Escherichia coli* DNA Ligase

The enzyme *E. coli* DNA ligase, also called poly(deoxyribonucleotide) : poly(deoxyribonucleotide) ligase (AMP-forming, NMN-forming) (E.C. 6.5.1.2) [37259-52-2], is obtained from *Escherichia coli*.

Properties *Escherichia coli* DNA ligase consists of a single polypeptide chain with a molecular mass of 74×10^3 dalton [1855], [1856]. The bacterial enzyme catalyzes phosphodiester-bond synthesis coupled to cleavage of the pyrophosphate group in NAD.

One unit of *E. coli* DNA ligase is defined as the enzyme activity that converts 100 nmol poly[d(AT)] to an exonuclease III-resistant form in 30 min at 30 °C [1857].

The NAD-dependent *E. coli* DNA ligase is much more specific than the ATP-dependent T4 DNA ligase. A number of ligations catalyzed by T4 DNA ligase are not catalyzed by *E. coli* DNA ligase, e.g., blunt-end ligation [1858], [1859]. The *E. coli* DNA ligase also does not act as an RNA ligase joining RNA molecules or DNA molecules containing RNA primer sequences.

Uses The *E. coli* DNA ligase is used in full-length cDNA synthesis [1724]. This method complements the present technique that employs nuclease S1 digestion of the hairpin loop prior to the second-strand synthesis. With this new technique, second-strand synthesis is mediated by the synchronous action of the enzymes *E. coli* DNA polymerase I, *E. coli* RNase H, and *E. coli* DNA ligase after first-strand synthesis with reverse transcriptase. The procedure uses a plasmid DNA vector which itself serves as a primer for the first- and, ultimately, the second-strand cDNA synthesis. Both steps are designed to enrich for recombinants containing full-length cDNAs over those with truncated cDNAs.

6.5.6.4 T4 Polynucleotide Kinase

The enzyme T4 polynucleotide kinase, also called ATP : 5′-dephosphopolynucleotide-5′-phosphotransferase (E.C. 2.7.1.78) [37211-65-7], is obtained from phage T4-infected *Escherichia coli*.

Properties The T4 polynucleotide kinase catalyzes the transfer for the terminal γ-phosphate group of ATP to the 5′-hydroxylated termini of polynucleotides like DNA or RNA. It also catalyzes the exchange of 5′-terminal phosphate groups [1860], [1839].

One unit of T4 polynucleotide kinase is defined as the enzyme activity required for the formation of 1 nmol of acid-precipitable ^{32}P in 30 min at 37 °C [1839].

The enzyme T4 polynucleotide kinase migrates as a single species in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The single band with a molecular mass of 33×10^3 dalton represents one of the four identical subunits of the active enzyme complex with a total molecular mass of 140×10^3 dalton [1861], [1847]. Maximum activity is obtained at pH 7.6 at 37 °C and requires Mg^{2+} ions and such reagents as dithiothreitol or 2-mercaptoethanol [1847]. The reported stimulating effect of higher ionic strength or polyamides like spermine or spermidine [1836], [1847] results from stabilization of the active oligomeric tertiary structure of the enzyme [1861]. Concentrations of ATP of at least 1 μmol/L and a ratio of 5 : 1 between ATP and protruding 5′-hydroxyl ends are required for optimal phosphorylation [1862]. The T4 polynucleotide kinase is inhibited to 50 % by 7 mmol/L of sodium or potassium phosphate and to 75 % by 7 mmol/L of ammonium sulfate [1847]. In addition to its kinase activity, T4 polynucleotide kinase also exhibits 3′-phosphatase activity [1863], [1864]. The pH optimum of this activity is between 5.0 and 6.0 and, thus, different from that of the kinase activity at pH 7.6.

Uses The enzyme T4 polynucleotide kinase is used to label DNA and RNA at its 5′-termini with ^{32}P residues by using γ-^{32}P-ATP as substrate [1865], [1862], [1839]. The 5′-terminus of DNA can be labeled with T4 polynucleotide kinase either by direct phosphorylation of 5′-hydroxyl groups or by exchange of DNA-bound, nonradioactively labeled 5′-phosphoryl groups and ^{32}P molecules [1866], [1868], [1818]. Alternative conditions for both reactions are also described for 5′-endlabeling of RNA [1829], [1831]. The T4 polynucleotide kinase is most commonly used in chemical sequence determination of DNA [1837], [1818] and RNA [1829], [1869], [1831], [1832]. Chemical DNA sequencing can determine the sequence of both the coding and the noncoding strands. Sequencing of the coding strand in the 5′–3′ direction is possible after labeling the 5′-end of DNA with T4 polynucleotide kinase and γ-^{32}P-ATP (Fig. 106). Sequencing of the complementary noncoding strand in the 3′-5′ direction is possible if a recessed 3′-end of dsDNA is elongated with Klenow enzyme and suitable α-^{32}P-dNTPs as substrates. 5′-Endlabeling is also used for mapping restriction sites by partial digestion [1870], [1871]; DNA [1831] or RNA fingerprinting [1872], [1873]; DNA footprinting via DNase protection [1874] or methylation protection [1875] ; hybridization studies [1876]; synthesis of substrates for DNA or RNA ligase [1877], [1878]; and sequence analysis of DNA [1879], [2015]. In addition, the 3′-phosphatase activity of T4 polynucleotide kinase may be used as a specific 3′-phosphatase [1863].

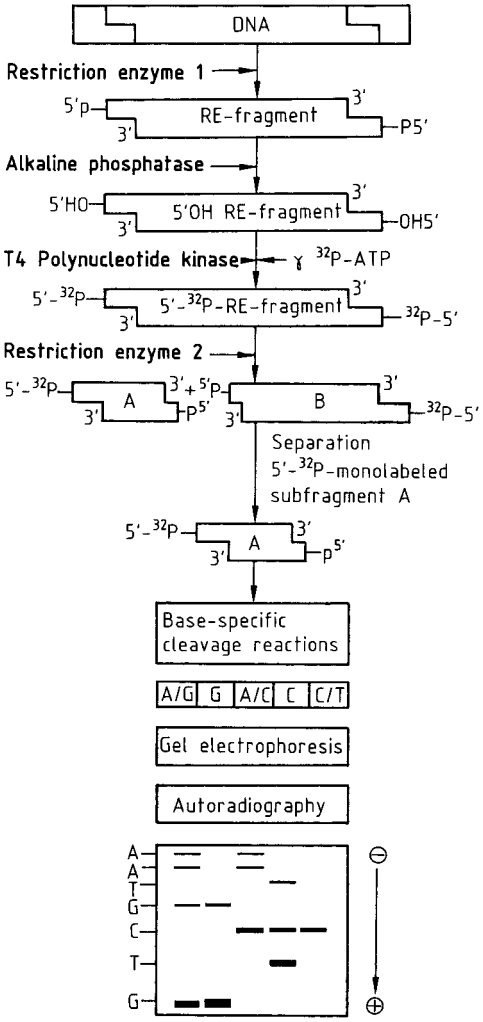


Fig. 106 Chemical DNA sequencing

6.5.6.5 T4 Polynucleotide Kinase, 3′-Phosphatase-Free

3′-Phosphatase-free T4 polynucleotide kinase, also called ATP : 5′-dephosphopolynucleotide 5′-phosphotransferase (E.C. 2.7.1.78) [37211-65-7], is obtained from phage T4 *pse*T1- infected *Escherichia coli*.

Properties 3′-Phosphatase-free T4 polynucleotide kinase is an altered T4 *pse* T1 gene product in which only the 3′-phosphatase activity but not the 5′-kinase activity has been affected [1863], [1864], [1880], [1881].

One unit of 3′-phosphatase-free T4 polynucleotide kinase is defined as the enzyme activity required for the formation of 1 nmol of acid-precipitable ^{32}P in 30 min at 37 °C [1839].

Maximum activity of the 5′-kinase reaction is obtained under the same conditions as those for wild-type T4 polynucleotide kinase. However, even under optimal conditions of the wild-type 3′-phosphatase activity, no removal of 3′-phosphatase groups of 3′-AMP is observed with the mutant T4 polynucleotide kinase at pH 5.5–6.0.

Uses 3′-Phosphatase-free T4 polynucleotide kinase is of special interest in RNA analysis. The absence of the 3′-phosphatase makes this enzyme extremely useful for the preparation of unique species of RNA phosphorylated at both the 5′- and the 3′-termini. Oligoribonucleotides with both 3′- and 5′-terminal phosphates are used as donors in the T4 RNA ligase reaction [1882], [1883]. Although the 3′-phosphatase is not required for activity with T4 RNA ligase, it is a convenient blocking group to prevent cyclization or self-addition of the donor. Thus, a unique intermolecular product is ensured.

Another use of 3′-phosphatase-free T4 polynucleotide kinase is the labeling of CMP to give 5′-^{32}P-Cp, which is commonly used for 3′-endlabeling of RNA with T4 RNA ligase [1884]. The 3′-terminally labeled RNA is very useful for fingerprinting and sequencing studies [1829], [1817], [1831].

6.5.6.6 Methylase *HpaII*

Methylase *Hpa*II, also called *S*-adenosyl-L-methionine:DNA (cytosine-5)-methyltransferase (E.C. 2.1.1.37) [9037-42-7], is obtained from *Haemophilus parainfluenzae*.

Properties Methylase *Hpa*II is isolated from *H. parainfluenzae* containing methylase *Hpa*I as well [1885], [1886]. Methylase *Hpa*I acts on double-stranded DNA by transferring methyl groups from *S*-adenosylmethionine to the palindromic recognition site

$$\begin{matrix} 5' & & 3' \\ & CCGG & \\ 3' & GGCC & 5' \end{matrix}$$

which results in 5-methylation of both internal cytosines [1885], [1887].

One unit of methylase *Hpa*II is defined as the enzyme activity required to protect 1 μg of λ DNA to $>$ 95 % against cleavage by the restriction endonuclease *Hpa*II in 1 h at 37 °C [1885]. Single-stranded DNA is methylated by methylase *Hpa*II with very low efficiency. However, methylase *Hpa*II is capable of methylating hemimethylated recognition sequences [2011]. Since divalent cations such as Mg^{2+} are not essential for its activity, methylation of DNA with methylase *Hpa*II may be carried out in the presence of ethylenediaminetetraacetic acid. Site-specific methylation of DNA by methylase *Hpa*II protects the DNA against digestion by the restriction endonuclease *Hpa*II which recognizes and digests the identical tetrameric sequence CCGG [1888]. However, the activity of restriction endonuclease *Msp*I, an isoschizomer of *Hpa*II, is not affected by methylation of CCGG residues with methylase *Hpa*II because *Msp*I is only sensitive to methylation of the external cytosine residues [1889], [1890]. In addition, the action of the restriction endonuclease *Sma*I whose recognition site CCCGGG is a subset of the *Hpa*II sites, is also inhibited by methylation of the internal cytosine residue with methylase *Hpa*II. In contrast, the activity of *Xma*I

(similar to *Msp*I) is not inhibited by methylation of *Hpa*II sites with methylase *Hpa*II [1891].

Uses Methylase *Hpa*II is a useful tool for studying the in vivo effect of distinctly methylated $^{5\prime m}CpG^{3\prime}$ residues toward gene expression in eukaryotic cells. These 5-methylcytosine residues are located predominantly in CpG sequences [1892], [1893], [1894] at a level of about 1 % of the total nucleotides [1895]. An inverse correlation between the amount of CpG residues and gene activity was postulated [1896], [1897]. These results were confirmed by the gene-stimulating effect of azacytidine in vivo, because incorporation of this analogue into DNA mimics an undermethylated state of DNA [1898]. To study the in vivo effects of distinct methylation patterns, methylase *Hpa*II is used to introduce in vitro methylated CpG residues at the specific CCGG sites of isolated DNA [1899]. The DNA, which was previously methylated with methylase *Hpa*II to different degrees, is microinjected into frog oocytes [1900], or eukaryotic cells are transformed with the modified DNA by DNA-mediated gene transfer [1901]. The in vivo effects of the distinct *Hpa*II methylation patterns on gene activity can be studied by analysis of gene-specific transcripts in Northern blots. To correlate the level of transcriptional activity with changes in the degree of methylation, the level of *Hpa*II-specific methylation sites within the transformed eukaryotic cells can be demonstrated directly by comparison of the restriction activities of isoschizomeric nucleases *Hpa*II and *Msp*I on extracted DNA in Southern blots [1902], [1900], [1903].

7
Enzyme Safety and Regulatory Considerations

This chapter reviews the safe handling of enzymes in the workplace and the regulation of enzyme-containing products. There are other aspects of both categories, including safety for the consumer of enzyme-containing products, labeling for safety and use, regulations pertaining to manufacturing, and some product regulatory categories that are not addressed. For information on these topics, the reader is referred to the trade association and other web sites listed.

7.1
Safe Handling of Enzymes

Much of our knowledge on the safe handling of enzymes has been developed and communicated by the detergent industry. In the late 1960s, proteases were first used on a large scale in detergent manufacturing. In the late 1960s and early 1970s there were reports of skin irritation and occupational asthma in detergent industry employees. These adverse health effects were attributed to enzyme exposure. In response to these health effects, the UK Soap and Detergent Industry Association (SDIA) formed a committee that developed industrial hygiene and employee health monitoring recommendations to reduce employee exposure to enzymes. The detergent industry and enzyme manufacturers worked together to reduce employee exposures to enzyme aerosols (dusts and mists) by the development of liquid and encapsulated enzymes [2154]. This has allowed the safe introduction and use of enzymes in many products and production processes, including detergents, processed foods, and textiles.

This chapter describes the elements of an enzyme safety program that are important for limiting exposure to enzymes and maintaining employee health and safety in the workplace. These include employee education, control measures, monitoring methods and medical surveillance. Many articles have been published on the various aspects of enzyme safety [2154–2157] and should be consulted for more detailed information.

7.1.1
Possible Health Effects

Data on the health hazards associated with enzymes substantiate a low degree of toxicity. The adverse effects induced by enzymes can be divided into two main types: (1) localized

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irritation and (2) respiratory allergy. There is no scientific evidence to suggest that exposure to enzymes can result in other manifestations of toxicity, including reproductive toxicity, developmental toxicity, genotoxicity, systemic toxicity, and carcinogenicity.

Respiratory Allergies As with any protein that is foreign to the respiratory tract, repeated inhalation of enzyme-containing dust or mists can lead to the development of respiratory allergy or allergic asthma. There are two main stages in the development of respiratory allergy, which is also called Type 1 hypersensitivity. The first stage is called induction. This occurs when the individual is first exposed by inhalation to an allergen such as an enzyme, household dust, or pollen. If enough enzyme is inhaled the body will begin to recognize it as a foreign material and will produce allergen-specific IgE antibodies. At the induction stage, there are no clinical symptoms.

The second stage, is called elicitation in some individuals, subsequent exposure to an antigen, such as an enzyme, can lead to clinical allergic symptoms. Typical clinical allergic symptoms include watery eyes, runny nose, and coughing, as well as tightness of chest and shortness of breath, or occupational asthma. Not all sensitized individuals will develop allergic symptoms when re-exposed to the enzyme. The development of allergies depends on the susceptibility of the individual, and the magnitude and length of exposure. Symptoms will only occur if the susceptible individual inhales enzyme aerosols, and the symptoms will disappear within hours or a few days.

Skin and Eye Contact Enzymes are not skin sensitizers. However, proteolytic enzymes may cause skin irritation after prolonged and repeated exposure. This adverse manifestation is due to the irritant characteristics of proteases and is not an allergic response. Skin irritation is heightened by the presence of moisture and is most likely to appear on areas of the body where perspiration occurs: hands, armpits, and feet. Gloves and protective clothing should be worn when there is a potential for skin contact. Good personal hygiene is also essential for preventing skin irritation.

Proteolytic enzymes may also cause eye irritation on direct exposure. Appropriate eye protection should be worn to prevent contact when working with enzyme preparations.

7.1.2 Control Technology

The primary goal in an enzyme safety program is to maintain exposure below a level that would cause adverse health effects. This can be accomplished by paying attention to the product's physical form and the implementation of engineering controls, work practices, and personal protective equipment. The detergent industry has established the following key strategies to prevent employee exposure [2157]. These can be applied to other industries as well.

- Containment at source of any dust or liquid aerosol formation.
- The avoidance of recurring routine or uncontrolled spillages.
- The avoidance of personal contamination.
- The handling of empty containers with appropriate controls.

Product Form The form of the product greatly influences the potential for aerosol formation. Because of this, product form often dictates the selection of engineering controls, handling procedures, and the type of protective equipment needed to provide adequate protection to the employee. Enzyme preparations are supplied in three main forms: liquids, granules, and powders. By their nature powdered enzyme preparations present the greatest chance for inhalation exposure because the fine dust can easily become airborne. When using powdered enzymes more stringent handling procedures and local exhaust ventilation are critical for ensuring safety in the workplace. Granular preparations are often provided with a protective coating which reduces the potential for formation of enzyme dust, but care must be given to avoid damaging and crushing the granules. When the granules have been crushed or damaged, the potential for exposure to enzyme-containing dust is increased. Enzymes in liquid form are nonvolatile; but there is still the chance of aerosol formation during material transfer, spills, mixing operations, and cleaning of equipment.

Engineering Controls Engineering controls are the preferred method of preventing the release of enzyme aerosols from manufacturing equipment. Because the form of the product determines what kind of engineering controls to use in the production facility, engineering controls should be designed for the specific product form and production process. Engineering controls in the form of enclosures and local exhaust ventilation are very effective and are the most suitable methods to control enzyme exposure. The process should be enclosed as much as possible to contain enzyme aerosols that are generated during production operations. Local exhaust ventilation should be used to supplement the enclosures. Using enclosures and local exhaust ventilation in conjunction with each other assists in the isolation of the enzyme preparation from the employee. For this reason, they should be used in the following areas:

- Locations where enzyme preparations are added into the process.
- Material transfer points.
- Where the enzyme containing product is packaged into containers.

Design specification, performance verification, system maintenance, and process change management are key factors in designing and implementing engineering controls.

Work-Practice Controls Work-practice controls include safe work practices, education, and good-housekeeping practices. Safe work practices should be instituted to prevent the generation of enzyme aerosols and prevent skin contact. Aerosols can form during equipment cleaning and spill-cleanup operations, such as sweeping and washing with high-pressure water. Vacuuming with a vacuum cleaner equipped with a high-efficiency particulate air (HEPA) filter is the preferred method of cleaning. Washing with high-pressure water and steam cleaning should be avoided. Workers need to be aware of any work practices that may create aerosols and how to minimize aerosol formation.

Employee and contractor education is paramount to a safe working environment. To achieve good compliance with work practices and the use of control technology, employees need to know the reasons for these control measures. Employees need to be informed of the potential health hazards, how and when to use control measures, emergency procedures and reasons for a medical surveillance program.

Personal Protective Equipment Personal protective equipment is utilized to supplement other control measures or as a primary control method in special situations such as spillage clean-up or equipment maintenance. Types of personal protective equipment include respiratory protection, protective clothes, and eye protection. A risk assessment should be conducted to determine when personal protective equipment should be worn and what type is necessary.

There are instances when engineering and work-practice controls need to be supplemented with respiratory protection. Cleaning operations and spillage clean-up may require respiratory protection, since it is difficult to control enzyme levels during these procedures. Important components of a respiratory protection program include selection of the appropriate respirator, training, fit testing, and medical surveillance.

Protective clothing and eye wear may be required in some operations such as maintenance operations, spillage clean-up, and cleaning operations. This skin and eye protection should be worn when there is a potential for skin and eye contact. This is especially true for proteolytic enzymes because of the irritation potential. Examples of eye and skin protection are safety glasses, goggles, face shields, gloves, and coveralls.

Air Monitoring Air monitoring is often performed in work environments to evaluate airborne levels of a particular substance. This is important for assessing the efficiency of engineering controls, whether respiratory protection is required, and whether the airborne levels meet occupational exposure limits. Components of an air-monitoring program include an air-sampling plan, measurement methods, air-sampling equipment, and methods to evaluate the results.

Exposure Limits The ACGIH has established a TLV for subtilisin of 60 ng/m^3. This is based on high-volume air monitoring for at least 60 min. Several countries have also established regulatory limits for subtilisin. Argentina, Canada, Denmark, Portugal, Spain, Switzerland, and the Netherlands, have adopted either a short-term exposure limit or ceiling of 60 ng/m^3 for subtilisins. The UK has established a TWA of 40 ng/m^3 for subtilisin.

Medical Surveillance Medical surveillance programs are implemented to monitor the health of employees for early detection of any potential health effects. Elements of a medical surveillance program include a baseline exam, routine medical monitoring, and evaluation of employees with symptoms. Components of the medical exams could include medical history, respiratory questionnaire, medical examination, pulmonary function test, and immunological testing for detection of allergen-specific IgE antibodies.

Immunological Tests Inhalation of enzymes can cause the production of allergen-specific IgE antibodies in susceptible individuals. The development of IgE antibody is not an adverse health effect but it is an indication that there was sufficient exposure to produce allergic antibodies. This individual is also at greater risk for developing a respiratory allergy. Further inhalation exposure to the same enzyme type may lead to the development of allergic symptoms. Allergen-specific IgE antibodies can be detected either by a laboratory test or by a skin prick test. An individual with a positive immunological test but no symptoms may continue to work with enzymes. An assessment of the workplace should be conducted to determine if appropriate engineering controls and work practices are in place to limit exposure. The immunological tests are also used or to aid in confirmation of an allergy to a particular enzyme.

Additional information can be obtained from enzyme suppliers and trade associations such as the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) http://www.amfep.org/amfep.html, Association Internationale de la Savonnerie et de la Detergence (AISE) http://www.aise-net.org/, US Soap and Detergent Association (SDA) http://www.sdahq.org/, and the Enzyme Technical Association (ETA) http://enzymetechnicalassoc.org/.

7.2
Product Regulatory Considerations

Enzyme products are regulated according to their application, i.e., whether they are used in food manufacture, as digestive aids, in animal feed, in detergents, for textile processing, etc. Regulations also differ country by country. This section gives an overview of many of the regulations and gives references for more detailed information; it specifically covers enzymes used as food ingredients and processing aids, in feed, and for industrial or chemical use. The information contained herein is not meant to give the reader a step-by-step guide on how to register an enzyme product for a particular use in a particular country. In addition to the information here, both the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) and the Enzyme Technical Association (ETA) maintain web sites with regulatory and safety information and links which may be of interest to the reader: http://www.amfep.org/amfep.html and http://enzymetechnicalassoc.org/. As with all of regulatory affairs, before seeking approval for a particular product in a country, it is important to contact the relevant authorities and discuss procedures and dossier content.

Enzyme products or preparations are generally characterized, and referred to according to their main enzymatic activity, i.e., as an α-amylase, subtilisin, glucoamylase, cellulase, etc. The IUB officially assigns E.C. numbers to enzymes (see http://www.chem.qmw.ac.uk/iubmb/enzyme/) as does the Chemical Abstracts Service (CAS, see http://www.cas.org/). Both the E.C. and CAS numbers are often referenced in specific regulations.

Whether there is specific legislation or not, it is prudent for the manufacturer of an enzyme preparation to assure that the potential product is safe for its intended use prior to introduction. For enzyme products intended for use in food, one needs to address the safety of the production organism (microorganism, plant, or animal), production

process, and product. There are several publications that discuss these issues and provide a framework for this assessment, including [2158–2162]. In addition, the Food Chemicals Codex [2163] and WHO/JECFA [2164] (see http://www.fao.org/es/ESN/Jecfa) have specifications for food-grade enzymes that should be regarded as the minimum specifications required for the regions they cover; several countries impose stricter specifications. For enzyme products intended for use in feed, similar safety requirements apply; in addition, the safety issues discussed by the Scientific Committee for Animal Nutrition (SCAN) and the Association of American Feed Control Officials (AAFCO) (see Section 7.2.2) need to be addressed. For enzymes used in industrial processes, such as textile processing, leather tanning, paper production, and consumer products, such as detergents and automatic dish washing products, the principal safety issues are discussed in the Section 7.2.3.

7.2.1 Food-Use Enzymes

Table 40 lists approvals needed by application and country for enzymes used in food processing.

The regulation of enzymes used in food processing is perhaps the most complex. Along with how enzymes are regulated in general country-by-country, one needs to be aware that enzymes produced using modern biotechnology techniques often have extra regulations, including the currently evolving ones for genetically modified organisms

Table 40. Approvals needed by application and country for enzymes used in food processing

Country/region	Traditional production	GM production organism
EU additive	Approval, acc. 89/107 Directive	Approval, acc. 89/107 Directive
EU processing aid	Not harmonized	Not harmonized
France	Article 8 (1989) Notification or full submission	Article 8 (1989) Notification or full submission
Denmark	Approval needed	Approval needed
UK	Voluntary	Voluntary
Poland	Approval needed	Approval needed
Switzerland	No approval needed	Approval needed
China	Approval needed if new	Approval needed if new
Thailand	Registration	Registration
Korea	Registration	Approval needed
Taiwan	Registration	Registration
Japan	Approval needed if new	Approval needed
Australia/New Zealand	Approval needed	Approval needed
Canada	Approval needed if new	Approval needed
USA	Company GRAS assessment, GRAS Notice, or Food Additive	Company GRAS assessment, GRAS Notice, or Food Additive
Brazil	Approval needed if new	Approval needed (if allowed)
Mexico	Approval needed if new	Approval needed if new

(GMO) food labeling. Because they are currently being developed and interpretation is key, GMO labeling regulations are not discussed in detail here. At present, the EU, Japan, and Australia/New Zealand, among others, have developed or are developing such regulations. The use of enzymes as digestive aids is also regulated specifically in some countries, e.g., the USA and the EU; these regulations are not discussed here.

In the EU, food enzymes are classified either as food additives or processing aids. If an enzyme has its technological function in the final food, it is considered a food additive and therefore regulated under the Food Additives Directive (95/2); there are only two enzymes currently regulated as such, invertase (E 1103) in confectionery and lysozyme (E 1105) in cheese. Certain other enzymes can be considered as food or food ingredients and therefore, if produced with novel methods, could fall under the Novel Foods Regulation 97/258. Most of the enzyme preparations used in food processing in the EU are considered processing aids, meaning that they have their technological function in the food-processing stage and not in the final food. They are excluded from the Food Additives Framework Directive according to its Article 1.3. Therefore, the use of most enzymes in food is not covered by a community regulation, but by widely divergent national provisions. In this context, it is important to mention that the European Commission, under pressure from some members who are not happy with the unharmonized situation, initiated a working group in 2000, the SCOOP Task Force 7.4. This Task Force 7.4 made an inventory of the enzymes available on the EU market, how they are regulated, how they are evaluated for safety, and how they should be classified. The final report of this Task Force was not published at the time when this paper was finalized.

To gain approval of an enzyme regulated as a food additive in the EU, a dossier needs to be submitted to the Scientific Committee for Food (SCF), which evaluates the safety of all food additives. The SCF issued Guidelines for the presentation of data on food enzymes on 11 April 1992 (see http://europa.eu.int/comm/food/fs/sc/scf/reports_en.html).

There are also two Vertical Directives in the EU that mention enzymes: the Fruit Juice and Wine Directives. In the Council Directive 93/77/EEC relating to fruit juices and certain similar products, pectolytic, proteolytic, and amylolytic enzymes are allowed. In the Council Regulations 822/87/EEC on the common organization of the market in wine, pectolytic enzymes are on the allowed ingredients list. If these Vertical Directives do not allow an enzyme, then the enzyme cannot be used in fruit juice or wine production.

Enzymes used as processing aids (not present with a technical effect in the final food) are regulated by national legislation in the EU. France, Denmark and the UK have legislation covering all food-use enzymes; France and Denmark require approval prior to use; in the UK, the approval is voluntary, but recommended.

The French Food Enzyme Law, Arrete du Septembre 1989 (J.O. du 01-10-89), stipulates that for a food enzyme to be used in France, either a Declaration de Vente (marketing notification) or a full dossier has to be submitted and approved. The responsibility of the review of the dossier lies with Agence Française de Sécurité Sanitaire des Aliments (AFSSA), the new Food Safety Agency in France established

in 2000 (see: http: www.afssa.fr). The regulations list enzymes by principal activity and use, and the authorities also track them by trade name. So, if a manufacturer wishes to change the name or add a use for an already listed enzyme, a Declaration de Vente must be filed and approved prior to sale. One important nuance is that after a dossier is approved, a Declaration de Vente must also be filed to list the enzyme product as an approved food additive on the positive list.

For approval of GMO-derived enzymes, the gene construction description has to be given; the production organism must be absent in the product and there should be no detectable (transformable) DNA in the product.

The French Enzyme law also sets purity requirements for food enzymes, as follows:

Cadmium	<0.5 mg/kg
Mercury	<0.5 mg/kg
Arsenic	<3 mg/kg
Lead	<10 mg/kg
Total microorganism count	<50.000/g
Salmonella	not present in 25 g
Coliforms	<30/g
Anaerobic SO_2 reducing	<30/g
Staphylococcus aureus	not present in 1 g
Antibiotic activity	not present
Aflatoxins	<0.005 mg/kg

In the UK, the regulation of enzymes is under the Department of Health (http://www.doh.gov.uk/), which is part of the Department for Environment, Food and Rural Affairs (DEFRA) (http://www.defra.gov.uk/). The Department of Health bases its approval of a ''new'' enzyme on the basis of its need and safety. The decision of ''need'' for a new enzyme is made by the Food Advisory Committee (FAC) (http://www.foodstandards.gov.uk/), and the safety is evaluated by the Committee on the Toxicity of Chemicals in Foods, Consumer Products and the Environment (COT) (http://www.foodstandards.gov.uk/committees/cot/summary.htm). COT published guidelines for the safety assessment of microbial enzyme preparations in 1993.

In Denmark, food enzymes are regulated in ''Bekendtgørelse om tils ætningsstoffer til levnedsmidler'' (Order on Additives for Foodstuffs) No. 942, issued December 11, 1997 by the Danish Ministry of Food, Agriculture and Fisheries (with amendments). The user or importer of an enzyme preparation is responsible for a notification under this scheme. The information to be contained in the notification should follow the guidelines laid down by the SCF in 1992. Notification is valid only for a specific brand or trade name and must be approved by the authorities prior to sale.

In Japan, the Ministry of Health, Labour and Welfare (MHLW) (http://www.mhlw.go.jp/english/) regulates the use of food enzymes. There is a positive list of approved enzymes (for English version, see Food Sanitation Law, Japan Food Additives Association, 1999) and all recombinantly derived enzymes must be approved, except for those derived from self-cloned microorganisms. (The MHLW will need to confirm the self-cloned status and should be contacted to determine information requirements.) The list of currently approved recombinant foods and food additives, including enzymes can be found at http://www.mhlw.go.jp/english/topics/food/index.html. The requirements for the approval of recombinantly derived enzymes can also be found at this site.

A system similar to that in Japan is currently under development in Korea.

Several countries, including Hungary, Taiwan, and Thailand have registration processes for food enzymes. These processes differ widely in what information is required to be provided prior to importation or sale of the enzyme product. It is important to note that Poland has a lengthy list of specifications that the products must meet, and Korea is developing regulations that will require approval of recombinantly derived food enzyme products.

In the USA, food ingredients, including food enzymes, are either regulated as food additives by the FDA or are Generally Recognized As Safe (GRAS). To be GRAS, an ingredient either needs to have been in commerce prior to 1958 or to have been determined to be safe for its intended use based upon scientific principles, and scientists knowledge about food safety need to acknowledge that it is safe. If an ingredient is not GRAS, it is regulated as a food additive. GRAS ingredients can be introduced into commerce without FDA approval, through what is commonly called a self-affirmation. FDA can also be notified of the GRAS determination through the voluntary GRAS Notification process (see: http://www.cfsan.fda.gov/~dms/opa-noti.html). FDA proposed the GRAS Notification process on April 17, 1997, and although the regulation has not been finalized, the GRAS Notice process is in use. When a GRAS Notice is filed, the FDA evaluates whether each submitted notice provides a sufficient basis for a GRAS determination and whether information in the notice or otherwise available to FDA raises issues that lead the agency to question whether use of the substance is GRAS. Following this evaluation, FDA responds to the notifier by letter, and posts the letter on the web site.

In general, FDA's response has been in one of three categories:

1. The agency does not question the basis for the notifier's GRAS determination.
2. The agency concludes that the notice does not provide a sufficient basis for a GRAS determination (e.g., because the notice does not include appropriate data and information or because the available data and information raise questions about the safety of the notified substance).
3. The response letter states that the agency has, at the notifier's request, ceased to evaluate the GRAS notice.

For a list of GRAS Notices and issued letters, see: http://www.cfsan.fda.gov/~rdb/opa-gras.html.

Food additives require prior approval by the FDA through a food additive petition (see: http://www.cfsan.fda.gov/~lrd/foodadd.html). The food additive petition process can take several years to result in a regulation indicating approval; food additives must be approved prior to introduction in commerce in the USA.

GMO-derived products are regulated by the same regulatory scheme as non-GMO-derived products. GMO-derived food enzymes can be introduced into commerce through self-affirmations, GRAS Notifications and/or food additive petitions, just as for non-GMO derived food enzymes.

Prior to the implementation of the GRAS Notice process, there was a petition process for GRAS substances in which the petitioner would ask FDA to affirm the product as GRAS. Several enzyme preparations have been affirmed as GRAS and most are listed in the Partial List of Enzymes that are Used in Foods, http://www.cfsan.fda.gov/~dms/opa-enzy.html, which also lists those regulated as food additives.

Products which are regulated by the Bureau of Alcohol, Tobacco, and Firearms (BATF; i.e., beer, wine, and distilled alcohol) and the U.S. Department of Agriculture (USDA; i.e., meat and poultry) require FDA sanction prior to the BATF or USDA approval. Historically, the only way to do this is through the petitioning process and having a regulation finalized on the product. Once the product is regulated by FDA, BATF will allow its use in wine (27 CFR §24.246) and distilled alcohol (27 CFR §24.247) without any further action, but it may need to be added to the Brewer's Adjunct Manual for use in beer. USDA will allow the use through a letter request. Both BATF and USDA have agreed to honor the GRAS Notification process, and that is currently the process a manufacturer would follow to gain the approval of a new GRAS food enzyme that is also regulated by BATF or USDA.

In Canada, Health Canada regulates food-use enzymes under the Food and Drugs Act. Each enzyme for sale in Canada must be listed in the regulation by enzyme activity, the specific source from which it is derived, and the allowed application(s) (see Part B, Div. 16, Table V, Food Additives that May be Used as Food Enzymes; http://www.hc-sc.gc.ca/food-aliment/english/publications/acts_and_regulations/food_and_ drugs_acts/c-tables.pdf). Listing is achieved via a food additive petition. Once Health Canada's staff has completed their safety review and are satisfied, it is possible to obtain provisional authorization to sell the food enzyme through an Interim Marketing Authorization. The full regulation of an approved food additive takes several years, with listing of the proposed regulation in Gazette I for comment and publication of the regulation in Gazette II after approval by Parliament. Submission requirements are similar to those in the USA. GMO regulations are currently being developed under Novel Food Legislation.

Australia and New Zealand regulate food enzymes through the Australia New Zealand Food Authority (ANZFA). Enzymes need to be approved prior to sale; for more information and how to seek approval of an enzyme, label it, etc., see: http://www.anzfa.gov.au/foodstandards/. Approved enzymes are listed in Standard 1.3.3, clauses 15–17 and are listed by enzyme activity and source (see: http://www.anzfa.gov.au/foodstandards/foodstandardscodecontents/standard13/standard133.cfm).

New food enzyme regulations are also being developed in Brazil; it is still unclear whether or not enzymes derived from recombinant production organisms will be allowed. In Mexico, enzyme preparations are approved by the Ministry of Health.

7.2.2 Feed-Use Enzymes

Table 41 lists approvals needed by application and country for enzymes used in animal feed and feed processing.

In the EU, enzymes used as feed additives are regulated under the Feed Additive Directive 70/524/EEC. Details on the criteria and the contents of dossiers to be submitted to achieve approvals for these enzymes are given in Directive 87/153/EEC, as amended by 94/40/EC and 95/11/EC (the so called guidelines directive). In addition to these criteria, a description of the production organism and process needs to be included and the production organism needs to be ''on deposit'' (i.e., the production organism needs to be deposited with a recognized culture collection); this requirement, along with others, then limits the amount of process improvement the manufacturer can implement without reverting to the authorities for approval. The Scientific Committee for Animal Nutrition (SCAN; see http://europa.eu.int/comm/food/fs/sc/scan/index_en.html) determines the requirements for approval and reviews dossiers for feed enzymes. Approved enzymes are added to Annex I of the directive, along with all feed additives; approval is considered production-strain-specific.

In a publication in August 1998, SCAN took the position that in the GMOs used for future feed enzyme products, antibiotic resistance markers (ARMs) should be avoided or removed. Therefore, it is prudent for industry developing microbial sources for the production of feed enzymes to avoid the use of ARMs. SCAN has also published several other opinions on enzymes used in feed that can be viewed at their web site.

In the USA, ingredients used in feed must be either GRAS and/or listed in the Official Publication of American Feed Control Officials [AAFCO Manual (see also http://www.aafco.org/)] in Table 30. Labels are approved state by state. Under the Enzyme Coordination Policy proposed in 1997 and adopted in 1998, FDA will review new enzyme products for use in feed and recommend listing in the AAFCO Manual

Table 41. Approvals needed by application and country for enzymes used in animal feed and feed processing

Country/area	
EU	Full dossiers required for new enzymes/products Notifications of changes for approved products
USA	GRAS or Feed Additive, AAFCO listing
Canada	Approval required

via a Letter of No Objection. Requirements for listing are spelled out in the Coordination Policy in the AAFCO Manual. Other related materials that may consist of or contain enzymes are also listed in the AAFCO Manual, §§ 36.11 and 36.12.

In Canada, feed use enzymes are regulated by the Feed Section, Animal Health and Production Division, Canadian Food Inspection Agency. Addition of a new enzyme requires safety and efficacy data, intended labeling, product formulation, and analytical data and methods. Enzymes must be approved prior to use in feed. Instructions for how to apply for feed enzyme approval, which enzymes are approved, and activity-testing criteria can be found in several of the trade memoranda at http://inspection.gc.ca/english/anima/feebet/trademem/trindxe.shtml.

7.2.3 Industrial-Use Enzymes

Table 42 lists approvals needed by application and country for enzymes used in industrial applications.

Enzymes used in industrial processes such at textile processing and paper manufacture and as ingredients in laundry and dishwashing detergents are generally regulated as other chemicals are in the countries that have such regulations. They are usually included in an inventory listing, as discussed below. In addition, the ingredients used in such enzyme products also need to be listed on the respective inventories.

In the EU, this regulation is harmonized, and the enzyme activity in question needs to be listed in the European Inventory of Existing Chemical Substances (EINECS) or approved as new through the European List of New Chemical Substances (ELINCS). (See also: http://ecb.ei.jrc.it.) The regulation of these enzymes is covered under Council Directive 67/548/ EEC as amended (for the 7th time by 92/32/EEC), hereafter referred to as ELINCS directive, and implementing national regulations. There are more than 300 enzyme entries in EINECS. Enzymes for all uses, whether used as an ingredient or processing aid, if used in the EU are regulated through this regulatory scheme.

Table 42. Approvals needed by application and country for enzymes used in industrial applications

Country/region	
Australia, Canada, EU*, Japan, Korea, China, Philippines, USA	Listed on Inventories**
Germany, Austria	Dossier for detergent use to obtain registration number

* Enzymes for all uses (food, feed, chemical, etc.) are regulated through this system; some uses, as noted above under food and feed enzymes, also require specific approvals through other regulatory systems.

** For example, European Inventory of New and Existing Chemical Substances (EINECS) and the U.S. Toxic Substances Control Act (TSCA) Inventory, enzymes listed by activity (CAS or IUB No.).

All the enzymes listed in EINECS are currently characterized solely on the basis of their catalytic activity and listed by CAS number; the source is not critical for determination of the EINECS status of any enzyme. This means that enzymes derived from GM microorganisms, including protein-engineered enzymes, are covered by the EINECS listing. The EU Commission is currently examining how enzymes are listed on EINECS, and this regulatory scheme may change in the future.

In the USA, enzymes and the intergeneric microorganisms (i.e., recombinant microorganisms created by combining DNA from more than one taxonomic genus) used to produce them are regulated under the EPA Toxic Substances Control Act (TSCA). EPA TSCA regulations are in 40 CFR parts 700–789 (see: http://www.access.gpo.gov/nara/cfr/waisidx_00/40cfrv23_00.html).

While there are over 100 enzymes explicitly listed on the TSCA Inventory by CAS number, current EPA policy (unwritten) is that enzymes are naturally occurring substances and are implicitly on the Inventory. If a manufacturer has intent to manufacture or import an enzyme product and wants to confirm with the Agency that the enzyme is on the Inventory, the manufacturer or importer can file a Bona Fide Intent to Manufacture, per 40 CFR §720.25. The EPA will assess the information provided and make a determination of whether or not the entity that is the subject of the Bona Fide is on the Inventory. This is also the procedure that is used to determine if an entity is in the confidential section of the Inventory.

Intergeneric microorganisms are regulated under the Microbial Products of Biotechnology; Final Regulation Under the Toxic Substances Control Act; Final Rule, published in 1997. For the text of the rule, guidance on how to use it, and a list of microorganisms cleared for use, see: http://www.epa.gov/biotech_rule/index.html. Research and development of enzymes conducted in the USA is exempt from TSCA under 40 CFR §720.36 for chemicals and 40 CFR §720.234 for contained use intergeneric microorganisms. The intergeneric microorganism is only subject to regulation if its manufacture or R&D is conducted in the United States. If the enzyme product is manufactured outside the USA, and the product does not contain the microorganism when imported, only the enzyme product and formulation ingredients are subject to TSCA.

In Canada, enzymes for these uses need to be listed on the Domestic Substances List (DSL), and microorganisms, whether recombinant or not, used to produce them in Canada also need to be on the DSL. Notification requirements, procedures and the DSL can be found at http://www.ec.gc.ca/cceb1/nsd/eng/index_e.htm.

Japan, Australia, Korea, and the Philippines also have chemical inventories that include enzymes and their ingredients. In Japan, enzymes are considered natural and are not explicitly listed on the Inventory. Before import or manufacture of an enzyme product in any of these countries, the competent environmental regulatory authorities should be consulted.

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604 WFK Forschungsinstitut für Reinigungstechnologie (Germany); URL: htpp://www.gewebe@wfk.de

605 Testfabrics, Inc.;URL: http://www.testfabrics.com

606 TNO Instituut voor Reinigings Techniek-Delft-Netherlands; URL: http://www.tno.nl

607 SDL International Textile Innovators; URL; http://www.textileinnovators.com

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