2 Enzymes

2.1 Foreword

Enzymes are proteins with powerful catalytic activity. They are synthesized by biological cells and in all organisms, they are involved in chemical reactions related to metabolism. Therefore, enzyme-catalyzed reactions also proceed in many foods and thus enhance or deteriorate food quality. Relevant to this phenomenon are the ripening of fruits and vegetables, the aging of meat and dairy products, and the processing steps involved in the making of dough from wheat or rye flours and the production of alcoholic beverages by fermentation technology.

Enzyme inactivation or changes in the distribution patterns of enzymes in subcellular particles of a tissue can occur during storage or thermal treatment of food. Since such changes are readily detected by analytical means, enzymes often serve as suitable indicators for revealing such treatment of food. Examples are the detection of pasteurization of milk, beer or honey, and differentiation between fresh and deep frozen meat or fish.

Enzyme properties are of interest to the food chemist since enzymes are available in increasing numbers for enzymatic food analysis or for utilization in industrial food processing. Examples of both aspects of their use are provided in this chapter in section 2.6.4 on food analysis and in section 2.7, which covers food processing.

Details of enzymes which play a role in food science are restricted in this chapter to only those enzyme properties which are able to provide an insight into the build-up or functionality of enzymes or can contribute to the understanding of enzyme utilization in food analysis or food processing and storage.

2.2 General Remarks, Isolation and Nomenclature

2.2.1 Catalysis

Let us consider the catalysis of an exergonic reaction:

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P \qquad (2.1)$$

with a most frequently occurring case in which the reaction does not proceed spontaneously. Reactant A is metastable, since the activation energy, E_A, required to reach the activated transition state in which chemical bonds are formed or cleaved in order to yield product P, is exceptionally high (Fig. 2.1).

The reaction is accelerated by the addition of a suitable catalyst. It transforms reactant A into intermediary products (EA and EP in Fig. 2.1), the transition states of which are at a lower energy level than the transition state of a noncatalyzed reaction (A$\neq$ in Fig. 2.1). The molecules of the

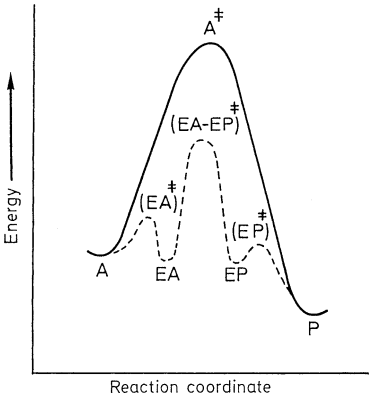


Fig. 2.1. Energy profile of an exergonic reaction A → P; — without and - - - with catalyst E

Table 2.1. Examples of catalyst activity

Reaction	Catalyst	Activation energy ($kJ \cdot mol^{-1}$)	k_{rel}(25 °C)
1. $H_2O_2 \rightarrow H_2O+1/2\ O_2$	Absent	75	1.0
	$I^{\oplus}$	56.5	$\sim 2.1 \cdot 10^3$
	Catalase	26.8	$\sim 3.5 \cdot 10^8$
2. Casein+n $H_2O \rightarrow$	$H^{\ominus}$	86	1.0
(n+1) Peptides	Trypsin	50	$\sim 2.1 \cdot 10^6$
3. Ethylbutyrate	$H^{\ominus}$	55	1.0
$+H_2O \rightarrow$ butyric acid+ethanol	Lipase	17.6	$\sim 4.2 \cdot 10^6$
4. Saccharose + $H_2O \rightarrow$	$H^{\ominus}$	107	1.0
Glucose+Fructose	Invertase	46	$\sim 5.6 \cdot 10^{10}$
5. Linoleic acid	Absent	150–270	1.0
$+O_2 \rightarrow$ Linoleic acid	Cu^{2+}	30–50	$\sim 10^2$
hydroperoxide	Lipoxygenase	16.7	$\sim 10^7$

species A contain enough energy to combine with the catalyst and, thus, to attain the "activated state" and to form or break the covalent bond that is necessary to give the intermediary product which is then released as product P along with free, unchanged catalyst. The reaction rate constants, k_{+1} and k_{-1}, are therefore increased in the presence of a catalyst. However, the equilibirum constant of the reaction, i. e. the ratio $k_{1+}/k_{-1} = K$, is not altered.

Activation energy levels for several reactions and the corresponding decreases of these energy levels in the presence of chemical or enzymatic catalysts are provided in Table 2.1. Changes in their reaction rates are also given. In contrast to reactions 1 and 5 (Table 2.1) which proceed at measurable rates even in the absence of catalysts, hydrolysis reactions 2, 3 and 4 occur only in the presence of protons as catalysts. However, all reaction rates observed in the case of inorganic catalysts are increased by a factor of at least several orders of magnitude in the presence of suitable enzymes. Because of the powerful activity of enzymes, their presence at levels of 10^{-8} to 10^{-6}mol/l is sufficient for *in vitro* experiments. However, the enzyme concentrations found in living cells are often substantially higher.

2.2.2 Specificity

In addition to an enzyme's ability to substantially increase reaction rates, there is a unique enzyme property related to its high specificity for both the compound to be converted (substrate specificity) and for the type of reaction to be catalysed (reaction specificity).

The activities of allosteric enzymes (cf. 2.5.1.3) are affected by specific regulators or effectors. Thus, the activities of such enzymes show an additional regulatory specificity.

2.2.2.1 Substrate Specificity

The substrate specificity of enzymes shows the following differences. The occurrence of a distinct functional group in the substrate is the only prerequisite for a few enzymes, such as some hydrolases. This is exemplified by nonspecific lipases (cf. Table 3.21) or peptidases (cf. 1.4.5.2.1) which generally act on an ester or peptide covalent bond.

More restricted specificity is found in other enzymes, the activities of which require that the substrate molecule contains a distinct structural feature in addition to the reactive functional group. Examples are the proteinases trypsin and chymotrypsin which cleave only ester or peptide bonds with the carbonyl group derived from lysyl or arginyl (trypsin) or tyrosyl, phenylalanyl or tryptophanyl residues (chymotrypsin). Many enzymes activate only one single substrate or preferentially catalyze the conversion of one substrate while other substrates are converted into products with a lower reaction rate (cf. ex-

Table 2.2. Substrate specificity of a legume α-glucosidase

Substrate	Relative activity (%)	Substrate	Relative activity (%)
Maltose	100	Cellobiose	0
Isomaltose	4.0	Saccharose	0
Maltotrisose	41.5	Phenyl-α-glucoside	3.1
Panose	3.5	Phenyl-α-maltoside	29.7
Amylose	30.9		
Amylopectin	4.4		

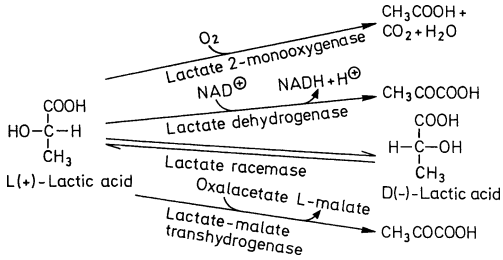


Fig. 2.2. Examples of reaction specificity of some enzymes

amples in Table 2.2 and 3.24). In the latter cases a reliable assessment of specificity is possible only when the enzyme is available in purified form, i. e. all other accompanying enzymes, as impurities, are completely removed.

An enzyme's substrate specificity for stereoisomers is remarkable. When a chiral center is present in the substrate in addition to the group to be activated, only one enantiomer will be converted to the product. Another example is the specificity for diastereoisomers, e. g. for cis-trans geometric isomers.

Enzymes with high substrate specificity are of special interest for enzymatic food analysis. They can be used for the selective analysis of individual food constituents, thus avoiding the time consuming separation techniques required for chemical analyses, which can result in losses.

2.2.2.2 Reaction Specificity

The substrate is specifically activated by the enzyme so that, among the several thermodynamically permissible reactions, only one occurs. This is illustrated by the following example: L(+)-lactic acid is recognized as a substrate by four enzymes, as shown in Fig. 2.2, although only lactate-2-monooxygenase decarboxylates the acid oxidatively to acetic acid. Lactate dehydrogenase and lactate-malate transhydrogenase form a common reaction product, pyruvate, but by different reaction pathways (Fig. 2.2). This may suggest that reaction specificity should be ascribed to the different cosubstrates, such as NAD^+ or oxalacetate. But this is not the case since a change in cosubstrates stops the reaction. Obviously, the enzyme's reaction specificity as well as the substrate specificity are predetermined by the structure and chemical properties of the protein moiety of the enzyme.

Of the four enzymes considered, only the lactate racemase reacts with either of the enantiomers of lactic acid, yielding a racemic mixture.

Therefore, enzyme reaction specificity rather than substrate specificity is considered as a basis for enzyme classification and nomenclature (cf. 2.2.6).

2.2.3 Structure

Enzymes are globular proteins with greatly differing particle sizes (cf. Table 1.26). As outlined in section 1.4.2, the protein structure is determined by its amino acid sequences and by its conformation, both secondary and tertiary, derived from this sequence. Larger enzyme molecules often consist of two or more peptide chains (subunits or protomers, cf. Table 1.26) arranged into a specified quaternary structure (cf. 1.4.2.3). Section 2.4.1 will show that the three dimensional shape of the enzyme molecule is actually responsible for its specificity and its effective role as a catalyst. On the other hand, the protein nature of the enzyme restricts its activity to a relatively narrow pH range (for pH optima, cf. 2.5.3) and heat treatment leads readily to loss of activity by denaturation (cf. 1.4.2.4 and 2.5.4.4).

Some enzymes are complexes consisting of a protein moiety bound firmly to a nonprotein component which is involved in catalysis, e. g. a "prosthetic" group (cf. 2.3.2). The activities of other enzymes require the presence of a cosubstrate which is reversibly bound to the protein moiety (cf. 2.3.1).

2.2.4 Isolation and Purification

Most of the enzyme properties are clearly and reliably revealed only with purified enzymes. As noted under enzyme isolation, prerequisites for the isolation of a pure enzyme are selected protein chemical separation methods carried out at 0–4 °C since enzymes are often not stable at higher temperatures.

Tissue Disintegration and Extraction. Disintegration and homogenization of biological tissue requires special precautions: procedures should be designed to rupture the majority of the cells in order to release their contents so that they become accessible for extraction. The tissue is usually homogenized in the presence of an extraction buffer which often contains an ingredient to protect the enzymes from oxidation and from traces of heavy metal ions. Particular difficulty is encountered during the isolation of enzymes which are bound tenaciously to membranes which are not readily solubilized. Extraction in the presence of tensides may help to isolate such enzymes. As a rule, large amounts of tissue have to be homogenized because the enzyme content in proportion to the total protein isolated is low and is usually further diminished by the additional purification of the crude enzyme isolate (cf. example in Table 2.3).

Enzyme Purification. Removal of protein impurities, usually by a stepwise process, is essentially the main approach in enzyme purification. As a first step, fractional precipitation, e. g. by ammonium sulfate saturation, is often used or the extracted proteins are fractionated by molecular weight e. g., column gel chromatography. The fractions containing the desired enzyme activity are collected and purified further, e. g., by ion-exchange chromatography. Additional options are also available, such as various forms of preparative electrophoresis, e. g. disc gel electrophoresis or isoelectric focusing. The purification procedure can be substantially shortened by using affinity column chromatography. In this case, the column is packed with a stationary phase to which is attached the substrate or a specific inhibitor of the enzyme. The enzyme is then selectively and reversibly bound and, thus, in contrast to the other inert proteins, its elution is delayed.

Control of Purity. Previously, the complete removal of protein impurities was confirmed by crystallization of the enzyme. This "proof" of pu-

Table 2.3. Isolation of a glucosidase from beans (*Phaseolus vidissimus*)

No. Isolation step	Protein (mg)	α-Glucosidase			
		Activity (μcat)	Specific activity (μcat/mg)	Enrichment (-fold)	Yield (%)
1. Extraction with 0.01 mol/L acetate buffer of pH 5.3					
2. Saturation to 90% with ammonium sulfate followed by solubilization in buffer of step 1	44,200	3840	0.087	1	100
3. Precipitation with polyethylene glycol (20%). Precipitate is then solubilized in 0.025 mol/L Tris-HCl buffer of pH 7.4	7610	3590	0.47	5.4	93
4. Chromatography on DEAE-cellulose column, an anion exchanger	1980	1650	0.83	9.5	43
5. Chromatography on SP-Sephadex C-50, a cation exchanger	130	845	6.5	75	22
6. Preparative isoelectric focusing	30	565	18.8	216	15

rity can be tedious and is open to criticism. Today, electrophoretic methods of high separation efficiency or HPLC are primarily used.
The behavior of the enzyme during chromatographic separation is an additional proof of purity. A purified enzyme is characterized by a symmetrical elution peak in which the positions of the protein absorbance and enzyme activity coincide and the specific activity (expressed as units per amount of protein) remains unchanged during repeated elutions.
During a purification procedure, the enzyme activities are recorded as shown in Table 2.3. They provide data which show the extent of purification achieved after each separation step and show the enzyme yield. Such a compilation of data readily reveals the undesired separation steps associated with loss of activity and suggests modifications or adoption of other steps.

2.2.5 Multiple Forms of Enzymes

Chromatographic or electrophoretic separations of an enzyme can occasionally result in separation of the enzyme into "*isoenzymes*", i. e. forms of the enzyme which catalyze the same reaction although they differ in their protein structure. The occurrence of multiple enzyme forms can be the result of the following:

a) Different compartments of the cell produce genetically independent enzymes with the same substrate and reaction specificity, but which differ in their primary structure. An example is glutamate-oxalacetate transaminase occurring in mitochondria and also in muscle tissue sarcoplasm. This is the indicator enzyme used to differentiate fresh from frozen meat (cf. 12.10.1.2).
b) Protomers associate to form polymers of differing size. An example is the glutamate dehydrogenase occurring in tissue as an equilibrium mixture of molecular weights $M_r = 2.5 \cdot 10^5 - 10^6$.
c) Different protomers combine in various amounts to form the enzyme. For example, lactate dehydrogenase is structured from a larger number of subunits with the reaction specificity given in Fig. 2.2. It consists of five forms (A_4, A_3B, A_2B_2, AB_3 and B_4), all derived from two protomers, A and B.

2.2.6 Nomenclature

The Nomenclature Commitee of the "International Union of Biochemistry and Molecular Biology" (IUBMB) adopted rules last amended in 1992 for the systematic classification and designation of enzymes based on reaction specificity. All enzymes are classified into six major classes according to the nature of the chemical reaction catalyzed:

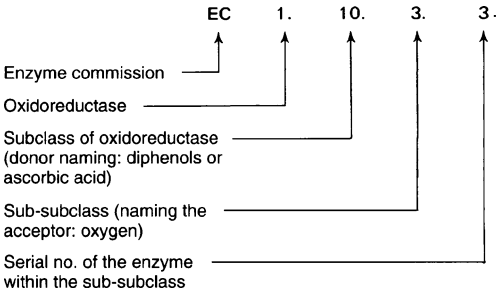
1. Oxidoreductases.
2. Transferases.
3. Hydrolases.
4. Lyases (cleave C−C, C−O, C−N, and other groups by elimination, leaving double bonds, or conversely adding groups to double bonds).
5. Isomerases (involved in the catalysis of isomerizations within one molecule).
6. Ligases (involved in the biosynthesis of a compound with the simultaneous hydrolysis of a pyrophosphate bond in ATP or a similar triphosphate).

Each class is then subdivided into subclasses which more specifically denote the type of reaction, e. g. by naming the electron donor of an oxidation-reduction reaction or by naming the functional group carried over by a transferase or cleaved by a hydrolase enzyme.
Each subclass is further divided into sub-subclasses. For example, sub-subclasses of oxidoreductases are denoted by naming the acceptor which accepts the electron from its respective donor.
Each enzyme is classified by adopting this system. An example will be analyzed. The enzyme ascorbic acid oxidase catalyzes the following reaction:

$$\text{L} - \text{Ascorbic acid} + \tfrac{1}{2}\,O_2 \rightleftharpoons \text{L} - \text{Dehydroascorbic acid} + H_2O \qquad (2.2)$$

Hence, its systematic name is L-ascorbate: oxygen oxidoreductase, and its systematic number is E.C. 1.1.10.3.3 (cf. Formula 2.3). The systematic names are often quite long. Therefore, the short, trivial names along with the systematic numbers are often convenient for enzyme designation. Since enzymes of different biological origin often differ in their properties, the source and, when known, the subcellular fraction used for iso-

lation are specified in addition to the name of the enzyme preparation; for example, "ascorbate oxidase (E.C. 1.1.10.3.3) from cucumber". When known, the subcellular fraction of origin (cytoplasmic, mitochondrial or peroxisomal) is also specified.



(2.3)

A number of enzymes of interest to food chemistry are described in Table 2.4. The number of the section in which an enzyme is dealt with is given in the last column.

2.2.7 Activity Units

The catalytic activity of enzymes is exhibited only under specific conditions, such as pH, ionic strength, buffer type, presence of cofactors and suitable temperature. Therefore, the rate of substrate conversion or product formation can be measured in a test system designed to follow the enzyme activity. The International System of Units (SI) designation is $\mathrm{mol\,s^{-1}}$ and its recommended designation is the "katal" (kat*). Decimal units are formed in the usual way, e. g.:

$$\mu\mathrm{kat} = 10^{-6}\,\mathrm{kat} = \mu\mathrm{mol}\cdot\mathrm{s}^{-1} \qquad (2.4)$$

Concentration of enzymatic activity is given as $\mu\mathrm{kat\,l^{-1}}$. The following activity units are derived from this:

a) The *specific catalytic activity*, i. e. the activity of the enzyme preparation in relation to the protein concentration.

b) The *molar catalytic activity*. This can be determined when the pure enzyme with a known molecular weight is available. It is expressed as "katal per mol of enzyme" ($\mathrm{kat\,mol^{-1}}$). When the enzyme has only one active site or center per molecule, the molar catalytic activity equals the "turnover number", which is defined as the number of substrate molecules converted per unit time by each active site of the enzyme molecule.

2.3 Enzyme Cofactors

Rigorous analysis has demonstrated that numerous enzymes are not pure proteins. In addition to protein, they contain metal ions and/or low molecular weight nonprotein organic molecules. These nonprotein hetero constituents are denoted as cofactors which are indispensable for enzyme activity.

According to the systematics (Fig. 2.3), an apoenzyme is the inactive protein without a cofactor. Metal ions and coenzymes participating in enzymatic activity belong to the cofactors which are subdivided into prosthetic groups and cosubstrates. The prosthetic group is bound firmly to the enzyme. It can not be removed by, e. g. dialysis, and during enzyme catalysis it remains attached to the enzyme molecule. Often, two substrates are converted by such enzymes, one substrate followed by the other, returning the prosthetic group to its original state. On the other

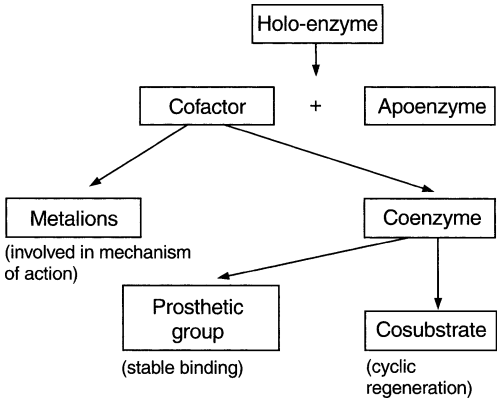


Fig. 2.3. Systematics of cofactor-containing enzymes (according to *Schellenberger*, 1989)

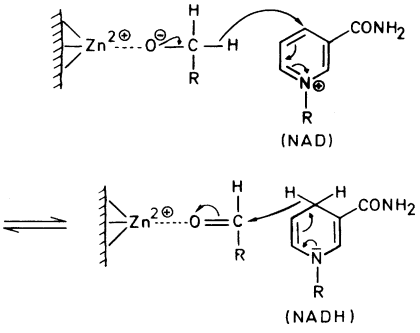
* The old definition in the literature may also be used: 1 enzyme unit (U) $\triangleq$ 1 $\mu\mathrm{mol\,min^{-1}}$ ($1\,\mathrm{U} \triangleq 16.67\cdot 10^{-9}\,\mathrm{kat}$).

hand, during metabolism, the cosubstrate reacts with at least two enzymes. It transfers the hydrogen or the functional group to another enzyme and, hence, is denoted as a "transport metabolite" or as an "intermediary substrate". It is distinguished from a true substrate by being regenerated in a subsequent reaction. Therefore the concentration of the intermediary substrates can be very low. In food analysis higher amounts of cosubstrates are often used without regeneration.
Only those cofactors with enzymatic activities of importance in enzymatic analysis of food and/or in food processing will be presented. Some cofactors are related to water-soluble vitamins (cf. 6.3). The metal ions are dealt with separately in section 2.3.3.

2.3.1 Cosubstrates

2.3.1.1 Nicotinamide Adenine Dinucleotide

Transhydrogenases (e. g. lactate dehydrogenase, alcohol dehydrogenase) dehydrogenate or hydrogenate their substrates with the help of a pyridine cosubstrate (Fig. 2.4); its nicotinamide residue accepts or donates a hydride ion (H^-) at position 4:



(2.5)

The reaction proceeds stereospecifically (cf. 2.4.1.2.1); ribose phosphate and the $-CONH_2$ group force that pyridine ring of the cosubstrate to become planar on the enzyme surface. The role of Zn^{2+} ions in this catalysis is outlined in section 2.3.3.1. The transhydrogenases differ according to the site on the pyridine ring involved in or accessible to H-transfer. For example, alcohol and lactate dehydrogenases transfer

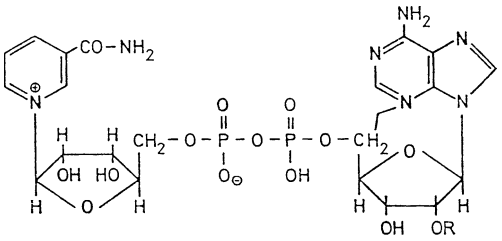


Fig. 2.4. Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP); R = H: NAD; R = PO_3H_2: NADP

the pro-R-hydrogen from the A* side, whereas glutamate or glucose dehydrogenases transfer the pro-S-hydrogen from the B* side*.
The oxidized and reduced forms of the pyridine cosubstrate are readily distinguished by absorbance readings at 340 nm (Fig. 2.5). Therefore, whenever possible, enzymatic reactions which are difficult to measure directly are coupled with an NAD(P)-dependent indicator reaction (cf. 2.6.1.1) for food analysis.

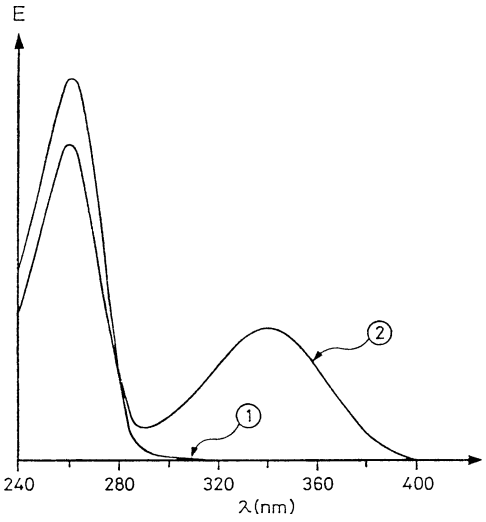


Fig. 2.5. Electron excitation spectra of NAD (1) and NADH (2)

* Until the absolute configuration of the chiral center is determined, the two sides of the pyridine ring are denoted as A and B.

Table 2.4. Systematic classification of some enzymes of importance to food chemistry

Class/subclass		Enzyme	EC-Number	In text found under
1.	*Oxidoreductases*			
1.1	CH−OH as donor			
1.1.1	With $NAD^{\oplus}$ or $NADP^{\oplus}$ as acceptor	Alcohol dehydrogenase	1.1.1.1	2.6.1
		Butanediol dehydrogenase	1.1.1.4	2.7.2.1.5
		L-Iditol 2-dehydrogenase	1.1.1.14	2.6.1
		L-Lactate dehydrogenase	1.1.1.27	2.6.1
		3-Hydroxyacyl-CoA dehydrogenase	1.1.1.35	12.10.1.2
		Malate dehydrogenase	1.1.1.37	2.6.1
		Galactose 1-dehydrogenase	1.1.1.48	2.6.1
		Glucose-6-phosphate 1-dehydrogenase	1.1.1.49	2.6.1
1.1.3	With oxygen as acceptor	Glucose oxidase	1.1.3.4 2.7.2.1.1	2.6.1 and
		Xanthine oxidase	1.1.3.22	2.3.3.2
1.2	Aldehyde group as donor			
1.2.1	With $NAD^{\oplus}$ or $NADP^{\oplus}$ as acceptor	Aldehyde dehydrogenase	1.2.1.3	2.7.2.1.4
1.8	S-Compound as donor			
1.8.5	With quinone or related compound as acceptor	Glutathione dehydrogenase (ascorbate)	1.8.5.1	15.2.2.7
1.10	Diphenol or endiol as donor			
1.10.3	With oxygen as acceptor	Ascorbate oxidase	1.10.3.3	2.2.6
1.11	Hydroperoxide as acceptor	Catalase	1.11.1.6	2.7.2.1.2
		Peroxidase	1.11.1.7	2.3.2.2 and 2.5.4.4
1.13	Acting on single donors			
1.13.11	Incorporation of molecular oxygen	Lipoxygenase	1.13.11.12	2.5.4.4 and 3.7.2.2
1.14	Acting on paired donors			
1.14.18	Incorporation of one oxygen atom	Monophenol monooxy genase (Polyphenol oxidase)	1.14.18.1	2.3.3.2
2.	*Transferases*			
2.3	Transfer of acyl groups			
2.3.2	Aminoacyl transferases	Transglutaminase	2.3.2.13	2.7.2.4
2.7	Transfer of phosphate			
2.7.1	HO-group as acceptor	Hexokinase	2.7.1.1	2.6.1
		Glycerol kinase	2.7.1.30	2.6.1
		Pyruvate kinase	2.7.1.40	2.6.1
2.7.3	N-group as acceptor	Creatine kinase	2.7.3.2	2.6.1
3.	*Hydrolases*			
3.1	Cleavage of ester bonds			
3.1.1	Carboxylester hydrolases	Carboxylesterase	3.1.1.1	3.7.1.1
		Triacylglycerol lipase	3.1.1.3	2.5.4.4 and 3.7.1.1
		Phospholipase A_2	3.1.1.4	3.7.1.2
		Acetylcholinesterase	3.1.1.7	2.4.2.5
		Pectinesterase	3.1.1.11	4.4.5.2
		Phospholipase A_1	3.1.1.32	3.7.1.2
3.1.3	Phosphoric monoester hydrolases	Alkaline phosphatase	3.1.3.1	2.5.4.4

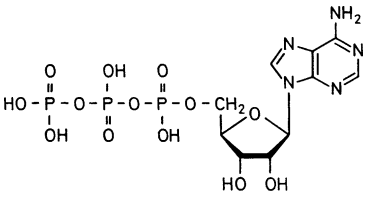
Table 2.4. Continued

Class/subclass		Enzyme	EC-Number	In text found under
3.1.4	Phosphoric diester hydrolases	Phospholipase C	3.1.4.3	3.7.1.2
		Phospholipase D	3.1.4.4	3.7.1.2
3.2	Hydrolyzing O-glycosyl compounds			
3.2.1	Glycosidases	α-Amylase	3.2.1.1	4.4.5.1.1
		β-Amylase	3.2.1.2	4.4.5.1.2
		Glucan-1,4-α-D-glucosidase (Glucoamylase)	3.2.1.3	4.4.5.1.3
		Cellulase	3.2.1.4	4.4.5.3
		Polygalacturonase	3.2.1.15	2.5.4.4 and 4.4.5.2
		Lysozyme	3.2.1.17	2.7.2.2.11 and 11.2.3.1.4
		α-D-Glucosidase (Maltase)	3.2.1.20	2.6.1
		β-D-Glucosidase	3.2.1.21	2.6.1
		α-D-Galactosidase	3.2.1.22	
		β-D-Galactosidase (Lactase)	3.2.1.23	2.7.2.2.7
		β-Fructofuranosidase (Invertase, saccharase)	3.2.1.26	2.7.2.2.8
		1,3-β-D-Xylanase	3.2.1.32	2.7.2.2.10
		α-L-Rhamnosidase	3.2.1.40	2.7.2.2.9
		Pullulanase	3.2.1.41	4.4.5.1.4
		Exopolygalacturonase	3.2.1.67	4.4.5.2
3.2.3	Hydrolysing S-glycosyl compounds	Thioglucosidase (Myrosinase)	3.2.3.1	2.7.2.2.12
3.4	Peptidases[a]			
3.4.21	Serine endopeptidases[a]	Microbial serine endopeptidases e. g. Subtilisin	3.4.21.62	1.4.5.2.1
3.4.22	Cysteine endopeptidases[a]	Papain	3.4.22.2	1.4.5.2.2
		Ficin	3.4.22.3	1.4.5.2.2
		Bromelain	3.4.22.33	1.4.5.2.2
3.4.23	Aspartic acid endopeptidases[a]	Chymosin (Rennin)	3.4.23.4	1.4.5.2.4
3.4.24	Metalloendopeptidases[a]	Thermolysin	3.4.24.27	1.4.5.2.3
3.5	Acting on C−N bonds, other than peptide bonds			
3.5.2	In cyclic amides	Creatininase	3.5.2.10	2.6.1
4.	Lyases			
4.2	C−O-Lyases			
4.2.2	Acting on polysaccharides	Pectate lyase	4.2.2.2	4.4.5.2
		Exopolygalacturonate lyase	4.2.2.9	4.4.5.2
		Pectin lyase	4.2.2.10	4.4.5.2
5.	*Isomerases*			
5.3	Intramolecular oxidoreductases			
5.3.1	Interconverting aldoses and ketoses	Xylose isomerase	5.3.1.5	2.7.2.3
		Glucose-6-phosphate isomerase	5.3.1.9	2.6.1

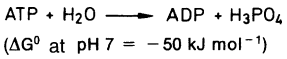
[a] cf. Table 1.33.

2.3.1.2 Adenosine Triphosphate

The nucleotide adenosine triphosphate (ATP) is an energy-rich compound. Various groups are cleaved and transferred to defined substrates during metabolism in the presence of ATP. One possibility, the transfer of orthophosphates by kinases, is utilized in the enzymatic analysis of food (cf. Table 2.16).



(2.6)



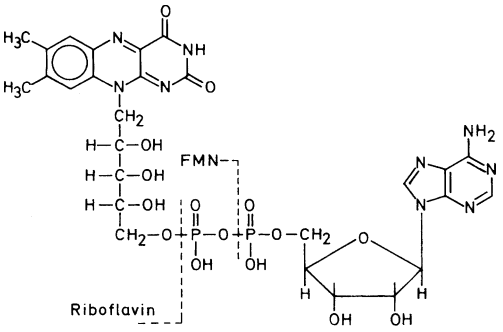
(2.7)

2.3.2 Prosthetic Groups

2.3.2.1 Flavins

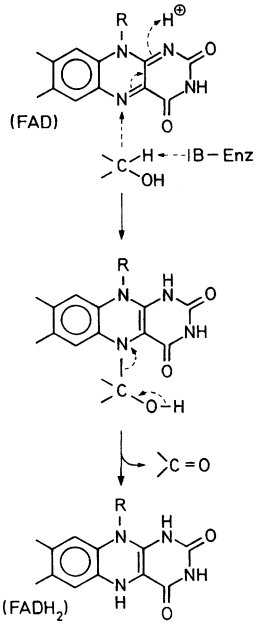
Riboflavin (7,8-dimethyl-10-ribityl-isoalloxazine), known as vitamin B_2 (cf. 6.3.2), is the building block of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Both act as prosthetic groups for electron transfer reactions in a number of enzymes.

Due to the much wider redox potential of the flavin enzymes, riboflavin is involved in the transfer of either one or two electrons. This is different from nicotinamides which participate in double electron transfer only. Values between +0.19 V (stronger oxidizing effect than $NAD^{\oplus}$) and −0.49 V (stronger reducing effect than NADH) have been reported.



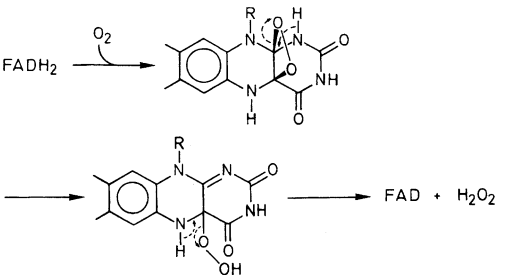
Flavin-adenine-dinucleotide (FAD) (2.8)

An example for a flavin enzyme is glucose oxidase, an enzyme often used in food processing to trap residual oxygen (cf. 2.7.2.1.1). The enzyme isolated and purified from *Aspergillus niger* is a dimer (M_r = 168,000) with two noncovalently bound FAD molecules. In contrast to xanthine oxidase (cf. 2.3.3.2), for example, this enzyme has no heavy metal ion. During oxidation of a substrate, such as the oxidation of β-D-glucose to δ-D- gluconolactone, the flavoquinone is reduced by two single electron transfers:



(2.9)

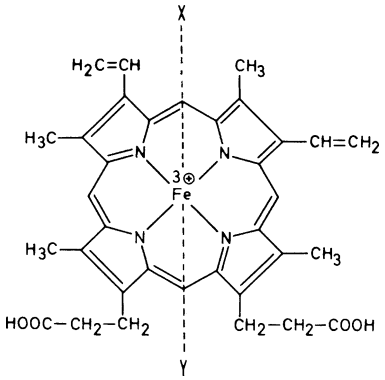
Like glucose oxidase, many flavin enzymes transfer the electrons to molecular oxygen, forming H_2O_2 and flavoquinone. The following intermediary products appear in this reaction:



(2.10)

2.3.2.2 Hemin

Peroxidases from food of plant origin and several catalases contain ferri-protoporphyrin IX (hemin, cf. Formula 2.11) as their prosthetic group and as the chromophore responsible for the brown color of the enzymes:

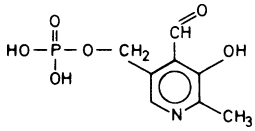


(2.11)

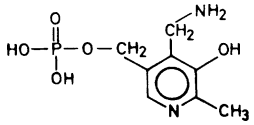
In catalytic reactions there is a change in the electron excitation spectra of the peroxidases (Fig. 2.6a) which is caused by a valence change of the iron ion (Fig. 2.6b). Intermediary compounds I (green) and II (pale red) are formed during this change by reaction with H_2O_2 and reducing agent AH. The reaction cycle is completed by another single electron transfer.
Some verdoperoxidases, which are green in color (as suggested by their name) and found in various foods of animal origin, e. g. milk, contain an unidentified Fe-protoporphyrin as their prosthetic group.

2.3.2.3 Pyridoxal Phosphate

Pyridoxal phosphate (Formula 2.12) and pyridoxamine (Formula 2.13), derived from it, are designated as vitamin B_6 (cf. 6.3.3) and are essential ingredients of food:



(2.12)



(2.13)

Coupled to the enzyme as a prosthetic group through a lysyl residue, pyridoxal phosphate is involved in conversion reactions of amino acids. In the first step of catalysis, the amino group of the amino acid substrate displaces the 6-amino group of lysine from the aldimine linkage (cf. Reaction 2.14). The positively charged pyridine ring then exerts an electron shift towards the α-C-atom of the amino acid substrate; the shift being supported by the release of one substituent of the α-C-atom. In Fig. 2.7 is shown how the ionization of the proton attached to the α-C-atom leads to *transamination*

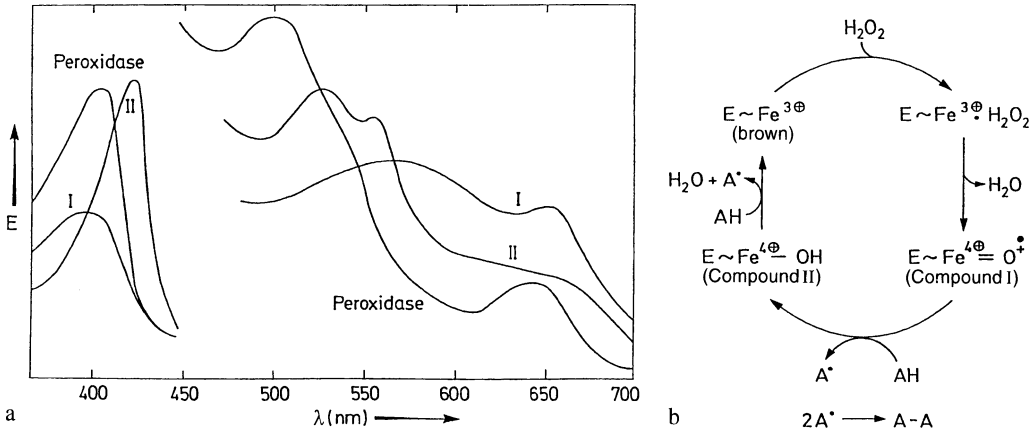
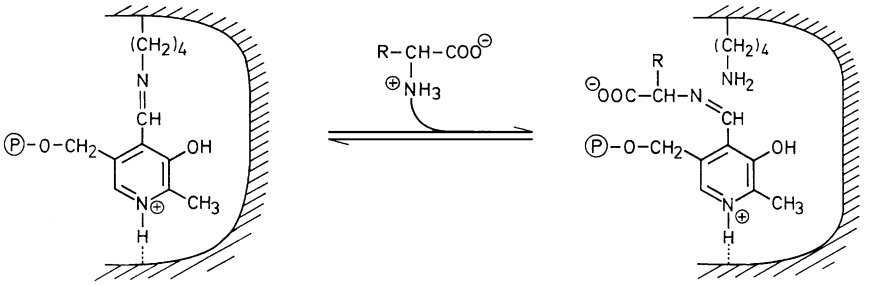


Fig. 2.6. Peroxidase reaction with H_2O_2 and a hydrogen donor (AH). **a** Electron excitation spectra of peroxidase and intermediates I and II; **b** mechanism of catalysis



(2.14)

of the amino acid with formation of an α-keto acid. The reaction may also proceed through a *decarboxylation* (Fig. 2.7) and yield an amine. Which of these two pathway options will prevail is decided by the structure of the protein moiety of the enzyme.

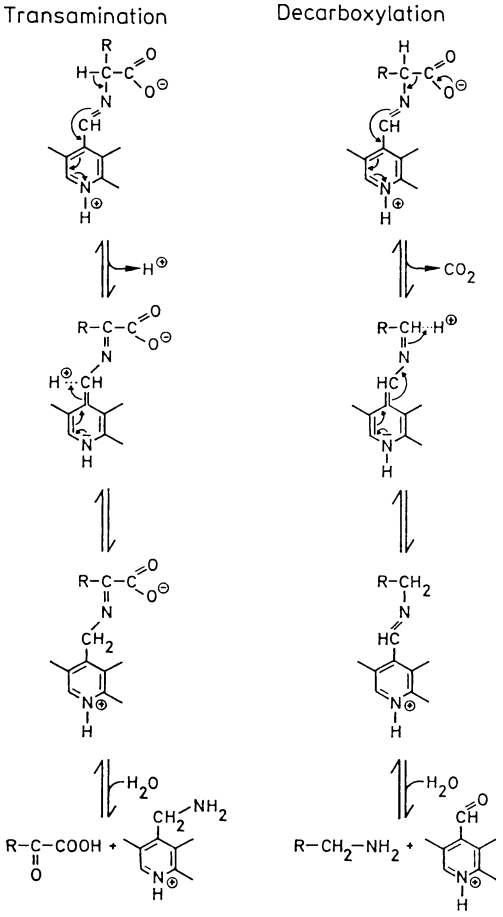


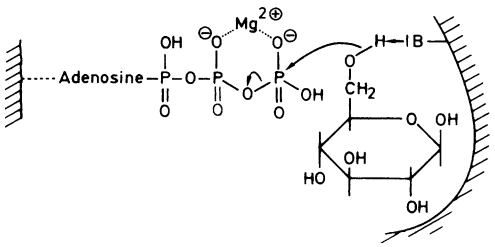
Fig. 2.7. The role of pyridoxal phosphate in transamination and decarboxylation of amino acids

2.3.3 Metal Ions

Metal ions are indispensable cofactors and stabilizers of the conformation of many enzymes. They are especially effective as cofactors with enzymes converting small molecules. They influence the substrate binding and participate in catalytic reactions in the form of a *Lewis* acid or play the role of an electron carrier. Only the most important ions will be discussed.

2.3.3.1 Magnesium, Calcium and Zinc

$Mg^{2\oplus}$ ions activate some enzymes which hydrolyze phosphoric acid ester bonds (e. g. phosphatases; cf. Table 2.4) or transfer phosphate residues from ATP to a suitable acceptor (e. g. kinases; cf. Table 2.4). In both cases, $Mg^{2\oplus}$ ions act as an electrophilic *Lewis* acid, polarize the P−O-linkage of the phosphate residue of the substrate or cosubstrate and, thus, facilitate a nucleophilic attack (water with hydrolases; ROH in the case of kinases). An example is the hexokinase enzyme (cf. Table 2.16) which, in glycolysis, is involved in catalyzing the phosphorylation of glucose to glucose-6-phosphate with ATP as cosubstrate. The effect of a $Mg^{2\oplus}$ ion within the enzyme-substrate complex is obvious from the following formulation:



(2.15)

$Ca^{2\oplus}$ ions are weaker *Lewis* acids than $Mg^{2\oplus}$ ions. Therefore, the replacement of $Mg^{2\oplus}$ by $Ca^{2\oplus}$ may result in an inhibition of the kinase enzymes. Enhancement of the activity of other enzymes by $Ca^{2\oplus}$ is based on the ability of the ion to interact with the negatively charged sites of amino acid residues and, thus, to bring about stabilization of the enzyme conformation (e. g. α-amylase; cf. 4.4.4.5.1). The activation of the enzyme may be also caused by the involvement of the $Ca^{2\oplus}$ ion in substrate binding (e. g. lipase; cf. 3.7.1.1).

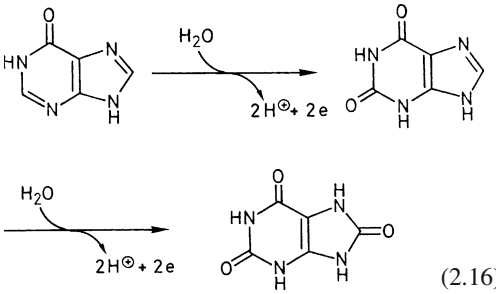
The $Zn^{2\oplus}$ ion, among the series of transition metals, is a cofactor which is not involved in redox reactions under physiological conditions. As a *Lewis* acid similar in strength to $Mg^{2\oplus}$, $Zn^{2\oplus}$ participates in similar reactions. Hence, substituting the $Zn^{2\oplus}$ ion for the $Mg^{2\oplus}$ ion in some enzymes is possible without loss of enzyme activity. Both metal ions can function as stabilizers of enzyme conformation and their direct participation in catalysis is readily revealed in the case of alcohol dehydrogenase. This enzyme isolated from horse liver consists of two identical polypeptide chains, each with one active site. Two of the four $Zn^{2\oplus}$ ions in the enzyme readily dissociate. Although this dissociation has no effect on the quaternary structure, the enzyme activity is lost. As described under section 2.3.1.1, both of these $Zn^{2\oplus}$ ions are involved in the formation of the active site. In catalysis they polarize the substrate's C−O linkage and, thus, facilitate the transfer of hydride ions from or to the cosubstrate. Unlike the dissociable ions, removal of the two residual $Zn^{2\oplus}$ ions is possible only under drastic conditions, namely disruption of the enzyme's quaternary structure which is maintained by these two ions.

2.3.3.2 Iron, Copper and Molybdenum

The redox system of $Fe^{3\oplus}/Fe^{2\oplus}$ covers a wide range of potentials (Table 2.5) depending on the attached ligands. Therefore, the system is exceptionally suitable for bridging large potential differences in a stepwise electron transport system. Such an example is encountered in the transfer of electrons by the cytochromes as members of the respiratory chain (cf. textbook of biochemistry) or in the biosynthesis of unsaturated fatty acids (cf. 3.2.4), and by some individual enzymes.

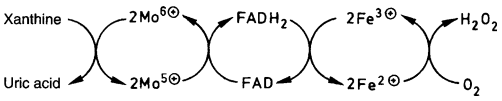
Iron-containing enzymes are attributed either to the heme (examples in 3.3.2.2) or to the non-heme Fe-containing proteins. The latter case is exemplified by lipoxygenase, for which the mechanism of activity is illustrated in section 3.7.2.2, or by xanthine oxidase.

Xanthine oxidase from milk ($M_r = 275{,}000$) reacts with many electron donors and acceptors. However, this enzyme is most active with substrates such as xanthine or hypoxanthine as electron donors and molecular oxygen as the electron acceptor. The enzyme is assumed to have two active sites per molecule, with each having 1 FAD moiety, 4 Fe-atoms and 1 Mo-atom. During the oxidation of xanthine to uric acid:



(2.16)

oxygen is reduced by two one-electron steps to H_2O_2 by an electron transfer system in which the following valence changes occur:



(2.17)

Under certain conditions the enzyme releases a portion of the oxygen when only one electron transfer has been completed. This yields $O_2^{\ominus}$, the superoxide radical anion, with one unpaired electron. This ion can initiate lipid peroxidation by a chain reaction (cf. 3.7.2.1.8).

Polyphenol oxidases and ascorbic acid oxidase, which occur in food, are known to have a $Cu^{2\oplus}/Cu^{1\oplus}$ redox system as a prosthetic group. Polyphenol oxidases play an important role in the quality of food of plant origin because they cause the "*enzymatic browning*" for example in potatoes, apples and mushrooms. Tyrosinases, catecholases, phenolases or cresolases are enzymes that react with oxygen and a large range of mono and diphenols.

Table 2.5. Redox potentials of Fe^{3+}/Fe^{2+} complex compounds at pH 7 (25 °C) as affected by the ligand

Redox-System	E'_0 (Volt)
$[Fe^{III}(o\text{-phen}^a)_3]^{3\oplus}/[Fe^{II}(o\text{-phen})_3]^{2\oplus}$	+1.10
$[Fe^{III}(OH_2)_6]^{3\oplus}/[Fe^{II}(OH_2)_6]^{2\oplus}$	+0.77
$[Fe^{III}(CN)_6]^{3\ominus}/[Fe^{II}(CN)_6]^{4\ominus}$	+0.36
Cytochrome a($Fe^{3\oplus}$)/Cytochrome a ($Fe^{2\oplus}$)	+0.29
Cytochrome c ($Fe^{3\oplus}$)/Cytochrome c ($Fe^{2\oplus}$)	+0.26
Hemoglobin ($Fe^{3\oplus}$)/Hemoglobin ($Fe^{2\oplus}$)	+0.17
Cytochrome b ($Fe^{3\oplus}$)/Cytochrome b ($Fe^{2\oplus}$)	+0.04
Myoglobin ($Fe^{3\oplus}$)/Myoglobin ($Fe^{2\oplus}$)	0.00
$(Fe^{III}EDTA)^{1\ominus}/(Fe^{II}EDTA)^{2\ominus}$	−0.12
$(Fe^{III}(oxin^b)_3)/(Fe^{II}(oxin)_3)^{1\ominus}$	−0.20
Ferredoxin ($Fe^{3\oplus}$)/Ferredoxin ($Fe^{2\oplus}$)	−0.40

[a] o-phen: o-Phenanthroline.
[b] oxin: 8-Hydroxyquinoline.

Polyphenol oxidase catalyzes two reactions: first the hydroxylation of a monophenol to o-diphenol (EC 1.14.18.1, monophenol monooxygenase) followed by an oxidation to o-quinone (EC 1.10.3.1, o-diphenol: oxygen oxidoreductase). Both activities are also known as cresolase and catecholase activity. At its active site, polyphenol oxidase contains two $Cu^{1\oplus}$ ions with two histidine residues each in the ligand field. In an "ordered mechanism" (cf. 2.5.1.2.1) the enzyme first binds oxygen and later monophenol with participation of the intermediates shown in Fig. 2.8. The Cu ions change their valency ($Cu^{1\oplus} \rightarrow Cu^{2\oplus}$). The newly formed complex ([] in Fig. 2.8) has a strongly polarized O−O=bonding, resulting in a hydroxylation to o-diphenol. The cycle closes with the oxidation of o-diphenol to o-quinone.

2.4 Theory of Enzyme Catalysis

It has been illustrated with several examples (Table 2.1) that enzymes are substantially better catalysts than are protons or other ionic species used in nonenzymatic reactions. Enzymes invariably surpass all chemical catalysts in relation to substrate and reaction specificities.
Theories have been developed to explain the exceptional efficiency of enzyme activity. They are based on findings which provide only indirect insight into enzyme catalysis. Examples are the identification of an enzyme's functional groups involved in catalysis, elucidation of their arrangement within the tertiary structure of the enzyme, and the detection of conformational changes induced by substrate binding. Complementary studies involve low molecular weight model substrates, the reactions of which shed light on the active sites or groups of the enzyme and their coordinated interaction with other factors affecting enzymatic catalysis.

2.4.1 Active Site

An enzyme molecule is, when compared to its substrate, often larger in size by a factor

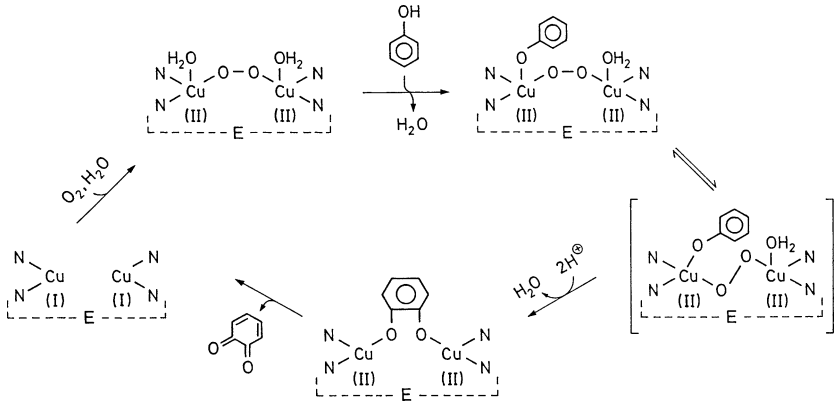


Fig. 2.8. Mechanism of polyphenol oxidase activity

of several orders of magnitude. For example, glucose oxidase ($M_r = 1.5 \cdot 10^5$) and glucose ($M_r = 180$). This strongly suggests that in catalysis only a small locus of an active site has direct contact with the substrate. Specific parts of the protein structure participate in the catalytic process from the substrate binding to the product release from the so-called *active site*. These parts are amino acid residues which bind substrate and, if required, cofactors and assist in conversion of substrate to product.

Investigations of the structure and function of the active site are conducted to identify the amino acid residues participating in catalysis, their steric arrangement and mobility, the surrounding microenvironment and the catalysis mechanism.

2.4.1.1 Active Site Localization

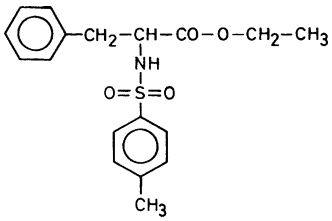
Several methods are generally used for the identification of amino acid residues present at the active site since data are often equivocal. Once obtained, the data must still be interpreted with a great deal of caution and insight.

The influence of pH on the activity assay (cf. 2.5.3) provides the first direct answer as to whether dissociable amino acid side chains, in charged or uncharged form, assist in catalysis. The data readily obtained from this assay must again be interpreted cautiously since neighboring charged groups, hydrogen bonds or the hydrophobic environment of the active site can affect the extent of dissociation of the amino acid residues and, thus, can shift their pK values (cf. 1.4.3.1).

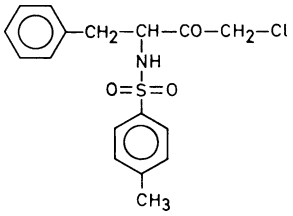
Selective labeling of side chains which form the active site is also possible by chemical modification. When an enzyme is incubated with reagents such as iodoacetic acid (cf. 1.2.4.3.5) or dinitrofluorobenzene (cf. 1.2.4.2.2), resulting in a decrease of activity, and subsequent analysis of the modified enzyme shows that only one of the several available functional groups is bound to reagent (e. g. one of several −SH groups), then this group is most probably part of the active site. Selective labeling data when an inhibiting substrate analogue is used are more convincing. Because of its similarity to the chemical structure of the substrate, the analogue will be bound covalently to the enzyme but not converted into product. We will consider the following examples:

N-tosyl-L-phenylalanine ethyl ester (Formula 2.18) is a suitable substrate for the proteinase chymotrypsin which hydrolyzes ester bonds.

When the ethoxy group is replaced by a chloromethyl group, an inhibitor whose structure is similar to the substrate is formed (N_α-tosyl-L-phenylalanine chloromethylketone, TPCK).

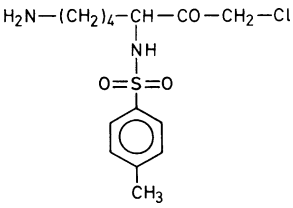


(2.18)



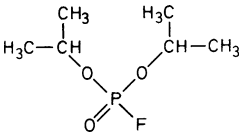
(2.19)

Thus, the substrate analogue binds specifically and irreversibly to the active site of chymotrypsin. Analysis of the enzyme inhibitor complex reveals that, of the two histidine residues present in chymotrypsin, only His^{57} is alkylated at one of its ring nitrogens. Hence, the modified His residue is part of the active site (cf. mechanism of chymotrypsin catalysis, Fig. 2.17). TPCK binds highly specifically, thus the proteinase trypsin is not inhibited. The corresponding inhibiting substrate analogue, which binds exclusively to trypsin, is N-tosyl-L-lysine chloromethylketone (TLCK):



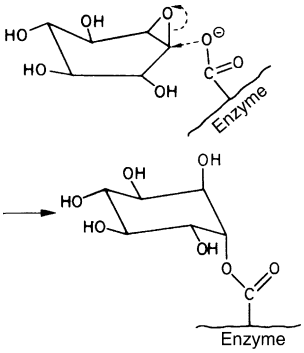
(2.20)

Reaction of diisopropylfluorophosphate (DIFP)



(2.21)

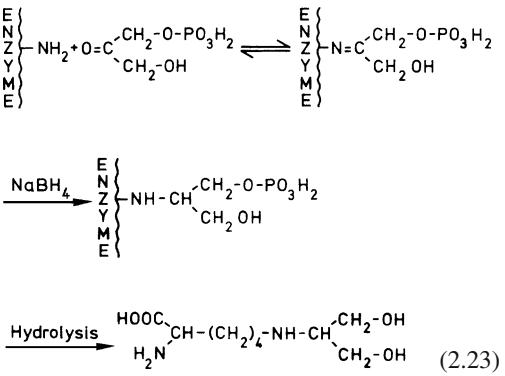
with a number of proteinases and esterases alkylates the unusually reactive serine residue at the active site. Thus, of the 28 serine residues present in chymotrypsin, only Ser^{195} is alkylated, while the other 27 residues are unaffected by the reagent. It appears that the reactivity of Ser^{195} is enhanced by its interaction with the neighboring His^{57} (cf. mechanism of catalysis in Fig. 2.17). The participation of a carboxyl group at the active site in β-glucosidase catalysis has been confirmed with the help of conduritol B-epoxide, an inhibiting substrate analogue:



(2.22)

A lysine residue is involved in enzyme catalysis in a number of lyase enzymes and in enzymes in which pyridoxal phosphate is the cosubstrate. An intermediary *Schiff* base product is formed between an ε-amino group of the enzyme and the substrate or pyridoxal phosphate (cf. 2.3.2.3). The reaction site is then identified by reduction of the *Schiff* base with $NaBH_4$.

An example of a "lysine" lyase is the aldolase enzyme isolated from rabbit muscle. The intermediary product formed with dihydroxyacetone phosphate (cf. mechanism in Fig. 2.19) is detected as follows:

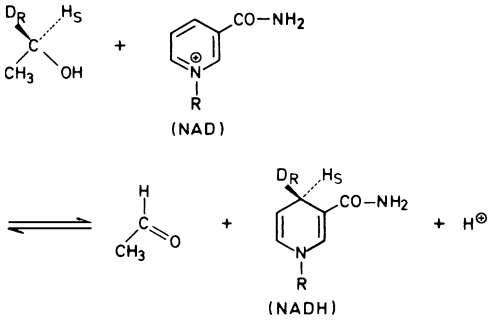


(2.23)

2.4.1.2 Substrate Binding

2.4.1.2.1 Stereospecificity

Enzymes react stereospecifically. Before being bound to the binding locus, the substrates are distinguished by their cis, trans-isomerism and also by their optical antipodes. The latter property was illustrated by the reactions of L(+)-lactic acid (Fig. 2.2). There are distinct recognition areas on the binding locus. Alcohol dehydrogenase will be used to demonstrate this. This enzyme removes two hydrogen atoms, one from the methylene group and the other from the hydroxyl group, to produce acetaldehyde. However, the enzyme recognizes the difference between the two methylene hydrogens since it always stereospecifically removes the same hydrogen atom. For example, yeast alcohol dehydrogenase always removes the pro-R-hydrogen from the C-1 position of a stereospecifically deuterated substrate and transfers it to the C-4 position of the nicotinamide ring of NAD:



(2.24)

To explain the stereospecificity, it has been assumed that the enzyme must bind simultaneously to more than one point of the molecule. Thus, when two substituents (e. g. the methyl and hydroxyl groups of ethanol; Fig. 2.9) of the prochiral site are attached to the enzyme surface at positions A and B, the position of the third substituent is fixed. Therefore, the same substituent will always be bound to reactive position C, e. g. one of the two methylene hydrogens in ethanol. In other words, the two equal substituents in a symmetrical molecule are differentiated by asymmetric binding to the enzyme.

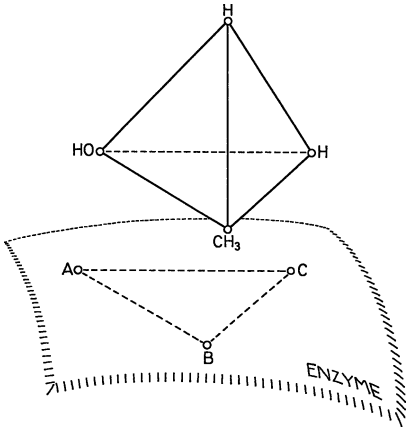


Fig. 2.9. A model for binding of a prochiral substrate (ethanol) by an enzyme

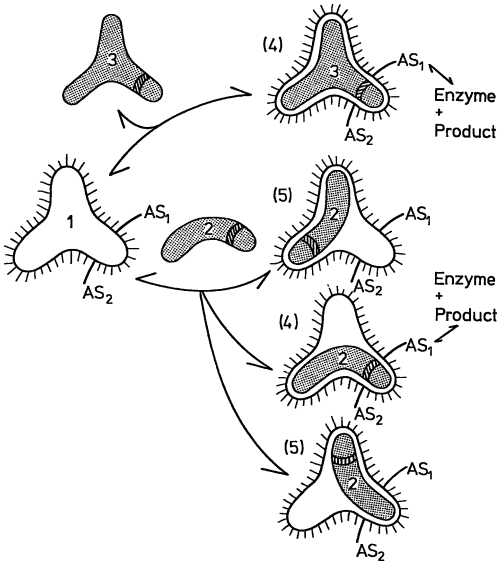


Fig. 2.10. Binding of a good (3) and of a bad substrate (2) by the active site (1) of the enzyme (according to *Jencks*, 1969). (4) A productive enzyme-substrate complex; (5) a nonproductive enzyme substrate complex. As_1 and As_2: reactive amino acid residues of the enzyme involved in conversion of substrate to product

2.4.1.2.2 "Lock and Key" Hypothesis

To explain substrate specificity, *E. Fischer* proposed a hypothesis a century ago in which he depicted the substrate as being analogous to a key and the enzyme as its lock. According to this model, the active site has a geometry which is complementary only to its substrate (Fig. 2.10). In contrast, there are many possibilities for a "bad" substrate to be bound to the enzyme, but only one provides the properly positoned enzyme-substrate complex, as illustrated in Fig. 2.10, which is converted to the product.

The proteinases chymotrypsin and trypsin are two enzymes for which secondary and tertiary structures have been elucidated by x-ray analysis and which have structures supporting the lock and key hypothesis to a certain extent. The binding site in chymotrypsin and trypsin is a three-dimensional hydrophobic pocket (Fig. 2.11). Bulky amino acid residues such as aromatic amino acids fit neatly into the pocket (chymotrypsin, Fig. 2.11a), as do substrates with lysyl or arginyl residues (trypsin, Fig. 2.11b). Instead of Ser^{189}, the trypsin peptide chain has Asp^{189} which is present in the deep cleft in the form of a carboxylate anion and which attracts the positively charged lysyl or arginyl residues of the substrate. Thus, the substrate is stabilized and realigned by its peptide bond to face the enzyme's Ser^{195} which participates in hydrolysis (transforming locus).

The peptide substrate is hydrolyzed by the enzyme elastase by the same mechanism as for chymotrypsin. However, here the pocket is closed to such an extent by the side chains of Val^{216} and Thr^{226} that only the methyl group of alanine can enter the cleft (Fig. 2.11c). Therefore, elastase has specificity for alanyl peptide bonds or alanyl ester bonds.

2.4.1.2.3 Induced-fit Model

The conformation of a number of enzymes is changed by the binding of the substrate. An example is carboxypeptidase A, in which the Try^{248} located in the active site moves approximately 12 Å towards the substrate, glycyl-L-phenylalanine, to establish contact. This and other observations support the dynamic induced-fit model proposed by *Koshland* (1964). Here, only the substrate has the power to induce a change in the tertiary structure to the active form of the enzyme. Thus, as the substrate molecule approaches the enzyme surface, the amino acid

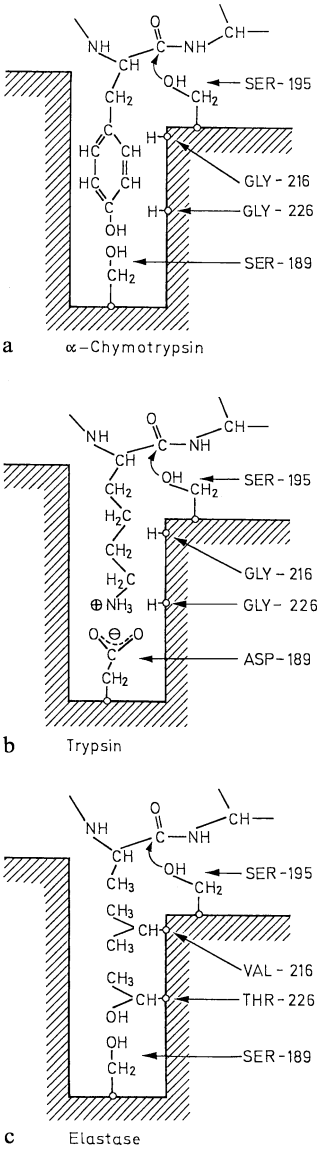


Fig. 2.11. A hypothesis for substrate binding by α-chymotrypsin, trypsin and elastase enzymes (according to *Shotton*, 1971)

residues A and B change their positions to conform closely to the shape of the substrate (I, in Fig. 2.12). Groups A and B are then in the necessary position for reaction with the substrate. Diagrams II and III (Fig. 2.12) illustrate the case when the added compound is not suitable as substrate. Although group C positioned the

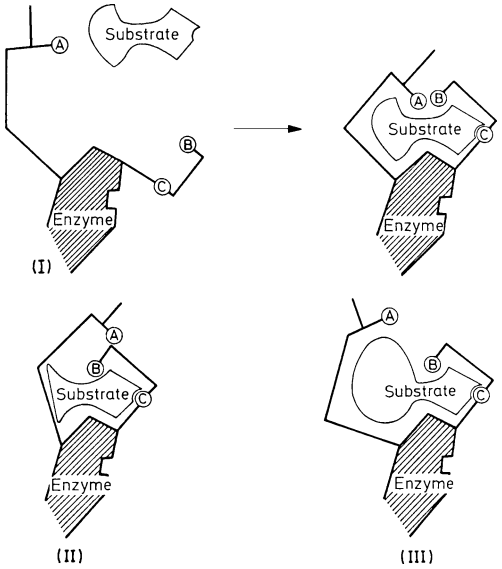


Fig. 2.12. A schematic presentation of "induced-fit model" for an active site of an enzyme (according to *Koshland*, 1964).
— Polypeptide chain of the enzyme with catalytically active residues of amino acids, A and B; the residue C binds the substrate

substrate correctly at its binding site, the shape of the compound prevents groups A and B from being aligned properly in their active positions and, thus, from generating the product.

In accordance with the mechanisms outlined above, one theory suitable for enzymes following the lock and key mechanism and the other theory for enzymes operating with the dynamic induced-fit model, the substrate specificity of any enzyme-catalyzed reaction can be explained satisfactorily.

In addition, the relationship between enzyme conformation and its catalytic activity thus outlined also accounts for the extreme sensitivity of the enzyme as catalyst. Even slight interferences imposed on their tertiary structure which affect the positioning of the functional groups result in loss of catalytic activity.

2.4.2 Reasons for Catalytic Activity

Even though the rates of enzymatically catalyzed reactions vary, they are very high compared to the

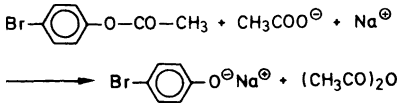
effectiveness of chemical catalysts (examples in Table 2.2). The factors responsible for the high increase in reaction rate are outlined below. They are of different importance for the individual enzymes.

2.4.2.1 Steric Effects – Orientation Effects

The specificity of substrate binding contributes substantially to the rate of an enzyme-catalyzed reaction.

Binding to the active site of the enzyme concentrates the reaction partners in comparison with a dilute substrate solution. In addition, the reaction is now the favored one since binding places the substrate's susceptible reactive group in the proximity of the catalytically active group of the enzyme.

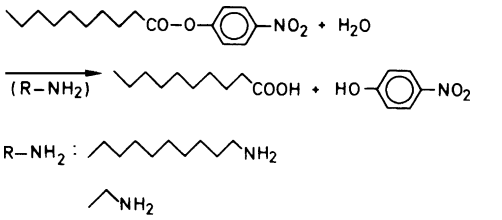
Therefore the contribution of substrate binding to the reaction rate is partially due to a change in the molecularity of the reaction. The intermolecular reaction of the two substrates is replaced by an intramolecular reaction of an enzyme-substrate complex. The consequences can be clarified by using model compounds which have all the reactive groups within their molecules and, thus, are subjected to an intramolecular reaction. Their reactivity can then be compared with that of the corresponding bimolecular system and the results expressed as a ratio of the reaction rates of the intramolecular (k_1) to the intermolecular (k_2) reactions. Based on their dimensions, they are denoted as "effective molarity". As an example, let us consider the cleavage of p-bromophenylacetate in the presence of acetate ions, yielding acetic acid anhydride:



(2.25)

Intramolecular hydrolysis is substantially faster than the intermolecular reaction (Table 2.6). The effective molarity sharply increases when the reactive carboxylate anion is in close proximity to the ester carbonyl group and, by its presence, retards the mobility of the carbonyl group. Thus, the effective molarity increases (Table 2.6) as the C−C bond mobility decreases. Two bonds can rotate in a glutaric acid ester, whereas only one can rotate in a succinic acid ester. The free rotation is effectively blocked in a bicyclic system. Hence, the reaction rate is sharply increased. Here, the rigid steric arrangement of the acetate ion and of the ester group provides a configuration that imitates that of a transition state.

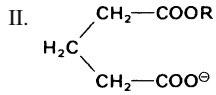
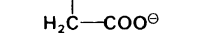
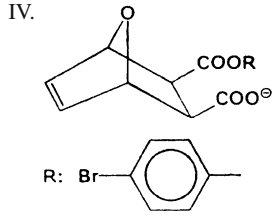
In contrast to the examples given in Table 2.6, examples should be mentioned in which substrates are not bound covalently by their enzymes. The following model will demonstrate that other interactions can also promote close positioning of the two reactants. Hydrolysis of p-nitrophenyldecanoic acid ester is catalyzed by an alkylamine:



(2.26)

The reaction rate in the presence of decylamine is faster than that in the presence of ethylamine by a factor of 700. This implies that the reactive amino group has been oriented very close to the susceptible carbonyl group of the ester by the establishment of a maximal number of hydrophobic contacts. Correspondingly, there is a decline in the reaction rate as the alkyl amine group is lengthened further.

Table 2.6. Relative reaction rate for the formation of acid anhydrides

I.		
II.		$9.5 \cdot 10^2$
III.		$2.2 \cdot 10^5$
IV.		$5 \cdot 10^7$

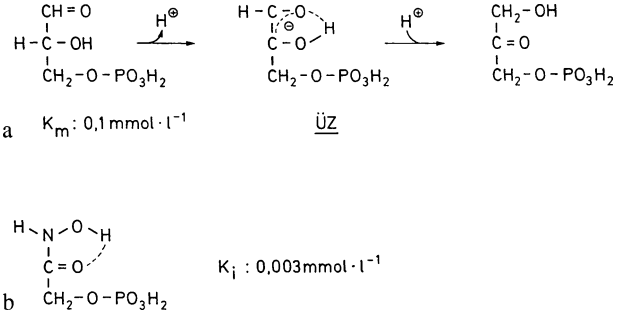


Fig. 2.13. Example of a transition state analog inhibitor **a** reaction of triosephosphate isomerase, TT: postulated transition state; **b** inhibitor

2.4.2.2 Structural Complementarity to Transition State

It is assumed that the active conformation of the enzyme matches the transition state of the reaction. This is supported by affinity studies which show that a compound with a structure analogous to the transition state of the reaction ("*transition state analogs*") is bound better than the substrate. Hydroxamic acid, for example, is such a transition state analog which inhibits the reaction of triosephosphate isomerase (Fig. 2.13). Comparisons between the *Michaelis* constant and the inhibitor constant show that the inhibitor has a 30 times higher affinity to the active site than the substrate.

The active site is complementary to the transition state of the reaction to be catalyzed. This assumption is supported by a reversion of the concept. It has been possible to produce catalytically active monoclonal antibodies directed against transition state analogs. The antibodies accelerate the reaction approximating the transition state of the analog. However, their catalytic activity is weaker compared to enzymes because only the environment of the antibody which is complementary to the transistion state causes the acceleration of the reaction.

Transition state analog inhibitors were used to show that in the binding the enzyme displaces the hydrate shell of the substrates. The reaction rate can be significantly increased by removing the hydrate shell between the participants.

Other important factors in catalytic reactions are the distortion of bonds and shifting of charges. The substrate's bonds will be strongly polarized by the enzyme, and thus highly reactive, through the precise positioning of an acid or base group or a metall ion (*Lewis* acid, cf. 2.3.3.1) (example see Formula 2.15). These hypotheses are supported by investigations using suitable transition state analog inhibitors.

2.4.2.3 Entropy Effect

An interpretation in thermodynamic terms takes into account that a loss of entropy occurs during catalysis due to the loss of freedom of rotation and translation of the reactants. This entropy effect is probably quite large in the case of the formation of an enzyme-substrate complex since the reactants are fairly rigidly positioned before the transition state is reached. Consequently, the conversion of the enzyme-substrate complex to the transition state is accompanied by little or no change of entropy. As an example, a reaction running at 27 °C with a decrease in entropy of $140\,J\,K^{-1}\,mol^{-1}$ is considered. Calculations indicate that this decrease leads to a reduction in free activation energy by about 43 kJ. This value falls in the range of the amount by which the activation energy of a reaction is lowered by an enzyme (cf. Table 2.1) and which can have the effect of increasing the reaction rate by a factor of 10^8

The catalysis by chymotrypsin, for example, shows how powerful the entropy effects can be. In section 2.4.2.5 we will see that this catalysis is a two-step event proceeding through an acylated enzyme intermediate. Here we will consider only the second step, deacylation, thereby distinguishing the following intermediates:

a) N-acetyl-L-tyrosyl-chymotrypsin
b) Acetyl-chymotrypsin.

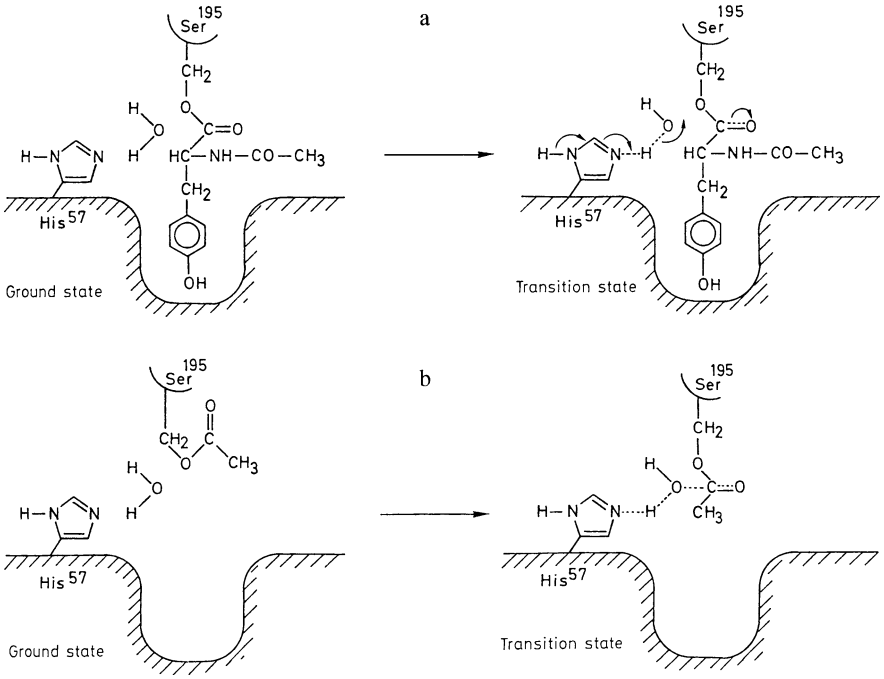


Fig. 2.14. Influence of the steric effect on deacylation of two acyl-chymotrypsins (according to *Bender* et al., 1964). **a** N-acetyl-L-tyrosyl-chymotrypsin, **b** acetyl-chymotrypsin

In case a) deacylation is faster by a factor of 3540 since the carbonyl group is immobilized by insertion of the bulky N-acetyl-L-tyrosyl group into a hydrophobic pocket on the enzyme (Fig. 2.14a) at the correct distance from the attacking nucleophilic $OH^{\ominus}$ ion derived from water (cf. 2.4.2.5). In case b) the immobilization of the small acetyl group is not possible (Fig. 2.14b) so that the difference between the ground and transition states is very large. The closer the ground state is to the transition state, the more positive will be the entropy of the transition state, $\Delta S^{\#}$; a fact that as mentioned before can lead to a considerable increase in reaction rate. The thermodynamic data in Table 2.7 show that the difference in reaction rates depends, above all, on an entropy effect; the enthalpies of the transition states scarcely differ.

Table 2.7. Thermodynamic data for transition states of twoacyl-chymotrypsins

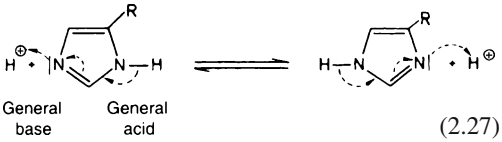
Acyl-enzyme	$\Delta G^{\#}$ ($kJ \cdot mol^{-1}$)	$\Delta H^{\#}$ ($kJ \cdot mol^{-1}$)	$\Delta S^{\#}$ ($J \cdot K^{-1} \cdot mol^{-1}$)
N-Acetyl-L-tyrosyl	59.6	43.0	−55.9
Acetyl	85.1	40.5	−149.7

2.4.2.4 General Acid–Base Catalysis

When the reaction rate is affected by the concentration of hydronium ($H_3O^{\oplus}$) or $OH^{\ominus}$ ions from water, the reaction is considered to be specifically acid or base catalyzed. In the so-called general acid or base catalysis the reaction rate is affected by prototropic groups located on the side chains of the amino acid residues. These groups involve proton donors (denoted as general acids) and proton acceptors (general bases). Most of the amino acids located on the active site of the enzyme influence the reaction rate by general acid-base catalysis.

As already mentioned, the amino acid residues in enzymes have prototropic groups which have the potential to act as a general acid or as a general base. Of these, the imidazole ring of histidine is of special interest since it can perform both func-

tions simultaneously:



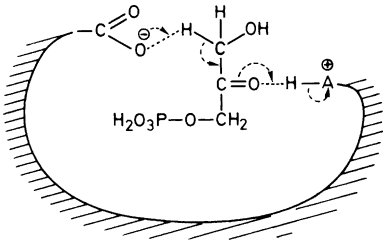
(2.27)

The imidazole ring ($pK_2 = 6.1$) can cover the range of the pH optima of many enzymes.

Thus, two histidine residues are involved in the catalytic activity of ribonuclease, a phosphodiesterase. The enzyme hydrolyzes pyrimidine-2′,3′-cyclic phosphoric acids. As shown in Fig. 2.15, cytidine-2′,3′-cyclic phosphoric acid is positioned between two imidazole groups at the binding locus of the acitive site.

His^{12} serves as a general base, removing the proton from a water molecule. This is followed by nucleophilic attack of the intermediary $OH^{\ominus}$ ions on the electrophilic phosphate group. This attack is supported by the concerted action of the general acid His^{119}.

Another concerted general acid-base catalysis is illustrated by triose phosphate isomerase, an enzyme involved in glycolysis. Here, the concerted action involves the carboxylate anion of a glutamic acid residue as a general base with a general acid which has not yet been identified:



(2.28)

The endiol formed from dihydroxyacetone-3-phosphate in the presence of enzyme isomerizes into glyceraldehyde-3-phosphate.

These two examples show clearly the significant differences to chemical reactions in solutions. The enzyme driven acid-base catalysis takes place selectively at a certain locus of the active site. The local concentration of the amino acid residue acting as acid or base is fairly high due to the perfect position relative to the substrate. On the other hand, in chemical reactions in solutions all reactive groups of the substrate are nonspecifically attacked by the acid or base.

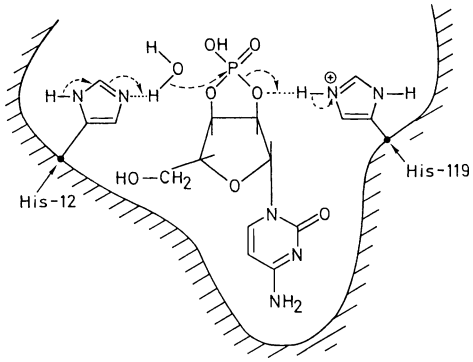


Fig. 2.15. Hydroylysis of cytidine-2′,3′-phosphate by ribonuclease (reaction mechanism according to *Findlay*, 1962)

2.4.2.5 Covalent Catalysis

Studies aimed at identifying the active site of an enzyme (cf. 2.4.1.1) have shown that, during catalysis, a number of enzymes bind the substrate by covalent linkages. Such covalent linked enzyme-substrate complexes form the corresponding products much faster than compared to the reaction rate in a non-catalyzed reaction.

Examples of enzyme functional groups which are involved in covalent bonding and are responsible for the transient intermediates of an enzyme-substrate complex are compiled in Table 2.8. Nucleophilic catalysis is dominant (examples 1–6, Table 2.8), since amino acid residues are present in the active site of these enzymes, which

Table 2.8. Examples of covalently linked enzymesubstrate intermediates

Enzyme	Reactive functional group	Intermediate
1. Chymotrypsin	HO-(Serine)	Acylated enzyme
2. Papain	HS-(Cysteine)	Acylated enzyme
3. β-Amylase	HS-(Cysteine)	Maltosylenzyme
4. Aldolase	ε-H_2N-(Lysine)	*Schiff* base
5. Alkaline phosphatase	HO-(Serine)	Phosphoenzyme
6. Glucose-6-phosphatase	Imidazole-(Histidine)	Phosphoenzyme
7. Histidine decarboxylase	O=C < (Pyruvate)	*Schiff* base

only react with substrate by donating an electron pair (nucleophilic catalysis). Electrophilic reactions occur mostly by involvement of carbonyl groups (example 7, Table 2.8) or with the help of metal ions.

A number of peptidase and esterase enzymes react covalently in substitution reactions by a two-step nucleophilic mechanism. In the first step, the enzyme is acylated; in the second step, it is deacylated. Chymotrypsin will be discussed as an example of this reaction mechanism. Its activity is dependent on His^{57} and Ser^{195}, which are positioned in close proximity within the active site of the enzyme because of folding of the peptide chain (Fig. 2.16).

Because Asp^{102} is located in hydrophobic surroundings, it can polarize the functional groups in close proximity to it. Thus, His^{57} acts as a strong general base and abstracts a proton from the OH-group of the neighboring Ser^{195} residue (step 'a', Fig. 2.17). The oxygen remaining on Ser^{195} thus becomes a strong nucleophile and attacks the carbon of the carbonyl group of the peptide bond of the substrate. At this stage an amine (the first product) is released (step 'b', Fig. 2.17) and the transient covalently-bound acyl enzyme is formed. A deacylation step follows. The previous position of the amine is occupied by a water molecule. Again, His^{57}, through support from Asp^{102}, serves as a general base, abstracting the proton from water (step 'c', Fig. 2.17). This is followed by nucleophilic attack of the resultant $OH^{\ominus}$ ion on the carbon of the carbonyl group of the acyl enzyme (step 'd', Fig. 2.17), resulting in free enzyme and the second product of the enzymic conversion.

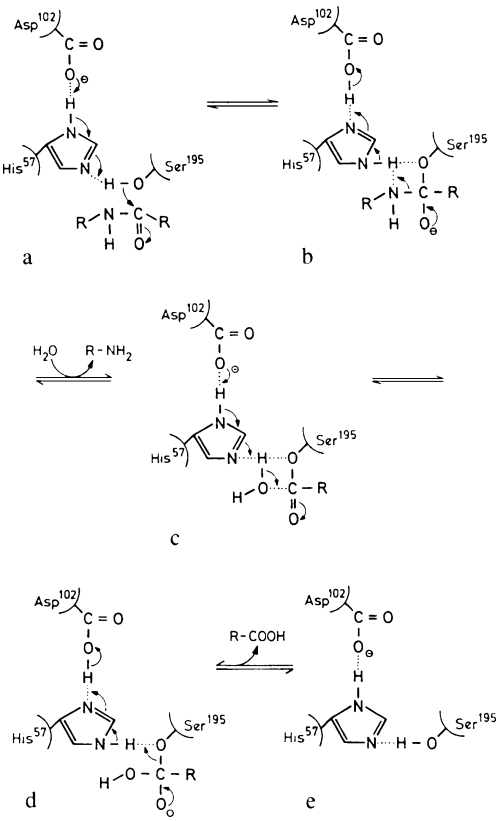


Fig. 2.17. Postulated reaction mechanism for chymotrypsin activity (according to *Blow* et al., 1969)

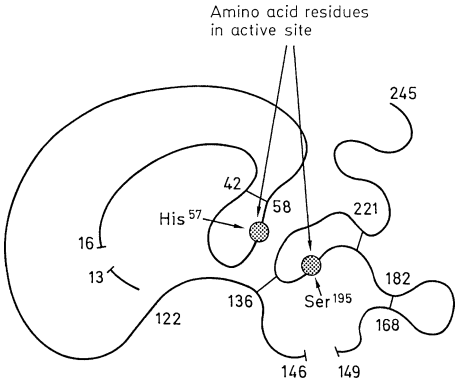


Fig. 2.16. Polypeptide chain conformation in the chymotrypsin molecule (according to *Lehninger*, 1977)

An exceptionally reactive serine residue has been identified in a great number of hydrolase enzymes, e. g., trypsin, subtilisin, elastase, acetylcholine esterase and some lipases. These enzymes appear to hydrolyze their substrates by a mechanism analogous to that of chymotrypsin. Hydrolases such as papain, ficin and bromelain, which are distributed in plants, have a cysteine residue instead of an "active" serine residue in their active sites. Thus, the transient intermediates are thioesters.

Enzymes involved in the cleavage of carbohydrates can also function by the above mechanism. Figure 2.18 shows that amylose hydrolysis by β-amylase occurs with the help of four functional groups in the active site. The enzyme-substrate complex is subjected to a nucleophilic attack

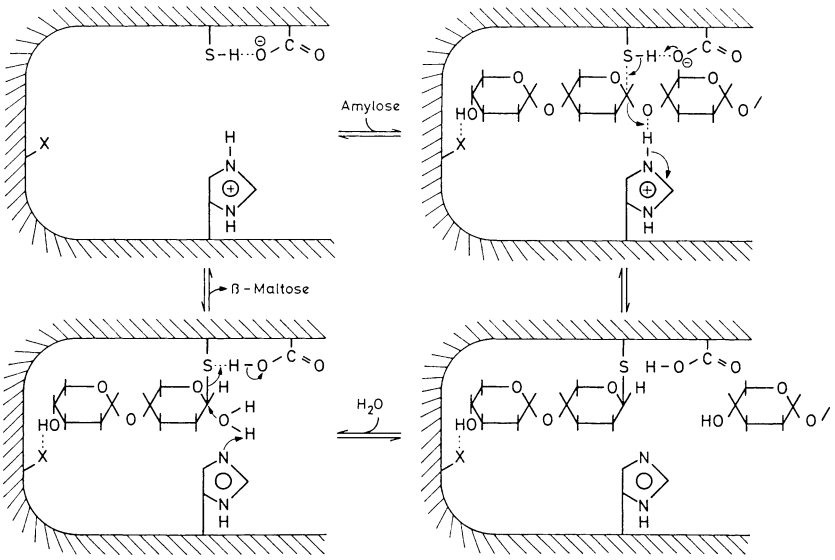


Fig. 2.18. Postulated mechanism for hydrolysis of amylose by β-amylase

by an SH-group on the carbon involved in the α-glycosidic bond. This transition step is facilitated by the carboxylate anion in the role of a general base and by the imidazole ring as an acid which donates a proton to glycosidic oxygen. In the second transition state the imidazole ring, as a general base in the presence of a water molecule, helps to release maltose from the maltosylenzyme intermediate.

Lysine is another amino acid residue actively involved in covalent enzyme catalysis (cf. 2.4.1.1). Many lyases react covalently with a substrate containing a carbonyl group. They catalyze, for example, aldol or retroaldol condensations important for the conversion and cleavage of monosaccharides or for decarboxylation reactions of β-keto acids. As an example, the details of the reaction involved will be considered for aldolase (Fig. 2.19). The enzyme-substrate complex is first stabilized by electrostatic interaction between the phosphate residues of the substrate and the charged groups present on the enzyme. A covalent intermediate, a *Schiff* base, is then formed by nucleophilic attack of the ε-amino group of the "active" lysine on a carbonyl group of the substrate. The *Schiff* base cation facilitates the retroaldol cleavage of the substrate, whereas a negatively charged group on the enzyme (e. g. a thiolate or carboxylate anion) acts as a general base, i. e. binds the free proton. Thus,

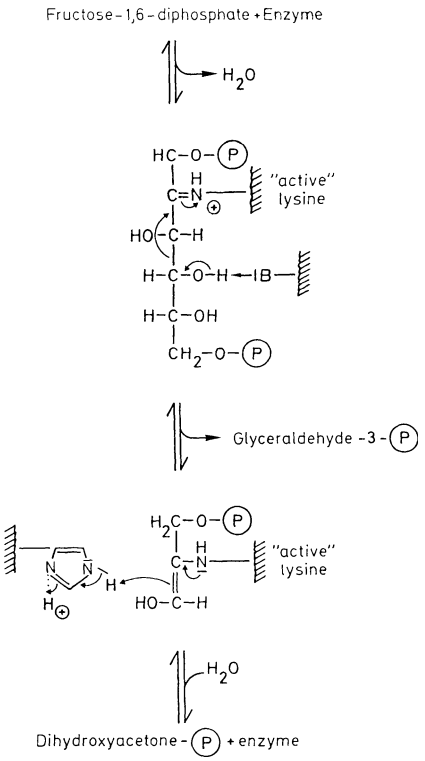
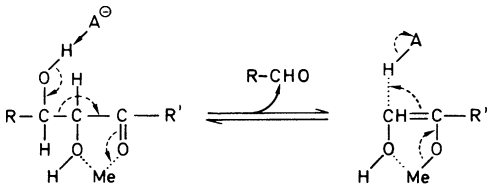


Fig. 2.19. Aldolase of rabbit muscle tissue. A model for its activity; P: PO_3H_2

the first product, glyceraldehyde-3-phosphate, is released. An enamine rearrangement into a ketimine structure is followed by release of dihydroxyacetone phosphate.

This is the mechanism of catalysis by aldolases which occur in plant and animal tissues (lysine aldolases or class I aldolases). A second group of these enzymes often produced by microorganisms contains a metal ion (metallo-aldolases). This group is involved in accelerating retroaldol condensations through electrophilic reactions with carbonyl groups:



(2.29)

Other examples of electrophilic metal catalysis are given under section 2.3.3.1. Electrophilic reactions are also carried out by enzymes which have an α-keto acid (pyruvic acid or α-keto butyric acid) at the transforming locus of the active site. One example of such an enzyme is histidine decarboxylase in which the N-terminal amino acid residue is bound to pyruvate. Histidine decarboxylation is initiated by the formation of a *Schiff* base by the reaction mechanism in Fig. 2.20.

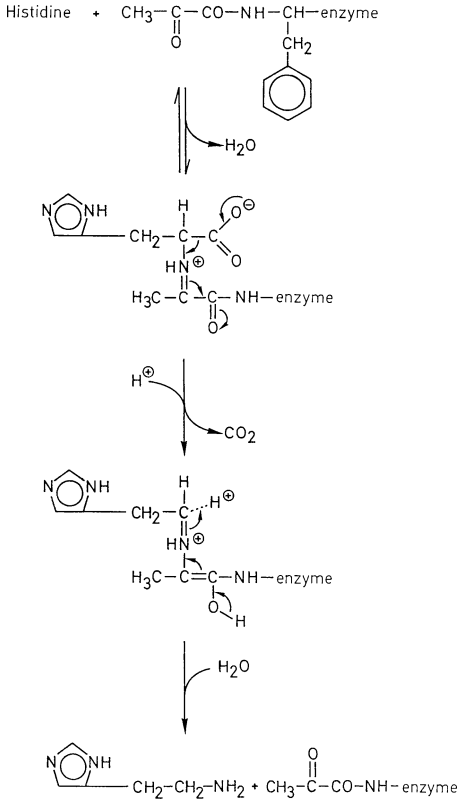


Fig. 2.20. A proposed mechanism for the reaction of histidine decarboxylase

2.4.3 Closing Remarks

The hypotheses discussed here allow some understanding of the fundamentals involved in the action of enzymes. However, the knowledge is far from the point where the individual or combined effects which regulate the rates of enzyme-catalyzed reactions can be calculated.

2.5 Kinetics of Enzyme-Catalyzed Reactions

Enzymes in food can be detected only indirectly by measuring their catalytic activity and, in this way, differentiated from other enzymes. This is the rationale for acquiring knowledge needed to analyze the parameters which influence or determine the rate of an enzyme-catalyzed reaction.

The reaction rate is dependent on the concentrations of the components involved in the reaction. Here we mean primarily the substrate and the enzyme. Also, the reaction can be influenced by the presence of activators and inhibitors. Finally, the pH, the ionic strength of the reaction medium, the dielectric constant of the solvent (usually water) and the temperature exert an effect.

2.5.1 Effect of Substrate Concentration

2.5.1.1 Single-Substrate Reactions

2.5.1.1.1 Michaelis–Menten Equation

Let us consider a single-substrate reaction. Enzyme E reacts with substrate A to form an in-

termediary enzyme-substrate complex, EA. The complex then forms the product P and releases the free enzyme:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} E + P \tag{2.30}$$

In order to determine the catalytic activity of the enzyme, the decrease in substrate concentration or the increase in product concentration as a function of time can be measured. The activity curve obtained (Fig. 2.21) has the following regions:

a) The maximum activity which occurs for a few msec until an equilibrium is reached between the rate of enzyme-substrate formation and rate of breakdown of this complex.
Measurements in this pre-steady state region which provide an insight into the reaction steps and mechanism of catalysis are difficult and time consuming. Hence, further analysis fo the pre-steady state will be ignored.

b) The usual procedure is to measure the enzyme activity when a steady state has been reached. In the steady state the intermediary complex concentration remains constant while the concentration of the substrate and end product are changing. For this state, the following is valid:

$$\frac{dEA}{dt} = -\frac{dEA}{dt} \tag{2.31}$$

c) The reaction rate continuously decreases in this region in spite of an excess of substrate. The decrease in the reaction rate can be considered to be a result of:
Enzyme denaturation which can readily occur, continuously decreasing the enzyme concentration in the reaction system, or the product formed increasingly inhibits enzyme activity or, after the concentration of the product increases, the reverse reaction takes place, converting the product back into the initial reactant.

Since such unpredictable effects should be avoided during analysis of enzyme activities, as a rule the initial reaction rate, v_0, is measured as soon as possible after the start of the reaction.
The basics of the kinetic properties of enzymes in the steady state were given by *Briggs* and *Haldane* (1925) and are supported by earlier mathematical models proposed by *Michaelis* and *Menten* (1913).

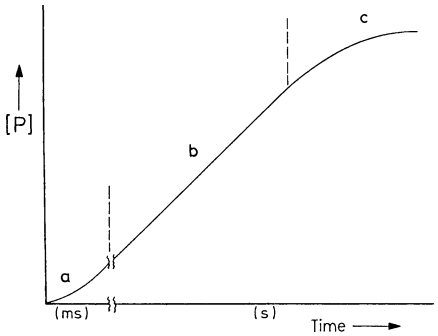


Fig. 2.21. Progress of an enzyme-catalyzed reaction

The following definitions and assumptions should be introduced in relation to the reaction in Equation 2.30:

$[E_0]$ = total enzyme concentration available at the start of the catalysis.
$[E]$ = concentration of free enzyme not bound to the enzyme-substrate complex, EA, i. e. $[E] = [E_0] - [EA]$.
$[A_0]$ = total substrate concentration available at the start of the reaction. Under these conditions, $[A_0] \gg [E_0]$. Since in catalysis only a small portion of A_0 reacts, the substrate concentration at any time, [A], is approximately equal to $[A_0]$.

When the initial reaction rate, v_0, is considered, the concentration of the product, [P], is 0. Thus, the reaction in Equation 2.30 takes the form:

$$\frac{dP}{dt} = \upsilon_0 = k_2(EA) \tag{2.32}$$

The concentration of enzyme-substrate complex, [EA], is unknown and can not be determined experimentally for Equation 2.32. Hence, it is calculated as follows: The rate of formation of EA, according to Equation 2.30, is:

$$\frac{d\,EA}{dt} = k_1(E)(A_0) \tag{2.33}$$

and the rate of EA breakdown is:

$$-\frac{d\,EA}{dt} = k_{-1}(EA) * k_2(EA) \tag{2.34}$$

Under steady-state conditions the rates of breakdown and formation of EA are equal (cf. Equation 2.31):

$$k_1(\mathrm{E})(\mathrm{A}_0) = (k_{-1} * k_2)\ (\mathrm{EA}) \tag{2.35}$$

Also, the concentration of free enzyme, [E], can not be readily determined experimentally. Hence, free enzyme concentration from the above relationship ($[\mathrm{E}] = [\mathrm{E}_0] - [\mathrm{EA}]$) is substituted in Equation 2.35:

$$k_1[(\mathrm{E}_0) - (\mathrm{EA})](\mathrm{A}_0) = (k_{-1} * k_2)(\mathrm{EA}) \tag{2.36}$$

Solving Equation 2.36 for the concentration of the enzyme-substrate complex, [EA], yields:

$$(\mathrm{E\,A}) = \frac{(\mathrm{E}_0)(\mathrm{A}_0)}{\frac{k_{-1}+k_2}{k_1} + (\mathrm{A}_0)} \tag{2.37}$$

The quotient of the rate constants in Equation 2.37 can be simplified by defining a new constant, $\mathrm{K_m}$, called the *Michaelis* constant:

$$(\mathrm{EA}) = \frac{(\mathrm{E}_0)(\mathrm{A}_0)}{\mathrm{K}_m + (\mathrm{A}_0)} \tag{2.38}$$

Substituting the value of [EA] from Equation 2.38 in Equation 2.32 gives the *Michaelis–Menten* equation for v_0 (initial reaction rate):

$$\upsilon_0 = \frac{k_2(\mathrm{E}_0)(\mathrm{A}_0)}{\mathrm{K_m} + (\mathrm{A}_0)} \tag{2.39}$$

Equation 2.39 contains a quantity, $[\mathrm{E}_0]$, which can be determined only when the enzyme is present in purified form. In order to be able to make kinetic measurements using impure enzymes, *Michaelis* and *Menten* introduced an approximation for Equation 2.39 as follows. In the presence of a large excess of substrate, $[\mathrm{A}_0] \gg \mathrm{K_m}$ in the denominator of Equation 2.39. Therefore, $\mathrm{K_m}$ can be neglected compared to $[\mathrm{A}_0]$:

$$\upsilon_0 = \frac{k_2(\mathrm{E}_0)(\mathrm{A}_0)}{(\mathrm{A}_0)} = \mathrm{V} \tag{2.40}$$

Thus, a zero order reaction rate is obtained. It is characterized by a rate of substrate breakdown or product formation which is independent of substrate concentration, i. e. the reaction rate, V, is dependent only on enzyme concentration. This rate, V, is denoted as the maximum velocity.
From Equation 2.40 it is obvious that the catalytic activity of the enzyme must be measured in the presence of a large excess of substrate.
To eliminate the $[\mathrm{E}_0]$ term, V is introduced into Equation 2.39 to yield:

$$v_0 = \frac{V(A_0)}{K_\mathrm{m} * (A_0)} \tag{2.41}$$

If $[\mathrm{A}_0] = \mathrm{K_m}$, the following is derived from Equation 2.41:

$$\upsilon_0 = \frac{\mathrm{V}}{2} \tag{2.42}$$

Thus, the *Michaelis* constant, $\mathrm{K_m}$, is equal to the substrate concentration at which the reaction rate is half of its maximal value. $\mathrm{K_m}$ is independent of enzyme concentration. The lower the value of $\mathrm{K_m}$, the higher the affinity of the enzyme for the substrate, i. e. the substrate will be bound more tightly by the enzyme and most probably will be more efficiently converted to product. Usually, the values of $\mathrm{K_m}$, are within the range of 10^{-2} to $10^{-5}\mathrm{mol} \cdot 1^{-1}$. From the definition of $\mathrm{K_m}$:

$$\mathrm{K_m} = \frac{k_{-1} + k_2}{k_1} \tag{2.43}$$

it follows that $\mathrm{K_m}$ approaches the enzymesubstrate dissociation constant, $\mathrm{K_s}$, only if

$\mathrm{k_{+2}} \ll \mathrm{k_{-1}}$.

$$k_2 \ll k_{-1} \frown \mathrm{K_m} \approx \frac{k_{-1}}{k_1} = \mathrm{K_S} \tag{2.44}$$

Some values for the constants $\mathrm{k_{+1}}$, $\mathrm{k_{-1}}$, and $\mathrm{k_0}$ are compiled in Table 2.9. In cases in which the catalysis proceeds over more steps than shown in Equation 2.30 the constant $\mathrm{k_{+2}}$ is replaced by $\mathrm{k_0}$. The rate constant, $\mathrm{k_{+1}}$, for the formation of the enzyme-substrate complex has values in the order of 10^6 to 10^8: in a few cases it approaches the maximum velocity ($\sim 10^9 1 \cdot \mathrm{mol^{-1}\,s^{-1}}$), especially when small molecules of substrate readily diffuse through the solution to the active site of the enzyme. The values for $\mathrm{k_{-1}}$ are substantially lower in most cases, whereas $\mathrm{k_0}$ values are in the range of 10^1 to 10^6 $\mathrm{s^{-1}}$.

Table 2.9. Rate constants for some enzyme catalyzed reactions

Enzyme	Substrate	k_1 ($l \cdot mol^{-1}\ s^{-1}$)	K_{-1} (s^{-1})	k_0 (s^{-1})
Fumarase	Fumarate	$> 10^9$	$4.5 \cdot 10^4$	10^3
Acetylcholine esterase	Acetylcholine	10^9		10^3
Alcohol dehydrogenase (liver)	NAD	$5.3 \cdot 10^5$	74	
	NADH	$1.1 \cdot 10^7$	3.1	
	Ethanol	$> 1.2 \cdot 10^4$	> 74	10^3
Catalase	H_2O_2	$5 \cdot 10^6$		10^7
Peroxidase	H_2O_2	$9 \cdot 10^6$	< 1.4	10^6
Hexokinase	Glucose	$3.7 \cdot 10^6$	$1.5 \cdot 10^3$	10^3
Urease	Urea	$> 5 \cdot 10^6$		10^4

Another special case to be considered is if $[A_0] \ll K_m$, which occurs at about $[A_0] < 0.05\,K_m$. Here $[A_0]$ in the denominator of Equation 2.39 can be neglected:

$$\upsilon_0 = \frac{k_2(E_0)(A_0)}{K_m} \tag{2.45}$$

and, considering that $k_2[E_0] = V$, it follows that:

$$\upsilon_0 = \frac{V}{K_m}(A_0) \tag{2.46}$$

In this case the *Michaelis–Menten* equation reflects a first-order reaction in which the rate of substrate breakdown depends on substrate concentration. In using a kinetic method for the determination of substrate concentration (cf. 2.6.1.3), the experimental conditions must be selected such that Equation 2.46 is valid.

2.5.1.1.2 Determination of K_m and V

In order to determine values of K_m and V, the catalytic activity of the enzyme preparation is measured as a function of substrate concentration. Very good results are obtained when $[A_0]$ is in the range of 0.1 K_m to10 K_m.

A graphical evaluation of the result is obtained by inserting the data into Equation 2.41. As can be seen from a plot of the data in Fig. 2.22, the equation corresponds to a rectangular hyperbola. This graphical approach yields correct values for K_m only when the maximum velocity, V, can be accurately determined.

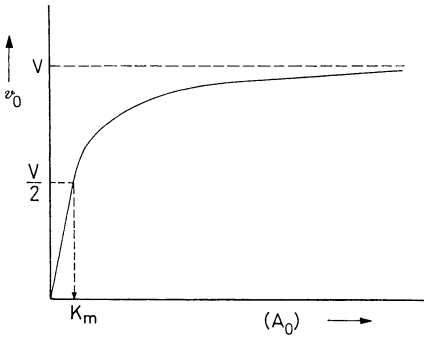


Fig. 2.22. Determination of *Michaelis* constant, K_m, according to Equation (2.41)

For a more reliable extrapolation of V, Equation 2.41 is transformed into a straight-line equation. Most frequently, the *Lineweaver–Burk* plot is used which is the reciprocal form of Equation 2.41:

$$\frac{1}{\upsilon_0} = \frac{K_m}{V} \cdot \frac{1}{(A_0)} + \frac{1}{V} \tag{2.47}$$

Figure 2.23 graphically depicts a plot of $1/v_0$ versus $1/[A_0]$. The values V and K_m are obtained from the intercepts of the ordinate $(1/V)$ and of the abscissa $(-1/K_m)$, respectively. If the data do not fit a straight line, then the system deviates from the required steady-state kinetics; e. g., there is inhibition by excess substrate or the system is influenced by allosteric effects (cf. 2.5.1.3; allosteric enzymes do not obey *Michaelis–Menten* kinetics).

A great disadvantage of the *Lineweaver–Burk* plot is the possibility of departure from a straight line since data taken in the region of saturating substrate concentrations or at low substrate concentrations can be slightly inflated. Thus, values taken from the straight line may be somewhat overestimated.

A procedure which yields a more uniform *distribution* of the data on the straight line is that proposed by *Hofstee* (the *Eadie–Hofstee* plot). In this procedure the *Michaelis–Menten* equation, 2.41, is algebraically rearranged into:

$$\upsilon_0(A_0) + \upsilon_0 K_m = V \cdot (A_0) \qquad \text{(a)}$$

$$\upsilon_0 + \frac{\upsilon_0}{(A_0)} \cdot K_m = V \qquad \text{(b)}$$

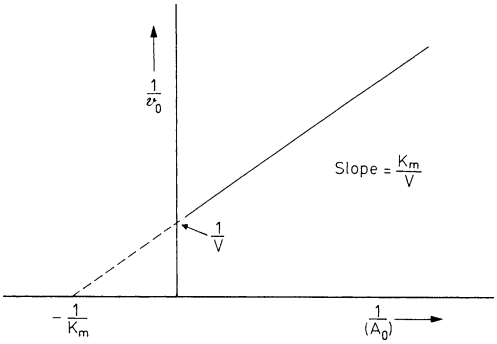


Fig. 2.23. Determination of K_m and V (according to *Lineweaver* and *Burk*)

$$\upsilon_0 = -K_m \frac{\upsilon_0}{(A_0)} \cdot V \qquad (c) \qquad (2.48)$$

When Equation 2.48c is plotted using the substrate-reaction velocity data, a straight line with a negative slope is obtained (Fig. 2.24) where y is v_0 and x is $v_0/[A_0]$. The y and x intercepts correspond to V and V/K_m, respectively.

Single-substrate reactions, for which the kinetics outlined above (with some exceptions, cf. 2.5.1.3) are particularly pertinent, are those catalyzed by lyase enzymes and certain isomerases. Hydrolysis by hydrolase enzymes can also be considered a single-substrate reaction when the water content remains unchanged, i. e., when it is present in high concentration (55.6 mol/μ). Thus, water, as a reactant, can be disregarded.

Characterization of an enzyme-substrate system by determining values for K_m and V is important in enzymatic food analysis (cf. 2.6.4) and for assessment of enzymatic reactions occurring in food (e. g. enzymatic browning of sliced potatoes, cf. 2.5.1.2.1) and for utilization of enzymes in food processing, e. g., aldehyde dehydrogenase (cf. 2.7.2.1.4).

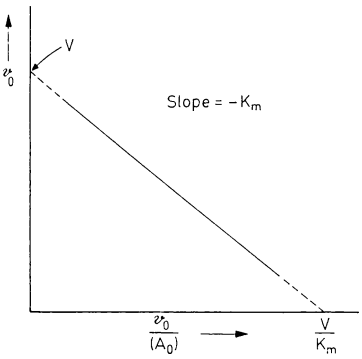


Fig. 2.24. Determination of K_m and V (according to *Hofstee*)

2.5.1.2 Two-Substrate Reactions

For many enzymes, for examples, oxidoreductase and ligase-catalyzed reactions, two or more substrates or cosubstrates are involved.

2.5.1.2.1 Order of Substrate Binding

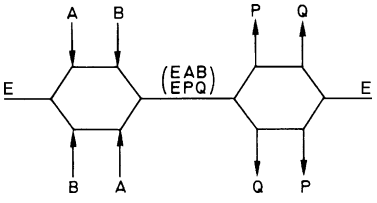
In the reaction of an enzyme with two substrates, the binding of the substrates can occur sequentially in a specific order. Thus, the binding mechanism can be divided into catalysis which proceeds through a ternary adsorption complex (enzyme + two substrates) or through a binary complex (enzyme + one substrate), i. e. when the enzyme binds only one of the two available substrates at a time.

A ternary enzyme-substrate complex can be formed in two ways. The substrates are bound to the enzyme in a random fashion ("random mechanism") or they are bound in a well-defined order ("ordered mechanism").

Let us consider the reaction

$$A + B \underset{}{\overset{E}{\rightleftharpoons}} P + Q \qquad (2.49)$$

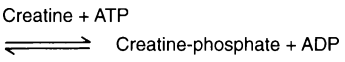
If the enzyme reacts by a "random mechanism", substrates A and B form the ternary enzyme-substrate complex, EAB, in a random fashion and the P and Q products dissociate randomly from the ternary enzyme-product complex, EPQ:



(2.50)

Creatine kinase from muscle (cf. 12.3.6) is an example of an enzyme which reacts by a random

mechanism:

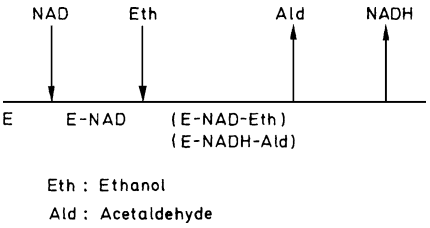


(2.51)

In an "ordered mechanism" the binding during the catalyzed reaction according to equation 2.49 is as follows:

(2.52)

Alcohol dehydrogenase reacts by an "*ordered mechanism*", although the order of the binding of substrates NAD^+ and ethanol is decided by the ethanol concentration. NAD^+ is absorbed first at low concentrations (< 4 mmol/l):



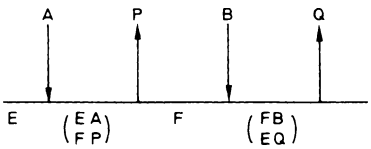
(2.53)

When the ethanol concentration is increased to 7–8 mmol/l, ethanol is absorbed first, followed by the cosubstrate. The order of removal of products (acetaldehyde and NADH) is, however, not altered.

Polyphenol oxidase from potato tubers also reacts by an "*ordered mechanism*". Oxygen is absorbed first, followed by phenolic substrates. The main substrates are chlorogenic acid and tyrosine. Enzyme affinity for tyrosine is greater and the reaction velocity is higher than for chlorogenic acid. The ratio of chlorogenic acid to tyrosine affects enzymatic browning to such an extent that it is considered to be the major problem in potato processing. The deep-brown colored melanoidins are formed quickly from tyrosine but not from chlorogenic acid. In assessing the processing quality of potato cultivars, the differences in phenol oxidase activity and the content of ascorbic acid in the tubers should also be considered in relation to "enzymatic browning". Ascorbic acid retards formation of melanoidins by its ability to reduce o-quinone, the initial product of enzymatic oxidation (cf. 18.1.2.5.8).

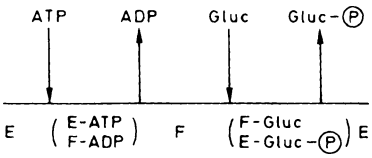
In enzymatic reactions where functional group transfers are involved, as a rule only binary enzyme-substrate complexes are formed by the so-called "*ping pong mechanism*".

A substrate is adsorbed by enzyme, E, and reacts during alteration of the enzyme (a change in the oxidation state of the prosthetic group, a conformational change, or only a change in covalent binding of a functional group). The modified enzyme, which is denoted F, binds the second substrate and the second reaction occurs, which regenerates the initial enzyme, E, and releases the second product:



(2.54)

The glycolytic enzyme hexokinase reacts by a "ping pong mechanism":



(2.55)

2.5.1.2.2 Rate Equations for a Two-Substrate Reaction

Here the reaction rate is distinguished by its dependence on two reactants, either two molecules of the same compound or two different compounds. The rate equations can be derived by the same procedures as used for single-substrate catalysis. Only the final forms of the equations will be considered.

When the catalysis proceeds through a ternary enzyme-substrate complex, EAB, the general equation is:

$$v_0 = \frac{V}{1 + \frac{K_a}{(A_0)} + \frac{K_b}{(B_0)} + \frac{K_{ja} \cdot k_b}{(A_0)(B_0)}} \qquad (2.56)$$

When compared to the rate equation for a single-substrate reaction (Equation 2.41), the difference becomes obvious when the equation for a single-

substrate reaction is expressed in the following form:

$$\upsilon_0 = \frac{V}{1 + \frac{K_a}{(A_0)}} \tag{2.57}$$

The constants K_a and K_b in Equation 2.56 are defined analogously to K_m, i. e. they yield the concentrations of A or B for $v_0 = V/2$ assuming that, at any given moment, the enzyme is saturated by the other substrate (B or A). Each of the constants, like K_m (cf. Equation 2.43), is composed of several rate constants. K_{ia} is the inhibitor constant for A.

When the binding of one substrate is not influenced by the other, each substrate occupies its own binding locus on the enzyme and the substrates form a ternary enzyme-substrate complex in a defined order ("*ordered mechanism*"), the following is valid:

$$K_{ia} \cdot K_b = K_a \cdot K_b \tag{2.58}$$

or from Equation 2.56:

$$\upsilon_0 = \frac{V}{1 + \frac{K_a}{(A_0)} + \frac{K_b}{(B_0)} + \frac{K_a \cdot K_b}{(A_0)(B_0)}} \tag{2.59}$$

However, when only a binary enzyme-substrate complex is formed, i. e. one substrate or one product is bound to the enzyme at a time by a "ping pong mechanism", the denominator term $K_{ia} \cdot K_b$ must be omitted since no ternary complex exists. Thus, Equation 2.56 is simplified to:

$$\upsilon_0 = \frac{V}{1 + \frac{K_a}{(A_0)} + \frac{K_b}{(B_0)}} \tag{2.60}$$

For the determination of rate constants, the initial rate of catalysis is measured as a function of the concentration of substrate B (or A) for several concentrations of A (or B). Evaluation can be done using the *Lineweaver–Burk* plot. Reshaping Equation 2.56 for a "*random mechanism*" leads to:

$$\frac{1}{\upsilon_0} = \left[\frac{K_b}{V} + \frac{K_{ia} \cdot K_b}{(A_0)V}\right] \frac{1}{(B_0)} + \left[1 + \frac{K_a}{(A_0)}\right] \frac{1}{V} \tag{2.61}$$

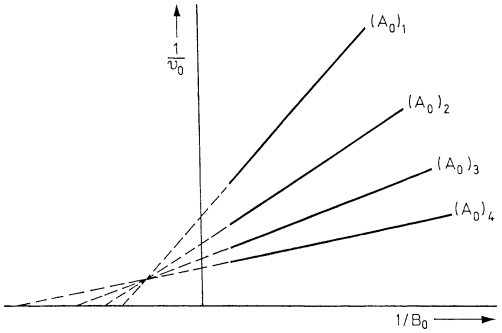


Fig. 2.25. Evaluation of a two-substrate reaction, proceeding through a ternary enzyme-substrate complex (according to *Lineweaver* and *Burk*). $[A_0]_4 > [A_0]_3 > [A_0]_2 > [A_0]_1$

First, $1/v_0$ is plotted against $1/[B_0]$. The corresponding slopes and ordinate intercepts are taken from the straight lines obtained at various values for $[A_0]$ (Fig. 2.25):

$$\text{Slope} = \frac{K_b}{V} + \frac{K_{ia}K_b}{V} \cdot \frac{1}{(A_0)}$$

$$\text{Ordinate intercept} = \frac{1}{V} + \frac{K_a}{V} \cdot \frac{1}{(A_0)} \tag{2.62}$$

and are then plotted against $1/[A_0]$. In this way two straight lines are obtained (Fig. 2.26a and b), with slopes and ordinate intercepts which provide data for calculating constants K_a, K_b, K_{ia}, and the maximum velocity, V. If the catalysis proceeds through a "ping pong mechanism", then plotting $1/v_0$ versus $1/[B_0]$ yields a family of parallel lines (Fig. 2.27) which are then subjected to the calculations described above.

A comparison of Figs. 2.25 and 2.27 leads to the conclusion that the dependence of the initial catalysis rate on substrate concentration allows the differentiation between a ternary and a binary enzyme-substrate complex. However, it is not possible to differentiate an "ordered" from a "random" reaction mechanism by this means.

2.5.1.3 Allosteric Enzymes

We are already acquainted with some enzymes consisting of several protomers (cf. Table 1.26). When the protomer activities are independent

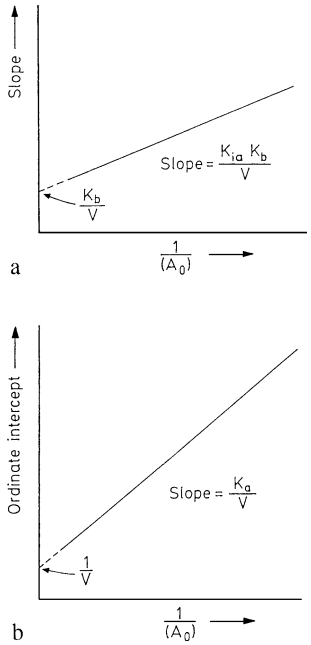


Fig. 2.26. Plotting slopes (**a**) and ordinate intercepts (**b**) from Fig. 2.25 versus $1/[A_0]$

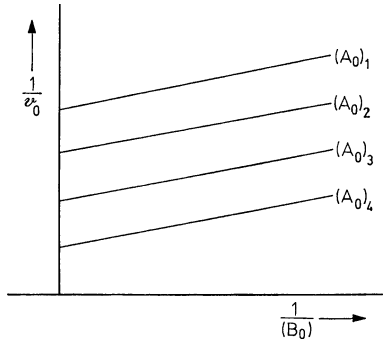


Fig. 2.27. Evaluation of a two-substrate reaction, proceeding through a binary enzyme-substrate complex (according to *Lineweaver* and *Burk*). $[A_0]_4 > [A_0]_3 > [A_0]_2 > [A_0]_1$

of each other in catalysis, the *Michaelis–Menten* kinetics, as outlined under sections 2.5.1.1 and 2.5.1.2, are valid. However, when the subunits cooperate, the enzymes deviate from these kinetics. This is particularly true in the case of positive cooperation when the enzyme is activated by the substrate. In this kind of plot, v_0 versus $[A_0]$ yields not a hyperbolic curve but a saturation curve with a sigmoidal shape (Fig. 2.28).

Thus, enzymes which do not obey the *Michaelis–Menten* model of kinetics are allosterically regulated. These enzymes have a site which reversibly binds the allosteric regulator (substrate, cosubstrate or low molecular weight compound) in addition to an active site with a binding and transforming locus. Allosteric enzymes are, as a rule, engaged at control sites of metabolism. An example is tetrameric phosphofructokinase, the key enzyme in glycolysis. In glycolysis and alcoholic fermentation it catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-diphosphate. The enzyme is activated by its substrate in the presence of ATP. The prior binding of a substrate molecule which enhances the binding of each succeeding substrate molecule is called positive cooperation. The two enzyme-catalyzed reactions, one which obeys *Michaelis–Menten* kinetics and the other which is regulated by allosteric effects, can be reliably distinguished experimentally by comparing the ratio of the substrate concentration needed to obtain the observed value of 0.9 V to that needed to obtain 0.1 V. This ratio, denoted as R_s, is a measure of the cooperativity of the interaction.

$$R_S = \frac{(A_0)_{0.9\,V}}{(A_0)_{0.1\,V}} \tag{2.63}$$

For all enzymes which obey *Michaelis–Menten* kinetics, $R_s = 81$ regardless of the value of K_m or V. The value of R_s is either lower or higher than 81 for allosteric enzymes. $R_s < 81$ is

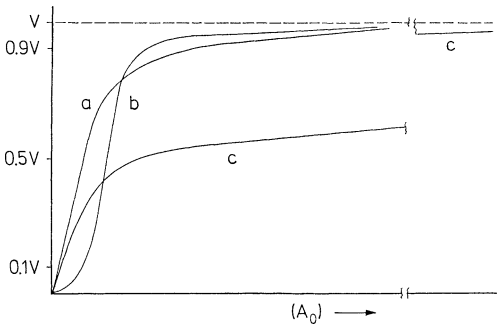
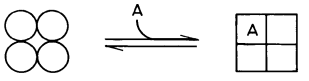


Fig. 2.28. The effect of substrate concentration on the catalytic reaction rate. **a** Enzyme obeying *Michaelis–Menten* kinetics; **b** allosterically regulated enzyme with positive cooperativity; **c** allosterically regulated enzyme with negative cooperativity

indicative of positive cooperation. Each substrate molecule, often called an effector, accelerates the binding of succeeding substrate molecules, thereby increasing the catalytic activity of the enzyme (case b in Fig. 2.28). When $R_s > 81$, the system shows negative cooperation. The effector (or allosteric inhibitor) decreases the binding of the next substrate molecule (case c in Fig. 2.28).

Various models have been developed in order to explain the allosteric effect. Only the symmetry model proposed by *Monod, Wyman* and *Changeux* (1965) will be described in its simplified form: specifically, when the substrate acts as a positive allosteric regulator or effector. Based on this model, the protomers of an allosteric enzyme exist in two conformations, one with a high affinity (R-form) and the other with a low affinity (T-form) for the substrate. These two forms are interconvertible. There is an interaction between protomers. Thus, binding of the allosteric regulator by one protomer induces a conformational change of all the subunits and greatly increases the activity of the enzyme.

Let us assume that the R- and T-forms of an enzyme consisting of four protomers are in an equilibrium which lies completely on the side of the T-form:



(2.64)

Addition of substrate, which here is synonymous to the allosteric effector, shifts the equilibrium from the low affinity T-form to the substantially more catalytically active R-form. Since one substrate molecule activates four catalytically active sites, the steep rise in enzyme activity after only a slight increase in substrate concentration is not unexpected. In this model it is important that the RT conformation is not permitted. All subunits must be in the same conformational state at one time to conserve the symmetry of the protomers. The equation given by *Hill* in 1913, derived from the sigmoidal absorption of oxygen by hemoglobin, is also suitable for a quantitative description of allosteric enzymes with sigmoidal behavior:

$$\upsilon_0 = \frac{V(A_0)^n}{K' + (A_0)^n} \qquad (2.65)$$

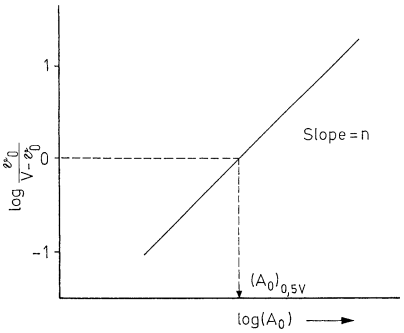


Fig. 2.29. Linear presentation of *Hill's* equation

The equation says that the catalytic rate increases by the nth power of the substrate concentration when $[A_0]$ is small in comparison to K. The *Hill* coefficient, n, is a measure of the sigmoidal character of the curve and, therefore, of the extent of the enzyme's cooperativity. For $n = 1$ (Equation 2.65) the reaction rate is transformed into the *Michaelis–Menten* equation, i. e. in which no cooperativity factor exists.

In order to assess the experimental data, Equation 2.65 is rearranged into an equation of a straight line:

$$\log = \frac{\upsilon_0}{V - \upsilon_0} = n \log(A_0) - \log K' \qquad (2.66)$$

The slope of the straight line obtained by plotting the substrate concentration as $\log[A_0]$ versus $\log[v_0/(V - v_0)]$ is the *Hill* coefficient, n (Fig. 2.29). The constant K incorporates all the individual K_m values involved in all the steps of substrate binding and transformation. The value of K_m is obtained by using the substrate concentration, denoted as $[A_0]_{0.5\,v}$, at which $v_0 = 0.5\,V$. Under these conditions, the following is derived from Equation 2.66):

$$\log \frac{0.5\,V}{0.5\,V} = 0 = n \cdot \log(A_0)_{0.5\,V} - \log K' \quad (a)$$
$$K' = (A_0)^n_{0.5\,V} \quad (b) \qquad (2.67)$$

2.5.2 Effect of Inhibitors

The catalytic activity of an enzyme, in addition to substrate concentration, is affected by the type

and concentration of inhibitors, i. e. compounds which decrease the rate of catalysis, and activators, which have the opposite effect. Metal ions and compounds which are active as prosthetic groups or which provide stabilization of the enzyme's conformation or of the enzyme-substrate complex (cf. 2.3.2 and 2.3.3) are activators. The effect of inhibitors will be discussed in more detail in this section.

Inhibitors are found among food constituents. Proteins which specifically inhibit the activity of certain peptidases (cf. 16.2.3), amylases or β-fructofuranosidase are examples. Furthermore, food contains substances which nonselectively inhibit a wide spectrum of enzymes. Phenolic constituents of food (cf. 18.1.2.5) and mustard oil (cf. 17.1.2.6.5) belong to this group. In addition, food might be contaminated with pesticides, heavy metal ions and other chemicals from a polluted environment (cf. Chapter 9) which can become inhibitors under some circumstances. These possibilities should be taken into account when enzymatic food analysis is performed.

Food is usually heat treated (cf. 2.5.4) to suppress undesired enzymatic reactions. As a rule, no inhibitors are used in food processing. An exception is the addition of, for example, SO_2 to inhibit the activity of phenolase (cf. 8.12.6).

Much data concerning the mechanism of action of enzyme inhibitors have been compiled in recent biochemical research. These data cover the elucidation of the effect of inhibitors on funtional groups of an enzyme, their effect on the active site and the clarification of the general mechanism involved in an enzymecatalyzed reaction (cf. 2.4.1.1).

Based on kinetic considerations, inhibitors are divided into two groups: inhibitors bound *irreversibly* to enzyme and those bound *reversibly*.

2.5.2.1 Irreversible Inhibition

In an irreversible inhibition the inhibitor binds mostly covalently to the enzyme; the EI complex formed does not dissociate:

$$E + I \xrightarrow{k_1} EI \qquad (2.68)$$

The rate of inhibition depends on the reaction rate constant k_1 in Equation 2.68, the enzyme concentration, [E], and the inhibitor concentration, [I]. Thus, irreversible inhibition is a function of reaction time. The reaction cannot be reversed by diluting the reaction medium. These criteria serve to distinguish irreversible from reversible inhibition.

Examples of irreversible inhibition are the reactions of SH-groups of an enzyme with iodoacetic acid:

$$Enz\text{-}SH + ICH_2COOH \longrightarrow Enz\text{-}S\text{-}CH_2\text{-}COOH + HI \qquad (2.69)$$

and other reactions with the inhibitors described in section 2.4.1.1.

2.5.2.2 Reversible Inhibition

Reversible inhibition is characterized by an equilibrium between enzyme and inhibitor:

$$E + I \rightleftharpoons EI \quad (a)$$

$$\frac{(E) \cdot (I)}{(EI)} = K_i \quad (b) \qquad (2.70)$$

The equilibrium constant or dissociation constant of the enzyme-inhibitor complex, K_i, also known as the inhibitor constant, is a measure of the extent of inhibition. The lower the value of K_i, the higher the affinity of the inhibitor for the enzyme. Kinetically, three kinds of reversible inhibition can be distinguished: competitive, non-competitive and uncompetitive inhibition (examples in Table 2.10). Other possible cases, such as allosteric inhibition and partial competitive or partial non-competitive inhibition, are omitted in this treatise.

2.5.2.2.1 Competitive Inhibition

Here the inhibitor binds to the active site of the free enzyme, thus preventing the substrate from binding. Hence, there is competition between substrate and inhibitor:

$$E + I \rightleftharpoons EI \ (a) \qquad E + A \rightleftharpoons EA \ (b) \qquad (2.71)$$

According to the steady-state theory for a single-substrate reaction, we have:

$$v_0 = \frac{V(A_0)}{K_m\left(1 + \frac{(I)}{K_i}\right) + (A_0)} \qquad (2.72)$$

Table 2.10. Examples of reversible enzyme inhibition

Enzyme	EC-Number	Sustrate	Inhibitor	Inhibition type[a]	K_i (mmol/l)
Glucose dehydrogenase	1.1.1.47	Glucose/NAD	Glucose-6-phosphate	C	$4.4 \cdot 10^{-5}$
Glucose-6-phosphate dehydrogenase	1.1.1.49	Glucose-6-phosphate/NADP	Phosphate	C	$1 \cdot 10^{-1}$
Succinate dehydrogenase	1.3.99.1	Succinate	Fumarate	C	$1.9 \cdot 10^{-3}$
Creatine kinase	2.7.3.2	Creatine/ATP	ADP	NC	$2 \cdot 10^{-3}$
Glucokinase	2.7.1.2	Glucose/ATP	D-Mannose	C	$1.4 \cdot 10^{-2}$
			2-Deoxyglucose	C	$1.6 \cdot 10^{-2}$
			D-Galactose	C	$6.7 \cdot 10^{-1}$
Fructose-biphosphatase	3.1.3.11	D-Fructose-1, 6-biphosphate	AMP	NC	$1.1 \cdot 10^{-4}$
α-Glucosidase	3.2.1.20	p-Nitrophenyl-α-D-glucopyranoside	Saccharose	C	$3.7 \cdot 10^{-2}$
			Turanose	C	$1.1 \cdot 10^{-2}$
Cytochrome c oxidase	1.9.3.1	Ferrocytochrome c	Azide	UC	

[a] C: competitive, NC: noncompetitive, and UC: uncompetitive.

In the presence of inhibitors, the *Michaelis* constant is apparently increased by the factor:

$$1 + \frac{(\mathrm{I})}{\mathrm{K_i}} \tag{2.73}$$

Such an effect can be useful in the case of enzymatic substrate determinations (cf. 2.6.1.3). When inhibitor activity is absent, i. e. $[\mathrm{I}] = 0$, Equation 2.72 is transformed into the *Michaelis–Menten* equation (Equation 2.41). The *Lineweaver–Burk* plot (Fig. 2.30a) shows that the intercept $1/\mathrm{V}$ with the ordinate is the same in the presence and in the absence of the inhibitor, i. e. the value of V is not affected although the slopes of the lines differ. This shows that the inhibitor can be fully dislodged by the substrate from the active site of the enzyme when the substrate is present in high concentration. In other words, inhibition can be overcome at high substrate concentrations (see application in Fig. 2.49). The inhibitor constant, $\mathrm{K_i}$, can be calculated from the corresponding intercepts with the abscissa in Fig. 2.30a by calculating the value of K_m from the abscissa intercept when $[\mathrm{I}] = 0$.

2.5.2.2.2 Non-Competitive Inhibition

The non-competitive inhibitor is not bound to the active site of the enzyme but to some other site. Therefore, the inhibitor can react equally with free enzyme or with enzyme-substrate complex. Thus, three processes occur in parallel:

$$\mathrm{E + A \rightleftharpoons EA\ (a)} \qquad \mathrm{E + I \rightleftharpoons EI\ (b)}$$
$$\mathrm{EA + I \rightleftharpoons EAI\ (c)} \tag{2.74}$$

Postulating that EAI and EI are catalytically inactive and the dissociation constants $\mathrm{K_i}$ and $\mathrm{K_{EAi}}$ are numerically equal, the following equation is obtained by rearrangement of the equation for a single-substrate reaction into its reciprocal form:

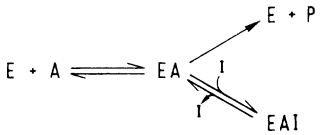
$$\frac{1}{\upsilon_0} = \frac{\mathrm{K_m}}{\mathrm{V}}\left(1 + \frac{(\mathrm{I})}{\mathrm{K_i}}\right)\frac{1}{(\mathrm{A_0})} + \frac{1}{\mathrm{V}}\left(1 + \frac{(\mathrm{I})}{\mathrm{K_i}}\right) \tag{2.75}$$

The double-reciprocal plot (Fig. 2.30b) shows that, in the presence of a noncompetitive inhi-

bitor; K_m is unchanged whereas the values of V are decreased such that V becomes $V/(1 + [I]/K_i)$, i. e. non-competitive inhibition can not be overcome by high concentrations of substrate.
This also indicates that, in the presence of inhibitor, the amount of enzyme available for catalysis is decreased.

2.5.2.2.3 Uncompetitive Inhibition

In this case the inhibitor reacts only with enzyme-substrate complex:



(2.76)

Rearranging Equation 2.76 into an equation for a straight line, the reaction rate becomes:

$$\frac{1}{\upsilon_0} = \frac{K_m}{V}\frac{1}{(A_0)} + \frac{1}{V}\left(1 + \frac{(I)}{K_i}\right) \qquad (2.77)$$

The double reciprocal plot (Fig. 2.30c) shows that in the presence of an uncompetitive inhibitor, both the maximum velocity, V, and K_m are changed but not the ratio of K_m/V. Hence the slopes of the lines are equal and in the presence of increasing amounts of inhibitor, the lines plotted are parallel. Uncompetitive inhibition is rarely found in single-substrate reactions. It occurs more often in two-substrate reactions.
In conclusion, it can be stated that the three types of reversible inhibition are kinetically distinguishable by plots of reaction rate versus substrate concentration using the procedure developed by *Lineweaver* and *Burk* (Fig. 2.30).

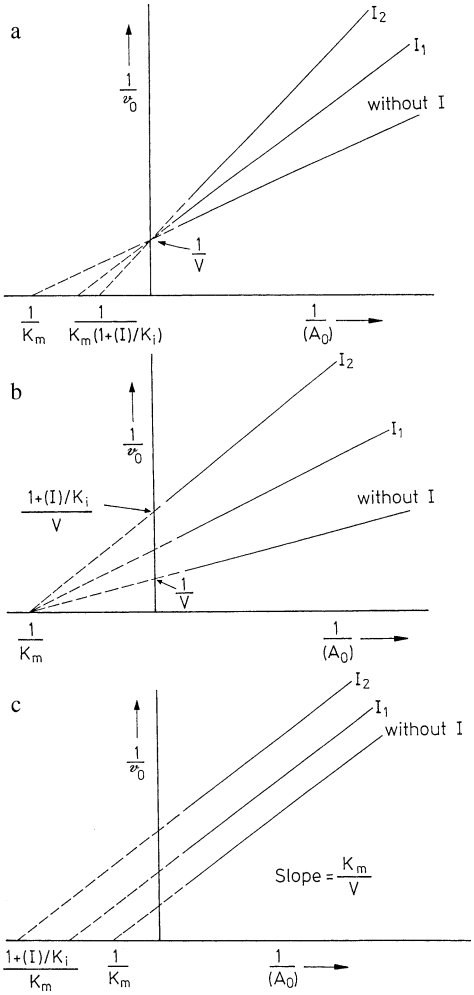


Fig. 2.30. Evaluation of inhibited enzyme-catalyzed reaction according to *Lineweaver* and *Burk*, $[I_1] < (I_2)$. **a** Competitive inhibition, **b** noncompetitive inhibition, **c** uncompetitive inhibition

2.5.3 Effect of pH on Enzyme Activity

Each enzyme is catalytically active only in a narrow pH range and, as a rule, each has a pH optimum which is often between pH 5.5 and 7.5 (Table 2.11).
The optimum pH is affected by the type and ionic strength of the buffer used in the assay. The reasons for the sensitivity of the enzyme to changes in pH are two-fold:

a) sensitivity is associated with a change in protein structure leading to irreversible denaturation,
b) the catalytic activity depends on the quantity of electrostatic charges on the enzyme's active site generated by the prototropic groups of the enzyme (cf. 2.4.2.4).

In addition the ionization of dissociable substrates as affected by pH can be of importance to

Table 2.11. pH Optima of various enzymes

Enzyme	Source	Substrate	pH Optimum
Pepsin	Stomach	Protein	2
Chymotrypsin	Pancreas	Protein	7.8
Papain	Tropical plants	Protein	7–8
Lipase	Microorganisms	Olive oil	5–8
α-Glucosidase (maltase)	Microorganisms	Maltose	6.6
β-Amylase	Malt	Starch	5.2
β-Fructofuranosidase (invertase)	Tomato	Saccharose	4.5
Pectin lyase	Microorganisms	Pectic acid	9.0–9.2
Xanthine oxidase	Milk	Xanthine	8.3
Lipoxygenase, type I[a]	Soybean	Linoleic acid	9.0
Lipoxygenase, type II[a]	Soybean	Linoleic acid	6.5

[a] See 3.7.2.2.

the reaction rate. However, such effects should be determined separately. Here, only the influences mentioned under b) will be considered with some simplifications.

An enzyme, E, its substrate, A, and the enzyme-substrate complex formed, EA, depending on pH, form the following equilibria:

$$E^{n+1} \underset{}{\overset{-H^{\oplus}}{\rightleftharpoons}} E^{n} \overset{-H^{\oplus}}{\rightleftharpoons} E^{n-1}$$

$$E^{n+1}A \overset{-H^{\oplus}}{\rightleftharpoons} E^{n}A \overset{-H^{\oplus}}{\rightleftharpoons} E^{n-1}A \qquad (2.78)$$

Which of the charged states of E and EA are involved in catalysis can be determined by following the effect of pH on V and K_m.

a) Plotting K_m versus pH reveals the type of prototropic groups involved in substrate binding and/or maintaining the conformation of the enzyme. The results of such a plot, as a rule, resemble one of the four diagrams shown in Fig. 2.31.
Figure 2.31a: K_m is independent of pH in the range of $4-9$. This means that the forms E^{n+1}, E^n, and E^{n-1}, i. e. enzyme forms which are neutral, positively or negatively charged on the active site, can bind substrate.
Figures 2.31b and c: K_m is dependent on one prototropic group, the pK value of which is below (Fig. 2.31b) or above (Fig. 2.31c) neutrality. In the former case, E^n and E^{n-1} are the active forms, while in the latter, E^{n+1} and E^n are the active enzyme forms in substrate binding.
Figure 2.31d: K_m is dependent on two prototropic groups; the active form in substrate binding is E^n.

b) The involvement of prototropic groups in the conversion of an enzyme-substrate complex into product occurs when the enzyme is saturated with substrate, i. e. when equation 2.40 which defines V is valid ($[A_0] \gg K_m$). Thus, a plot of V versus pH provides essentially the same four possibilities presented in Fig. 2.31, the difference being that, here, the prototropic groups of EA, which are involved in the conversion to product, are revealed.

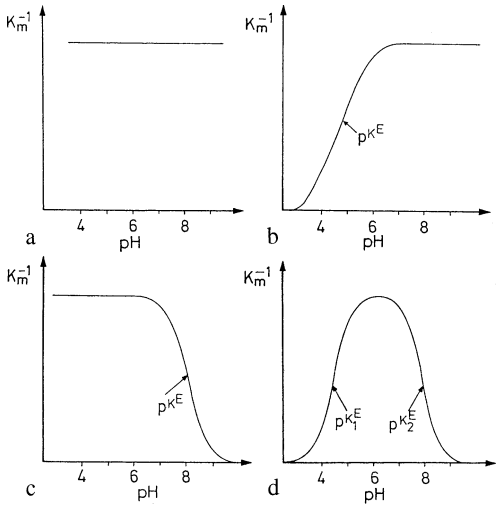
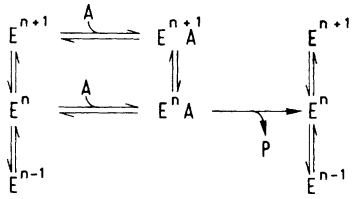


Fig. 2.31. The possible effects of pH on the *Michaelis* constant, K_m

In order to better understand the form of the enzyme involved in catalysis, a hypothetical enzyme-substrate system will be assayed and interpreted. We will start from the assumption that data are available for v_0 (initial velocity) as a function of substrate concentration at several pH's, e. g., for the *Lineweaver* and *Burk*. The values for K_m and V are obtained from the family of straight lines (Fig. 2.32) and plotted against pH. The diagram of $K_m^{-1} = f(pH)$ depicted in Fig. 2.33a corresponds to Fig. 2.31c which implies that neutral (E^n) and positively charged (E^{n+1}) enzyme forms are active in binding the substrate.

Figure 2.33b: V is dependent on one prototropic group, the pK value of which is below neutrality. Therefore, of the two enzyme-substrate complexes, $E^{n+1}A$ and E^nA, present in the equilibrium state, only the latter complex is involved in the conversion of A to the product.

In the example given above, the overall effect of pH on enzyme catalysis can be illustrated as follows:



(2.79)

This schematic presentation is also in agreement with the diagram of $V/K_m = f(pH)$ (Fig. 2.33c)

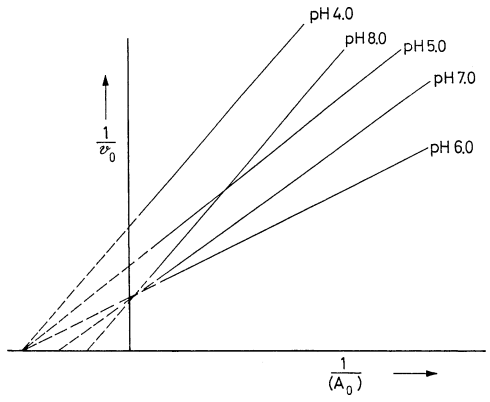


Fig. 2.32. Determination of V and K_m at different pH values

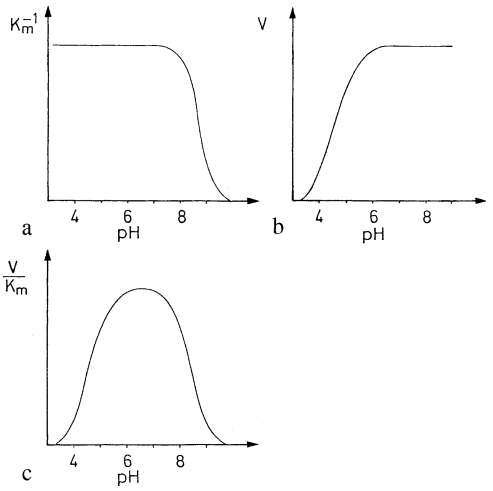


Fig. 2.33. Evaluation of K_m and V versus pH for a hypothetical case

which reveals that, overall, two prototropic groups are involved in the enzymecatalyzed reaction.

An accurate determination of the pK values of prototropic groups involved in enzyme-catalyzed reactions is possible using other assays (cf. *J.R. Whitaker*, 1972). However, identification of these groups solely on the basis of pK values is not possible since the pK value is often strongly influenced by surrounding groups. Pertinent to this claim is our recollection that the pH of acetic acid in water is 4.75, whereas in 80% acetone it is about 7. Therefore, the enzyme activity data as related to pH have to be considered only as preliminary data which must be supported and verified by supplementary investigations.

2.5.4 Influence of Temperature

Thermal processes are important factors in the processing and storage of food because they allow the control of chemical, enzymatic and microbial changes. Undesired changes can be delayed or stopped by refrigerated storage. Heat treatment may either accelerate desirable chemical or enzymatic reactions or inhibit undesirable changes by inactivation of enzymes or microorganisms. Table 2.12 informs about quality dete-

Table 2.12. Thermal inactivation of enzymes to prevent deterioration of food quality

Food product	Enzyme	Quality loss
Potato products, apple products	Monophenol oxidase	Enzymatic browning
Semi-ripe peas	Lipoxygenase, peroxidase	Flavor defects; bleaching
Fish products	Proteinase, thiaminase	Texture (liquefaction), loss of vitamine B_1
Tomato purée	Polygalacturonase	Texture (liquefaction)
Apricot products	β-Glucosidase	Color defects
Oat flakes	Lipase, lipoxygenase	Flavor defects (bitter taste)
Broccoli	Cystathionine	Off-flavor
Cauliflower	β-Lyase (cystine-lyase)	

rioration caused by enzymes which can be eliminated e. g., by thermal inactivation.
Temperature and time are two parameters responsible for the effects of a thermal treatment. They should be selected carefully to make sure that all necessary changes, e. g., killing of pathogens, are guaranteed, but still all undesired changes such as degradation of vitamins are kept as low as possible.

2.5.4.1 Time Dependence of Effects

The reaction rates for different types of enzymatic reactions have been discussed in section 2.5.1. The inactivation of enzymes and the killing of microorganisms can be depicted as a reaction of 1st order:

$$c_t = c_0 e^{-kt} \tag{2.80}$$

with c_0 and c_t = concentrations (activities, germ counts) at times 0 and t, and k = rate constant for the reaction. For c_t and t follows from equation 2.80:

$$\log c_t = -\frac{k}{2,3} \cdot t + \log c_0 \tag{2.81}$$

$$t = \frac{2,3}{k} \log \frac{c_0}{c_t} \tag{2.82}$$

$c_0/c_t = 10$ gives:

$$t = \frac{2,3}{k} = D \tag{2.83}$$

The co-called "D-value" represents the time needed to reduce the initial concentration (activity, germ count) by one power of ten. It refers to a certain temperature which has to be stated in each case. For example: *Bacillus cereus* $D_{121°C} = 2.3$ s, *Clostridium botulinum* $D_{121°C} = 12.25$ s. For a heat treatment process, the D-value allows the easy determination of the holding time required to reduce the germ count to a certain level. If the germ count of *B. cereus* or *Cl. botulinum* in a certain food should be reduced by seven powers of ten, the required holding times are $2.3 \times 7 = 16.1$ s and $12.25 \times 7 = 85.8$ s.

2.5.4.2 Temperature Dependence of Effects

A relationship exists for the dependence of reaction rate on temperature. It is expressed by an equation of *Arrhenius:*

$$k = A \cdot e^{-E_a/RT} \tag{2.84}$$

with k = rate constant for the reaction rate, E_a = activation energy, R = general gas constant and A = Arrhenius factor. For the relationship between k and T, the *Arrhenius* equation is only an approximation. According to the theory of the transition state (cf. 2.2.1), A is transferred via the active state $A^{\neq}$ into P. A and $A^{\neq}$ are in equilibrium.

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A^{\neq} \longrightarrow P \tag{2.85}$$

For the reaction rate follows:

$$k = M \cdot \frac{A}{A^{\neq}} = M \cdot \frac{k_1}{k_{-1}} = M \cdot K^{\neq} \tag{2.86}$$

with

$$M = \frac{K_B \cdot T}{h} = \frac{R \cdot T}{N_A \cdot h} \tag{2.87}$$

($K^{\#}$ equilibrium constant, k_B *Boltzmann* constant, h: *Planck* constant, N_A: *Avogadro* number).
For the equilibrium constant follows:

$$K^{\neq} = e^{-\Delta G^{\neq}/RT} \tag{2.88}$$

Resulting for the equilibrium constant in:

$$k = \frac{k_B \cdot T}{h} e^{-\Delta G^{\neq}/RT} \tag{2.89}$$

and for the free activation enthalpy:

$$\Delta G^{\neq} = -RT \ln \frac{k \cdot h}{k_B \cdot T} \tag{2.90}$$

If k is known for any temperature, $\Delta G^{\neq}$ can be calculated according to equation 2.90. Furthermore, the following is valid:

$$\Delta G^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq} \tag{2.91}$$

A combination with equation 2.90 results in:

$$-RT \ln \frac{k \cdot h}{k_B \cdot T} = \Delta H^{\neq} - T\Delta S^{\neq} \tag{2.92}$$

and

$$\log \frac{k}{T} = -\log \frac{h}{k_B} - \frac{\Delta H^{\neq}}{2.3RT} + \frac{T\Delta S^{\neq}}{2.3R} \tag{2.93}$$

It is possible to determine $\Delta H^{\neq}$ graphically based on the above equation if k is known for several temperatures and $\log k/T$ is plotted against $1/T$. If $\Delta G^{\neq}$ and $\Delta H^{\neq}$ are known, $\Delta S^{\neq}$ can be calculated from equation 2.91.
The activation entropy is contained in the *Arrhenius* factor A as can be seen by comparing the empirical *Arrhenius* equation 2.84 with equation 2.89 which is based on the transition state hypothesis:

$$k = A \cdot e^{-E_a}/RT \tag{2.94a}$$

$$k = \frac{k_B}{h} \cdot e^{-\Delta S^{\neq}/R} \cdot T \cdot e^{-\Delta H^{\neq}/RT} \tag{2.94b}$$

Activation energy E_a and activation enthalpy $\Delta H^{\neq}$ are linked with each other as follows:

$$\frac{d \ln k}{dT} = \frac{E_a}{RT^2} \tag{2.95}$$

$$\frac{d \ln k}{dT} = \frac{1}{T} + \frac{\Delta H^{\neq}}{RT^2} = \frac{RT + \Delta H^{\neq}}{RT^2} \tag{2.96}$$

$$E_a = \Delta H^{\neq} + RT \tag{2.97}$$

Using plots of $\log k$ against $1/T$, the activation energy of the *Arrhenius* equation can be determined. For enzyme catalyzed reactions, E_a is 10–60, for chemical reactions this value is 50–150 and for the inactivation of enzymes, the unfolding of proteins, and the killing of microorganisms, 250–350 kJ/mol are required.
For enzymes which are able to convert more than one substrate or compound into product, the activation energy may be dependent on the substrate. One example is alcohol dehydrogenase, an important enzyme for aroma formation in semiripened peas (Table 2.13). In this case the activation energy for the reverse reaction is only slightly influenced by substrate.
Under consideration of the temperature dependence of the rate constant k in equation 2.80, the implementation of the expression from *Arrhenius* equation 2.84 leads to:

$$c_1 = c_0 \cdot e^{-k_0 \cdot t \cdot e^{-E_a/RT}} \tag{2.98}$$

For a constant effect follows:

$$\frac{c_t}{c_0} = \text{const.} = e^{-k_0 \cdot t \cdot e^{-E_a/RT}} \tag{2.99}$$

Table 2.13. Alcohol dehydrogenase from pea seeds: activation energy of alcohol dehydrogenation and aldehyde reduction

Alcohol	E_a (kJ · mole^{-1})	Aldehyde	E_a (kJ · mole^{-1})
Ethanol	20		
n-Propanol	37	n-Propanal	20
2-Propenol	18		
n-Butanol	40	n-Butanal	21
n-Hexanol	37	n-Hexanal	18
2-trans-hexenol	15	2-trans-Hexenal	19
		2-trans-Heptenal	18

and

$$\ln t = +\frac{E_a}{RT} + \text{const.} \tag{2.100}$$

When plotting ln t against 1/T, a family of parallel lines results for each of different activation energies E_a with each line from a family corresponding to a constant effect c_t/c_0 (cf. equation 2.99) (Fig. 2.34).

For very narrow temperature ranges, sometimes a diagram representing log t against temperature δ (in °C) is favourable. It corresponds to:

$$\log \frac{t}{t_B} = -\frac{E_a}{2.3R \cdot T_B \cdot T}(\vartheta - \vartheta_B) = \frac{1}{z}(\vartheta - \vartheta_B) \tag{2.101}$$

with t_B as reference time and T_B or δ_B as reference temperature in K respectively °C. For $\log t/t_B$ the following is valid:

$$z = \frac{2.3R \cdot T_B \cdot T}{E_a} \tag{2.102}$$

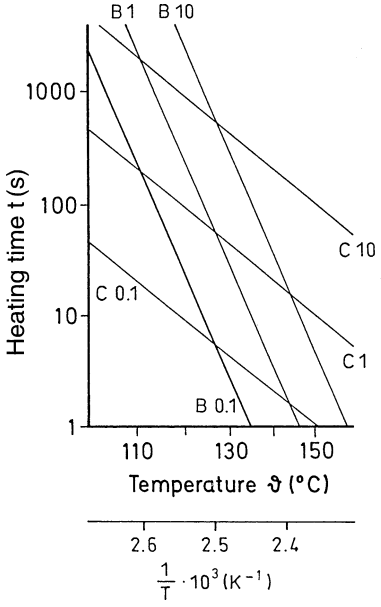


Fig. 2.34. Lines of equal microbiological and chemical effects for heat-treated milk (lines B10, B1, and BO.1 correspond to a reduction in thermophilic spores by 90, 9, and 1 power of ten compared to the initial load; lines C10, C1, and CO.1 correspond to a thiamine degradation of 30%, 3%, and 0.3%; according to *Kessler*, 1988)

This z-value, used in practice, states the temperature increase in °C required to achieve a certain effect in only one tenth of the time usually needed at the reference temperature. However, due to the temperature dependence of the z-value (equation 2.101), linearity can be expected for a very narrow temperature range only. A plot according to equation 2.100 is therefore more favourable.

In the literature, the effect of thermal processes is often described by the Q_{10} value. It refers to the ratio between the rates of a reaction at temperatures δ + 10(°C) and δ(°C):

$$Q_{10} = \frac{k_{\vartheta+10}}{k_\vartheta} = \frac{t_\vartheta}{t_{\vartheta+10}} \tag{2.103}$$

The combination of equations 2.101 and 2.103 shows the relationship between the Q_{10} value and z-value:

$$\frac{\log Q_{10}}{10} = \frac{E_a}{2.3RT^2} = \frac{1}{z} \tag{2.104}$$

2.5.4.3 Temperature Optimum

Contrary to common chemical reactions, enzyme-catalyzed reactions as well as growth of microorganisms show a so-called temperature optimum, which is a temperature-dependent maximum resulting from the overlapping of two counter effects with significantly different activation energies (cf. 2.5.4.2):

- increase in reaction or growth rate
- increase in inactivation or killing rate

For starch hydrolysis by microbial α-amylase, the following activation energies, which lie between the limits stated in section 2.5.4.2, were derived from e. g. the *Arrhenius* diagram (Fig. 2.35):

- E_a (hydrolysis) = 20 kJ · mol^{-1}
- E_a (inactivation) = 295 kJ · mol^{-1}

As a consequence of the difference in activation energies, the rate of enzyme inactivation is substantially faster with increasing temperature than the rate of enzyme catalysis. Based on activation energies for the above example, the following relative rates are obtained (Table 2.14). Increasing δ from 0 to 60 °C increases the hydrolysis rate by a factor of 5, while the rate of inactivation is accelerated by more than 10 powers of ten.

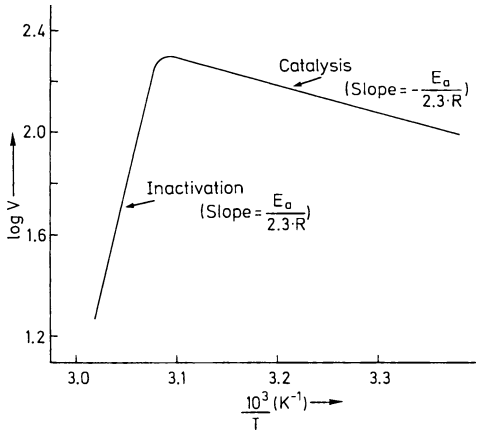


Fig. 2.35. Fungal α-amylase. Amylose hydrolysis versus temperature. *Arrhenius* diagram for assessing the activation energy of enzyme catalysis and enzyme inactivation; V = total reaction rate

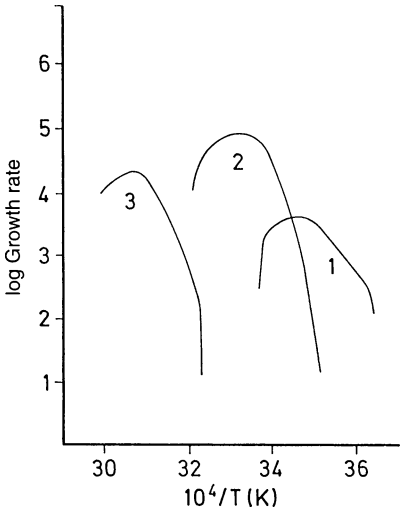


Fig. 2.36. Growth rate and temperature for 1) psychrophilic (*Vibrio AF-1*), 2) mesophilic (*E. coli K-12*) and 3) thermophilic (*Bacillus cereus*) microorganisms (according to *Herbert*, 1989)

Table 2.14. α-Amylase activity as affected by temperature: relative rates of hydrolysis and enzyme inactivation

Temperature (°C)	Relative rate[a]	
	hydrolysis	inactivation
0	1.0	1.0
10	1.35	$1.0 \cdot 10^2$
20	1.8	$0.7 \cdot 10^4$
40	3.0	$1.8 \cdot 10^7$
60	4.8	$1.5 \cdot 10^{10}$

[a] Activation energies of 20 kJ · mole^{-1} for hydrolysis and 295 kJ · mole^{-1} for enzyme inactivation were used for calculation according to *Whitaker* (1972).

The growth of microorganisms follows a similar temperature dependence and can also be depicted according to the *Arrhenius* equation (Fig. 2.36) by replacing the value k by the growth rate and assuming E_a is the reference value μ of the temperature for growth.

For maintaining food quality, detailed knowledge of the relationship between microbial growth rate and temperature is important for optimum production processes (heating, cooling, freezing).

The highly differing activation energies for killing microorganisms and for normal chemical reactions have triggered a trend in food technology towards the use of high-temperature short-time (HTST) processes in production. These are based on the findings that at higher temperatures the desired killing rate of microorganisms is higher than the occurrence of undesired chemical reactions.

2.5.4.4 Thermal Stability

The thermal stability of enzymes is quite variable. Some enzymes lose their catalytic activity at lower temperatures, while others are capable of withstanding – at least for a short period of time – a stronger thermal treatment. In a few cases enzyme stability is lower at low temperatures than in the medium temperature range.

Lipase and alkaline phosphatase in milk are thermolabile (Fig. 2.37), whereas acid phosphatase is relatively stable. Therefore, alkaline phosphatase is used to distinguish raw from pasteurized milk because its activity is easier to determine than that of lipase. Of all the enzymes in the potato tuber (Fig. 2.38), peroxidase is the last one to be thermally inactivated. Such inactivation patterns are often found among enzymes in vegetables. In such cases, peroxidase is a suitable indicator for controlling the total inactivation of all the enzymes e. g., in assessing the adequacy of a blanching process. However, newer developments aim to limit the enzyme inactivation to

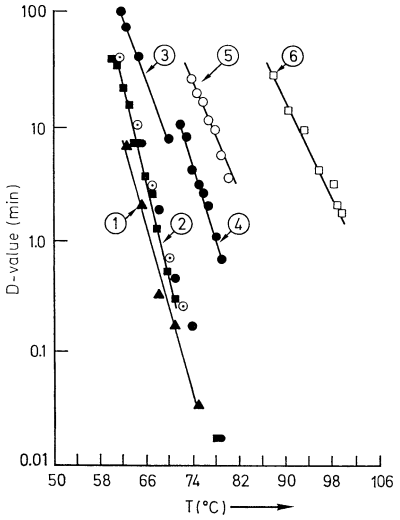


Fig. 2.37. Thermal inactivation of enzymes of milk. 1 Lipase (inactivation extent, 90%), 2 alkaline phosphatase (90%), 3 catalase (80%), 4 xanthine oxidase (90%), 5 peroxidase (90%), and 6 acid phosphatase (99%)

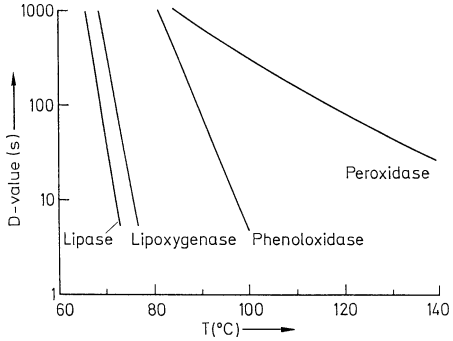


Fig. 2.38. Thermal inactivation (90%) of enzymes present in potato tuber

such enzymes responsible for quality deterioration during storage. For example semiripened pea seeds in which lipoxygenase is responsible for spoilage. However, lipoxygenase is more sensitive than peroxidase, thus a sufficient but gentle blanching requires the inactivation of lipoxygenase only. Inactivation of peroxidase is not necessary.

All the changes which occur in proteins outlined in section 1.4.2.4 also occur during the heating of enzymes. It the case of enzymes the consequences are even more readily observed since a slight conformational change at the active site can result in total loss of activity.

The inactivation or killing rates for enzymes and microorganisms depend on several factors. Most significant is the pH. Lipoxygenase isolated from pea seeds (Fig. 2.39) denatures most slowly at its isoelectric point (pH 5.9) as do many other enzymes.

Table 2.21 contains a list of technically useful proteinases and their thermal stability. However, these data were determined using isolated enzymes. They may not be transferrable to the same enzymes in food because in its natural environment an enzyme usually is much more stable. In additional studies, mostly related to heat transfer in food, some successful procedures to calculate the degree of enzyme inactivation based on thermal stabilty data of isolated enzymes have been developed. An example for the agreement between calculated and experimental results is presented in Fig. 2.40.

Peroxidase activity can partially reappear during storage of vegetables previously subjected to a blanching process to inactivate enzymes. The reason for this recurrence, which is also observed for alkaline phosphatase of milk, is not known yet.

Enzymes behave differently below the freezing point. Changes in activity depend on the type of enzyme and on a number of other factors which are partly contrary. The activity is positively influenced by increasing the concentration of enzyme

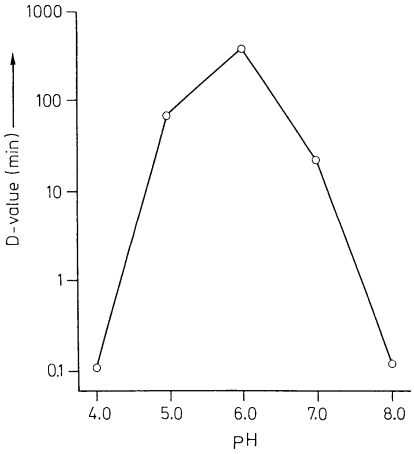


Fig. 2.39. Pea seed lipoxygenase. Inactivation extent at 65 °C as affected by pH

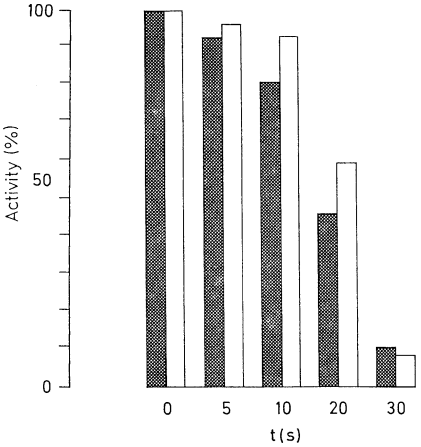


Fig. 2.40. Blanching of semiripened peas at 95 °C; lipoxygenase inactivation (according to *Svensson*, 1977). ■ Experimentally found, □ calculated

and substrate due to formation of ice crystals. A positive or negative change might be caused by changes in pH. Viscosity increase of the medium results in negative changes because the diffusion of the substrate is restricted. In completely frozen food (T < phase transition temperature T_g', cf. 0.3.3 and Table 0.8), a state reached only during deep-freezing, the catalytic activity stops temporarily. Relatively few enzymes are irreversibly destroyed by freezing.

2.5.5 Influence of Pressure

The application of high pressures can inhibit the growth of microorganisms and the activity of enzymes. This allows the protection of sensitive nutrients and aroma substances in foods. Some products preserved in this gentle way are now marketable. Microorganisms are relatively sensitive to high pressure because their growth is inhibited at pressures of 300–600 MPa and lower pH values increase this effect. However, bacterial spores withstand pressures of >1200 MPa.

In contrast to thermal treatment, high pressure does not attack the primary structure of proteins at room temperature. Only H-bridges, ionic bonds and hydrophobic interactions are disrupted. Quaternary structures are dissociated into subunits by comparatively low pressures (<150 MPa). Higher pressures (>1200 MPa) change the tertiary structure and very high pressures disrupt the H-bridges which stabilize the secondary structure. The hydration of proteins is also changed by high pressure because water molecules are pressed into cavities which can exist in the hydrophobic interior of proteins. In general, proteins are irreversibly denatured at room temperature by the application of pressures above 300 MPa while lower pressures cause only reversible changes in the protein structure.

In the case of enzymes, even slight changes in the steric arrangement and mobility of the amino acid residues which participate in catalysis can lead to loss of activity. Nevertheless, a relatively high pressure is often required to inhibit enzymes. But the pressure required can be reduced by increasing the temperature, as shown in Fig. 2.41 for α-amylase. While a pressure of 550 MPa is required at 25 °C to inactivate the enzyme with a rate constant (first order reaction) of $k = 0.01\ \text{min}^{-1}$, a pressure of only 340 MPa is required at 50 °C.

It is remarkable that enzymes can also be activated by changes in the conformation of the polypeptide chain, which are initiated especially by low pressures around 100 MPa. In the application of the pressure technique for the production of stable food, intact tissue, and not isolated enzymes, is exposed to high pressures. Thus, the enzyme activity can increase instead of decreasing when cells or membranes are disintegrated with the release of enzyme and/or substrate.

Some examples are presented here to show the pressures required to inhibit the enzyme activity which can negatively effect the quality of foods.

- Pectin methylesterase (EC 3.1.1.11) causes the flocculation of pectic acid (cf. 2.7.2.2.13) in orange juices and reduces the consistency of tomato products. In orange juice, irreversible enzyme inactivation reaches 90% at a pressure of 600 MPa. Even though the enzyme in tomatoes is more stable, increasing the temperature to 59–60 °C causes inactivation at 400 MPa and at 100 MPa after the removal of Ca^{2+} ions.
- Peroxidases (EC 1.11.1.3) induce undesirable aroma changes in plant foods. In green beans, enzyme inactivation reached 88% in 10 min after pressure treatment at 900 MPa. At pressures above 400 MPa (32 °C), the activity of this enzyme in oranges fell continuously

to 50%. However, very high pressures increased the activity at 32–60 °C. It is possible that high pressure denatures peroxidase to a heme(in) catalyst (cf. 3.7.2.1.7).
- Lipoxygenase from soybeans (cf. 3.7.2.2). This enzyme was inactivated in 5 min at pH 8.3 by pressures up to 750 MPa and temperatures in the range 0–75 °C. The pressure stability was reduced by gassing with CO_2 and reducing the pH to 5.4.
- Polyphenol oxidases (cf. 0.3.3) in mushrooms and potatoes require pressures of 800–900 MPa for inactivation. The addition of glutathione (5 mmol/l) increases the pressure sensitivity of the mushroom enzyme. In this case, the inactivation is obviously supported by the reduction of disulfide bonds.

2.5.6 Influence of Water

Up to a certain extent, enzymes need to be hydrated in order to develop activity. Hydration of e. g. lysozyme was determined by IR and NMR spectroscopy. As can be seen in Table 2.15, first the charged polar groups of the side chains hydrate, followed by the uncharged ones. Enzymatic activity starts at a water content of 0.2 g/g protein, which means even before a monomolecular layer of the polar groups with water has taken place. Increase in hydration resulting in a monomolecular layer of the whole available enzyme surface at 0.4 g/g protein raises the activity to a limiting value reached at a water content of 0.9 g/g protein. Here the diffusion of the substrate to the enzyme's active site seems to be completely guaranteed.

Table 2.15. Hydration of Lysozyme

g Water / g Protein	Hydration sequence	Molecular changes
0.0	Charged groups	Relocation of protons
	Uncharged, polar groups (formation of clusters)	New orientation of disulfide bonds
0.1	Saturation of COOH groups Saturation of polar groups in side chains	Change in conformation
0.2	Peptide-NH	Start of enzymatic activity
0.3	Peptide-CO Monomolecular hydration of polar groups Apolar side chains	
0.4	Complete enzyme hydration	

For preservation of food it is mandatory to inhibit enzymatic activity completely if the storage temperature is below the phase transition temperature T_g or T'_g (cf. 0.3.3). With help of a model system containing glucose oxidase, glucose and water as well as sucrose and maltodextrin (10 DE) for adjustment of T'_g values in the range of -9.5 to -32 °C, it was found that glucose was enzymatically oxidized only in such samples that were stored for two months above the T'_g value and not in those kept at storage temperatures below T'_g.

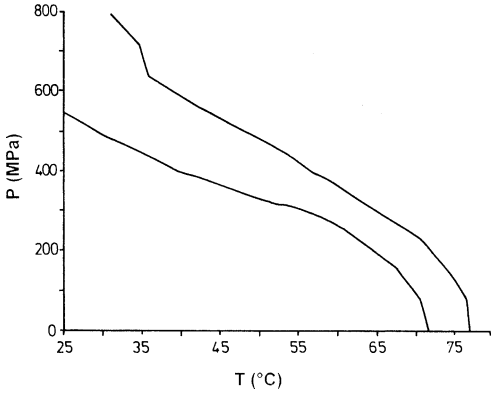


Fig. 2.41. Pressure–temperature diagram for the inactivation kinetics of α-amylase from *Bacillus subtilis* at pH 8.6 (according to *Ludikhuyze* et al., 1997). Range of the rate constants: $k = 0.01\ min^{-1}$ (lower line) to $k = 0.07\ min^{-1}$ (upper line)

2.6 Enzymatic Analysis

Enzymatic food analysis involves the determination of food constituents, which can be both substrates or inhibitors of enzymes, and the determination of enzyme activity in food.

2.6.1 Substrate Determination

2.6.1.1 Principles

Qualitative and quantitative analysis of food constituents using enzymes can be rapid, highly sensitive, selective and accurate (examples in Table 2.16). Prior purification and separation steps, as a rule, are not necessary in the enzymatic analysis of food.

In an enzymatic assay, spectrophotometric or electrochemical determination of the reactant or the product is the preferred approach. When this is not applicable, the determination is performed by a coupled enzyme assay. The coupled reaction includes an auxiliary reaction in which the food constituent is the reactant to be converted to product, and an indicator reaction which involves an indicator enzyme and its reactant or product, the formation or breakdown of which can be readily followed analytically. In most cases, the indicator reaction follows the auxiliary reaction:

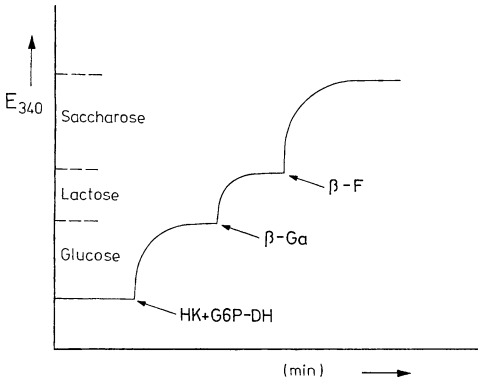


Fig. 2.42. Enzymatic determination of glucose, saccharose and lactose in one run. After adding cosubstrates, ATP and NADP, the enzymes are added in the order: hexokinase (HK), glucose-6-phosphate dehydrogenase (G6P-DH), β-galactosidase (β-Ga) and β-fructosidase (β-F)

$$\begin{aligned}
&\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glucose-6P} && \text{(a)}\\
&\text{Glucose-6P} + \text{NADP}^{\oplus} \xrightarrow{\text{G6P-DH}} \text{6-Phosphogluconate} + \text{NADPH} + \text{H}^{\oplus} && \text{(b)}\\
&\text{Lactose} \xrightarrow{\beta\text{-Ga}} \text{Glucose} + \text{Galactose} && \text{(c)}\\
&\text{Saccharose} \xrightarrow{\beta\text{-F}} \text{Glucose} + \text{Fructose} && \text{(d)}
\end{aligned} \qquad (2.105)$$

$$\begin{aligned}
&\text{A} + \text{B} \xrightleftharpoons{\text{Auxiliary reaction}} \text{P} + \text{Q}\\
&\text{P} + \text{C} \xrightleftharpoons{\text{Indicator reaction}} \text{R} + \text{S}
\end{aligned} \qquad (2.106)$$

Reactant A is the food constituent which is being analyzed. C or R or S is measured. The equilibrium state of the coupled indicator reaction is concentration dependent. The reaction has to be adjusted in some way in order to remove, for example, P from the auxiliary reaction before an equilibrium is achieved. By using several sequential auxiliary reactions with one indicator reaction, it is possible to simultaneously determine several constituents in one assay. An example is the analysis of glucose, lactose and saccharose (cf. Reaction 2.105).

First, glucose is phosphorylated with ATP in an auxiliary reaction (a). The product, glucose-6-phosphate, is the substrate of the NADP-dependent indicator reaction (b). Addition of β-galactosidase starts the lactose analysis (c) in which the released glucose, after phosphorylation, is again measured through the indicator reaction [(b) of Reaction 2.105 and also Fig. 2.42]. Finally, after addition of β-fructosidase, saccharose is cleaved (d) and the released glucose is again measured through reactions (a) and (b) as illustrated in Fig. 2.42.

2.6.1.2 End-Point Method

This procedure is reliable when the reaction proceeds virtually to completion. If the substrate is only partly consumed, the equilibrium is displaced in favor of the products by increasing the concentration of reactant or by removing one of the products of the reaction. If it is not possible to achieve this, a standard curve must

Table 2.16. Examples of enzymatic analysis of food constituents[a]

Constituent	Auxiliary reaction	Indicator reaction
Glucose	β-D-Glucose[b] + O_2 $\overset{\text{Glucose oxidase}}{\rightleftharpoons}$ ∂-D-Gluconolactone + H_2O_2(a_H)	o-Dianisidine + H_2O_2 $\overset{\text{Peroxidase}}{\rightleftharpoons}$ Oxid. o-dianisidine (a_I)
	Glucose + ATP $\overset{\text{Hexo-kinase}}{\rightleftharpoons}$ Glucose-6P(b_H)	Glucose-6P + $NADP^{\oplus}$ $\overset{\text{Glucose-6P dehydrogenase}}{\rightleftharpoons}$ Gluconate-6P + NADPH + $H^{\oplus}$(b_I)
Fructose	Fructose + ATP $\overset{\text{Hexo-kinase}}{\rightleftharpoons}$ Fructose-6P	
	Fructose-6P $\overset{\text{Glucosephosphate isomerase}}{\rightleftharpoons}$ Glucose-6P	As glucose-6P (b_I)
Sorbitol	D-Sorbitol + NAD $\overset{\text{Sorbitol dehydrogenase}}{\rightleftharpoons}$ Fructose + NADH + $H^{\oplus}$	
Maltose	Maltose + H_2O $\overset{\text{α-Glucosidase}}{\rightleftharpoons}$ 2 Glucose	As glucose ($b_H + b_I$)
Starch	Starch + (n − 1) H_2O $\overset{\text{Amylo-glucosidase}}{\rightleftharpoons}$ n-Glucose	As glucose ($b_H + b_I$)
Galactose	β-D-Galactose + $NAD^{\oplus}$ $\overset{\text{Galactose dehydrogenase}}{\rightleftharpoons}$ D-Galactono-γ-lactone + NADH + $H^{\oplus}$	
Ethanol	Ethanol + $NAD^{\oplus}$ $\overset{\text{Alcohol dehydrogenase}}{\rightleftharpoons}$ Acetaldehyde + NADH + $H^{\oplus}$	
Glycerol	Glycerol + ATP $\overset{\text{Glycerol kinase}}{\rightleftharpoons}$ sn-Glycerol-3P + ATP	ADP + Phosphoenolpyruvate $\overset{\text{Pyruvate kinase}}{\rightleftharpoons}$ ATP + Pyruvate (c)
		Pyruvate + NADH + $H^{\oplus}$ $\overset{\text{Lactate dehydrogenase}}{\rightleftharpoons}$ Lactate + $NAD^{\oplus}$ (d)
Lactate	L-Lactate assay is achieved by a reversed reaction of d), and D-lactate assay with a dehydrogenase specific for D-enantiomer.	
Creatinine and Creatine	Creatinine + H_2O $\overset{\text{Creatininase}}{\rightleftharpoons}$ Creatine	
	Creatine + ATP $\overset{\text{Creatine kinase}}{\rightleftharpoons}$ Creatine-P + ADP; ADP is determined through c) and d)	
Individual amino acids	R–CH(NH_2)COOH $\overset{\text{Amino acid decarboxylase[d]}}{\rightleftharpoons}$ R–CH_2–NH_2 + CO_2	
L-Malate	L-Malate + $NAD^{\oplus}$ $\overset{\text{Malate dehydrogenase}}{\rightleftharpoons}$ Oxalacetate + NADH + $H^{\oplus}$	

[a] For saccharose and lactose see Fig. 2.42.
[b] The content of α-anomeric form is accessible through mutarotation.
[c] After hydrolysis this method is suitable for the assay of acylglycerols.
[d] Specific decarboxylases are availabe as exemplified by those for L-tryosine, L-lysine, L-glutamic acid, L-aspartic acid, or L-arginine.

Table 2.17. Enzyme concentrations used in the end-point method of enzymatic food analysis

Substrate	Enzyme	K_m (mol/l)	Enzyme concentration (µcat/l)
Glucose	Hexokinase	$1.0 \cdot 10^{-4}$ (30 °C)	1.67
Glycerol	Glycerol kinase	$5.0 \cdot 10^{-5}$ (25 °C)	0.83
Uric acid	Urate oxidase	$1.7 \cdot 10^{-5}$ (20 °C)	0.28
Fumaric	Fumarase acid	$1.7 \cdot 10^{-6}$ (21 °C)	0.03

be prepared. In contrast to kinetic methods (see below), the concentration of substrate which is to be analyzed in food must not be lower than the *Michaelis* constant of the enzyme catalyzing the auxiliary reaction. The reaction time is readily calculated when the reaction rate follows first-order kinetics for the greater part of the enzymatic reaction.

In a two-substrate reaction the enzyme is saturated with the second substrate. Since Equation 2.41 is valid under these conditions, the catalytic activity of the enzyme needed for the assay can be determined for both one- and two-substrate reactions. The examples shown in Table 2.17 suggest that enzymes with low K_m values are desirable in order to handle the substrate concentrations for the end-point method with greater flexibility.

Data for K_m and V are needed in order to calculate the reaction time required. A prerequisite is a reaction in which the equilibrium state is displaced toward formation of product with a conversion efficiency of 99%.

2.6.1.3 Kinetic Method

Substrate concentration is obtained using a method based on kinetics by measuring the reaction rate. To reduce the time required per assay, the requirement for the quantitative conversion of substrate is abandoned. Since kinetic methods are less susceptible to interference than the endpoint method, they are advantageous for automated methods of enzymatic analysis.

The determination of substrate using kinetic methods is possible only as long as Equation 2.46 is valid. Hence, the following is required to perform the assay:

a) For a two-substrate reaction, the concentration of the second reactant must be so high that the rate of reaction depends only on the concentration of the substrate which is being analyzed.
b) Enzymes with high *Michaelis* constants are required; this enables relatively high substrate concentrations to be determined.
c) If enzymes with high *Michaelis* constants are not available, the apparent K_m is increased by using competitive inhibitors.

In order to explain requirement c), let us consider the example of the determination of glycerol as given in Table 2.16. This reaction allows the determination of only low concentrations of glycerol since the K_m values for participating enzymes are low: 6×10^{-5} mol/l to 3×10^{-4} mol/l. In the reaction sequence the enzyme pyruvate kinase is competitively inhibited by ATP with respect to ADP. The expression $K_m(1 + (I)/K_I)$ (cf. 2.5.2.2.1) may in these circumstances assume a value of 6×10^{-3} mol/l, for example. This corresponds to an apparent increase by a factor of 20 for the K_m of ADP (3×10^{-4} mol/l). The ratio $(S)/K_m(1 + [I]/K_I)$ therefore becomes 1×10^{-3} to 3×10^{-2}. Under these conditions, the auxiliary reaction (Table 2.16) with pyruvate kinase follows pseudo-first-order kinetics with respect to ADP over a wide range of concentrations and, as a result of the inhibition by ATP, it is also the rate-determining step of the overall reaction. It is then possible to kinetically determine higher concentrations of glycerol.

2.6.2 Determination of Enzyme Activity

In the foreword of this chapter it was emphasized that enzymes are suitable indicators for identifying heat-treated food. However, the determination of enzyme activity reaches far beyond this possibility: it is being used to an increasing extent for the evaluation of the quality of raw food and for optimizing the parameters of particular food processes. In addition, the activities of enzyme

preparations have to be controlled prior to use in processing or in enzymatic food analysis.

The measure of the catalytic activity of an enzyme is the rate of the reaction catalyzed by the enzyme. The conditions of an enzyme activity assay are optimized with relation to: type and ionic strength of the buffer, pH, and concentrations of substrate, cosubstrate and activators used. The closely controlled assay conditions, including the temperature, are critical because, in contrast to substrate analysis, the reliability of the results in this case often can not be verified by using a weighed standard sample.

Temperature is a particularly important parameter which strongly influences the enzyme assay. Temperature fluctuations significantly affect the reaction rate (cf. 2.5.4); e. g., a 1 °C increase in temperature results in about a 10% increase in activity. Whenever possible, the incubation temperature should by maintained at 25 °C.

The substrate concentration in the assay is adjusted ideally so that Equation 2.40 is valid, i. e. $[A_0] \gg K_m$. Difficulties often arise while trying to achieve this condition: the substrate's solubility is limited; spectrophotometric readings become unreliable because of high light absorbance by the substrate; or the high concentration of the substrate inhibits enzyme activity. For such cases procedures exist to assess the optimum substrate concentration which will support a reliable activity assay.

2.6.3 Enzyme Immunoassay

Food compounds can be determined specifically and sensitively by immunological methods. These are based on the specific reaction of an antibody containing antiserum with the antigen, the substance to be determined. The antiserum is produced by immunization of rabbits for example. Because only compounds with a high molecular weight ($M_r > 5000$) display immunological activity, for low molecular compounds (haptens) covalent coupling to a protein is necessary. The antiserum produced with the "conjugate" contains antibodies with activities against the protein as well as the hapten.

Prior to the application, the antiserum is tested for its specificity against all proteins present in the food to be analyzed. As far as possible all unspecificities are removed. For example, it is possible to treat an antiserum intended to be used for the determination of peanut protein with proteins from other nuts in such a way that it specifically reacts with peanut protein only. However, there are also cases in which the specificity could not be increased because of the close immunochemical relationship between the proteins. This happens, for example, with proteins from almonds, peach and apricot kernels.

The general principle of the competitive immunoassay is shown in Fig. 2.43. Excess amounts of marked and unmarked antigens compete for the antibodies present. The concentration of the unmarked antigen to be determined is the only variable if the concentration of the marked antigen and the antibody concentration are kept on a constant level during the examination. Following the principle of mass action, the unknown antigen concentration can be calculated indirectly based on the proportion of free marked antigen. Older methods still require the formation of a precipitate for the detection of an antibody–antigen reaction (cf. 12.10.2.3.2). Immunoassays are much faster and more sensitive.

Radioisotopes (^3H, ^{14}C) and enzymes are used to mark antigens. Furthermore, fluorescent and luminescent dyes as well as stable radicals are important. Horseradish peroxidase, alkaline phosphatase from calf stomach, and β-D-galactosidase from *E. coli* are often used as indicator enzymes because they are available in high purity, are very stable and their activity can be determined sensitively and precisely. Enzymes are bound to antigens or haptens by covalent bonds, e. g., by reaction with glutaraldehyde or carbodiimide.

Enzyme immunoassays are increasingly used in food analysis (examples see Table 2.18). Laboratories employing these methods need no specific

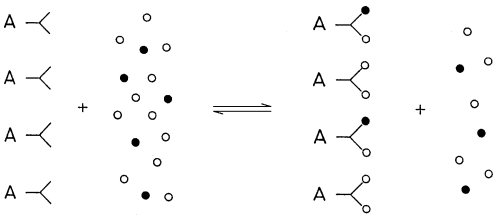


Fig. 2.43. Principle of an immunoassay. Marked antigens (●) and unmarked antigens (○) compete for the binding sites of the antibodies A

Table 2.18. Examples for application of enzyme immunoassay in food analysis

Detection and quantification
Type of meat
Soya protein in meat products
Myosin in muscle meat
Cereal proteins as well as papain in beer
Gliadins (absence of gluten in foods)
Veterinary drugs and fattening aids, e. g. penicillin in milk, natural or synthetic estrogens in meat
Toxins (aflatoxins, enterotoxins, ochratoxins) in food
Pesticides (atrazine, aldicarb, carbofuran)
Glycoalkaloids in potatoes

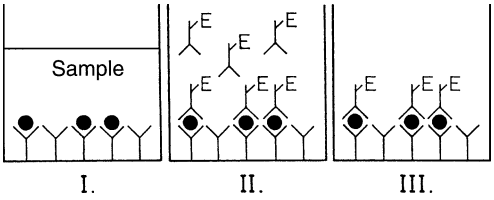


Fig. 2.44. Principle of non-competitive ELISA (sandwich ELISA)
Y Immobilized antibody,
● antigen,
YE enzyme-marked antibody

equipment contrary to use of radio immunological methods (RIA). Furthermore, for radio immunoassays free antigens have always to be separated from the ones bound to antibodies (heterogeneous immunoassay) while an enzyme immunoassay is suitable for homogeneous tests if the activity of the indicator enzyme is inhibited by the formation of an antigen–antibody-complex.
In food analysis, the ELISA *test* (*enzyme linked immunosorbent assay*) is the most important immunochemical method. In fact, two experimental procedures are applied: the competitive ELISA, as shown in Fig. 2.43, and the sandwich ELISA.
While the competitive ELISA is directed at the detection of low-molecular substances, the sandwich ELISA is suitable only for analytes (antigens) larger than a certain minimum size. The antigen must have at least two antibody binding sites (epitopes) which are spatially so far apart that it can bind two different antibodies. The principle of the sandwich ELISA is shown in Fig. 2.44. A plastic carrier holds the antibodies, e. g. against a toxin, by adsorption. When the sample is added, the toxin (antigen) reacts with the excess amount of antibodies (I in Fig. 2.44). The second antibody marked with an enzyme (e. g. alkaline phosphatase, peroxidase, glucose oxidase or luciferase) and with specificity for the antigen forms a sandwich complex (II). Unbound enzyme-marked antibodies are washed out. The remaining enzyme activity is determined (III). It is directly proportional to the antigen concentration in the sample which can be calculated based on measured standards and a calibration curve.

2.6.4 Polymerase Chain Reaction

With the polymerase chain reaction (PCR), a few molecules of any DNA sequence can be multiplied by a factor of 10^6 to 10^8 in a very short time. The sequence is multiplied in a highly specific way until it becomes visible electrophoretically. Based on PCR, analytical techniques have been developed for species identification in the case of animal and plant foods and microorganisms. It is

Table 2.19. Examples of approved genetically modified crops (as of 2003)[a]

Crop	Property
Cauliflower	Herbicide tolerance
Broccoli	Herbicide tolerance
Chicory	Herbicide tolerance
Cucumber	Fungal resistance
Potato	Insect and virus resistance
Pumpkin	Virus resistance
Corn	Herbicide tolerance, insect resistance
Melon	Virus resistance, delayed ripening
Papaya	Virus resistance
Paprika	Virus resistance
Rape seed	Higher concentrations of 12:0 und 14:0, herbicide resistance
Rice	Virus resistance
Red Bean	Insect resistance
Soybean	Altered fatty acid spectrum (cf. 14.3.2.2.5), herbicide tolerance
Tomato	Delayed ripening, increased pectin content
Wheat	Herbicide tolerance
Sugar beet	Herbicide tolerance

[a] The crop is approved in at least one country.

of special interest that PCR allows the detection of genetically modified food (genetically modified organism, GMO). Thus, it is possible to control the labeling of GMOs, which is required by law. In fact, the number of GMOs among food crops is increasing steadily (cf. Table 2.19); cf. survey by Anklam et al. (2002).

2.6.4.1 Principle of PCR

The first steps of a PCR reaction are shown schematically in Fig. 2.45. First, the extract which contains, among other substances, the DNA fragment (analyte) to be identified is briefly heated to 95 °C. This causes denaturation and separation into single strands. After cooling to 54 °C, two oligodeoxynucleotides (primer 1 and 2 having base sequences complementary to the target DNA) which flank the DNA sequence to be multiplied are added in excess. These primers, which are 15–30 nucleotides long and made with a synthesizer, hybridize with the complementary segments on the single strands. The temperature is increased to 72 °C and a mixture of the four deoxynucleoside 5′-triphosphates (dATP, dCTP, dGTP, dTTP, for structures of the bases cf. Formula 2.107) and a thermostable DNA polymerse, e. g., *Taq polymerase* from *Thermus aquaticus*, are added. The polymerase synthesizes new complementary strands starting from the primers in the $5' \rightarrow 3'$ direction using the deoxynucleotides. In the subsequent heating step, these strands are separated, in addition to the denatured target DNA which is no longer shown in Fig. 2.45. In the second cycle, the primers hybridize with the single strands which end with the nucleotide sequence of the other primer in each case. The PCR yields two DNA segments (a and b in Fig. 2.45) which are bounded by the nucleotide sequences of the primer. The DNA segment is amplified by repeating the steps denaturation – addition of primer – PCR 20 to 30 times, and is then electophoretically analyzed.

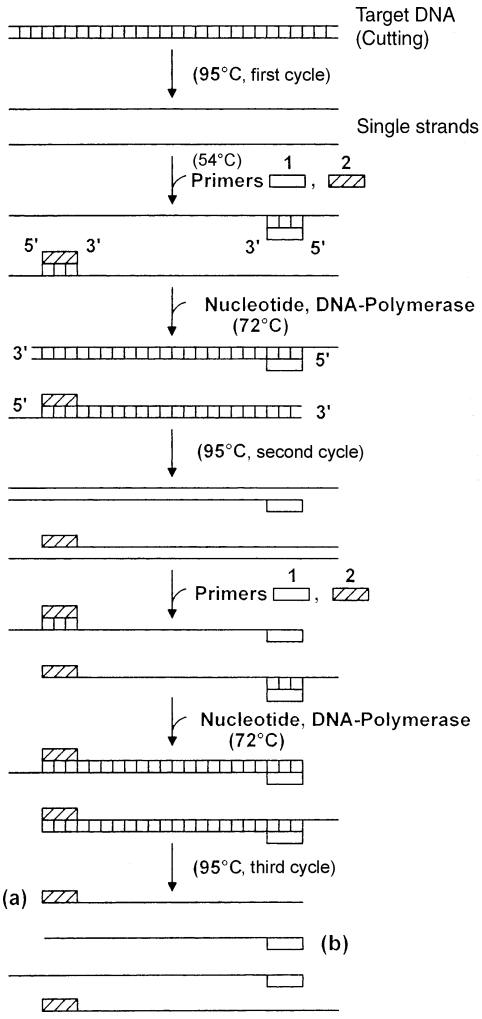
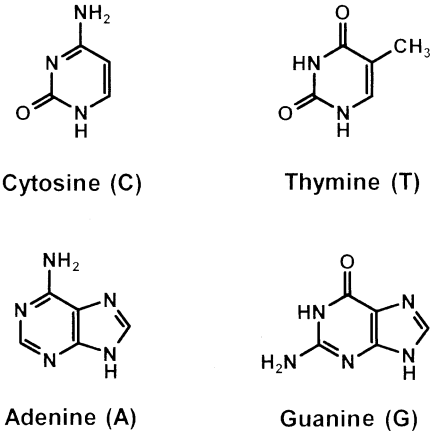


Fig. 2.45. Principle of PCR

Base:



(2.107)

In comparison with protein analysis, DNA analysis is more sensitive by several orders of mag-

nitude due to amplification which is millionfold after 20 cycles and billionfold after 30 cycles. Heated food can be analyzed because DNA is considerably more stable than proteins. It is also possible to detect GMOs which do not contain altered or added proteins identifiable by chemical methods. Acidic foods can cause problems when they are strongly heated, e. g., tomato products. In this case, the DNA is hydrolyzed to such an extent that the characteristic sequences are lost. The exceptional sensitivity of this method can also give incorrect positive results. For this reason, it is important that the PCR is quantitatively evaluated, especially when controlling limiting values. A known amount of a synthetic DNA is added to the sample and amplified competitively with the analyte. For calibration, mixtures of the target and competing DNA are subjected to PCR analysis.

2.6.4.2 Examples

2.6.4.2.1 Addition of Soybean

The addition of soybean protein to meat and other foods can be detected with the help of the primers GMO3 (5′-GCCCTCTACTCCACCCCCATCC-3′) and GMO4 (5′-GCCCATCTGCAAGCCTTTTTGTG-3′). They label a small but still sufficiently specific sequence of 118 base pairs (bp) of the gene for a lectin occurring in soybean. A small amplicon is of advantage since the DNA gets partially fragmented when meat preparations are heated.

2.6.4.2.2 Genetically Modified Soybeans

Genetically modified soybeans are resistant to the herbicide glyphosphate (cf. 9.4.3), which inhibits the key enzyme, 5-enolpyruvylshikimi-3-phosphate synthase (EPSPS), in the metabolism of aromatic amino acids in plants. However, glyphosphate is inactive against the EPSPS of bacteria. Hence transgenic soybeans contain a genetic segment which codes for an EPSPS from *Agrobacterium sp.* and a peptide for the transport of this enzyme. To detect this segment and, consequently, genetically modified soybeans, primers are used which induce the amplification of a segment of 172 bp in the PCR.

2.6.4.2.3 Genetically Modified Tomatoes

During ripening and storage, tomatoes soften due to the activity of an endogenous enzyme polygalacturonase (PG). The expression of the gene for PG is specifically inhibited in a particular tomato, resulting in extended storage life and better aroma. PCR methods have been developed to detect these transgenic tomatoes. However, this detection can fail if the DNA is too strongly hydrolyzed on heating the tomato products.

2.6.4.2.4 Species Differentiation

If specific primers fail, a PCR with universal primers can be applied in certain cases, followed by an RFLP analysis (restriction fragment length polymorphism). The DNA of a meat sample is first determined with a primer pair which exhibits a high degree of correspondence in its binding sites to the DNA of many animal species. In the case of various animal species, the PCR yields equally long products which should be relatively large (ca. 300–500 bp). The amplicon is cleaved in the subsequent RFLP analysis with different restriction endonucleases. After electrophoretic separation, the pattern of the resulting DNA fragments can be assigned to individual animal species. This method is suitable for samples of one type of meat. Preparations containing meat of several animal species or DNA which is more strongly fragmented on heating can be reliably analyzed only with animal species-specific primers.

2.7 Enzyme Utilization in the Food Industry

Enzyme-catalyzed reactions in food processing have been used unintentionally since ancient times. The enzymes are either an integral part of the food or are obtained from microorganisms. Addition of enriched or purified enzyme preparations of animal, plant or, especially, microbial origin is a recent practice. Most of these enzymes come from microorganisms, which have been genetically modified in view of their economic production. Such intentionally used

additives provide a number of advantages in food processing: exceptionally pronounced substrate specificity (cf. 2.2.2), high reaction rate under mild reaction conditions (temperature, pH), and a fast and continuous, readily controlled reaction process with generally modest operational costs and investment. Examples for the application of microbial enzymes in food processing are given in Table 2.20.

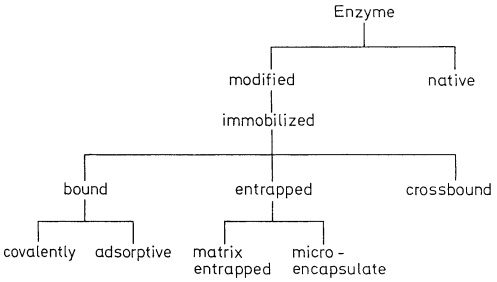


Fig. 2.46. Forms of immobilized enzymes

2.7.1 Technical Enzyme Preparations

2.7.1.1 Production

The methods used for industrial-scale enzyme isolation are outlined in principle under section 2.2.4. In contrast to the production of highly purified enzymes for analytical use, the production of enzymes for technical purposes is directed to removing the interfering activities which would be detrimental to processing and to staying within economically acceptable costs. Selective enzyme precipitation by changing the ionic strength and/or pH, adsorption on inorganic gels such as calcium phosphate gel or hydroxyl apatite, chromatography on porous gel columns and ultrafiltration through membranes are among the fractionation methods commonly used. Ionexchange chromatography, affinity chromatography (cf. 2.2.4) and preparative electrophoresis are relatively expensive and are seldom used. A few temperature-stable enzymes are heat treated to remove the other contaminating and undesired enzyme activities.

Commercial enzyme preparations are available with defined catalytic activity. The activity is usually adusted by the addition of suitable inert fillers such as salts or carbohydrates. The amount of active enzyme is relatively low, e. g., proteinase preparations contain 5–10% proteinase, whereas amylase preparations used for treamtent of flour contain only 0.1% pure fungal α-amylase.

2.7.1.2 Immobilized Enzymes

Enzymes in solution are usually used only once. The repeated use of enzymes fixed to a carrier is more economical. The use of enzymes in a continous process, for example, immobilized enzymes used in the form of a stationary phase which fills a reaction column where the reaction can be controlled simply by adjustment of the flow rate, is the most advanced technique. Immobilized enzymes are produced by various methods (Fig. 2.46).

2.7.1.2.1 Bound Enzymes

An enzyme can be bound to a carrier by covalent chemical linkages, or in many cases, by physical forces such as adsorption, by charge attraction, H-bond formation and/or hydrophobic interactions. The covalent attachment to a carrier, in this case an activated matrix, is usually achieved by methods employed in peptide and protein chemistry. First, the matrix is activated. In the next step, the enzyme is coupled under mild conditions to the reactive site on the matrix, usually by reaction with a free amino group. This is illustrated by using cellulose as a matrix (Fig. 2.47). Another possibility is a process of copolymerization with suitable monomers. Generally, covalent attachment of the enzyme prevents leaching or "bleeding".

2.7.1.2.2 Enzyme Entrapment

An enzyme can be entrapped or enclosed in the cavities of a polymer network by polymerization of a monomer such as acrylamide or N,N′-methylene-bis-acrylamide in the presence of enzyme, and still remain accessible to substrate through the network of pores. Furthermore,

Table 2.20. Examples for the use of microbial enzymes in food processing

EC Number	Enzyme[a]	Biological Origin	Application[b]
Oxidoreductases			
1.1.1.39	Malate dehydrogenase (decarboxylating)	*Leuconostoc oenos*	10
1.1.3.4	Glucose oxidase	*Aspergillus niger*	7, 10, 16
1.11.1.6	Catalase	*Micrococcus lysodeicticus*	
		Aspergillus niger	1, 2, 7, 10, 16
Transferases			
2.7.2.4	Transglutaminase	*Streptoverticillium*	5, 8
Hydrolases			
3.1.1.1	Carboxylesterase	*Mucor miehei*	2, 3
3.1.1.3	Triacylglycerol lipase	*Aspergillus niger, A. oryzae, Candida lipolytica, Mucor javanicus, M. miehei, Rhizopus arrhizus, R. niveus*	2, 3
3.1.1.11	Pectinesterase	*Aspergillus niger*	9, 10, 17
3.1.1.20	Tannase	*Aspergillus niger, A. oryzae*	10
3.2.1.1	α-Amylase	*Bacillus licheniformis, B. subtilis, Aspergillus oryzae*	3, 8, 9, 10, 12 14, 15
		Aspergillus niger, Rhizopus delemar, R. oryzae	8, 9, 10, 12, 14, 15
3.2.1.2	β-Amylase	*Bacillus cereus,*	8, 10
3.2.1.3	Glucan-1,4-α-D-gluco-sidase (glucoamylase)	*B. magatherium, B. subtilis Aspergillus oryzae*	3, 9, 10, 12, 14 15, 18
		Aspergillus niger, Rhizopus arrhizus, R. delemar, R. niveus, R. oryzae, Trichoderma reesei	9, 10, 12, 14, 15, 18
3.2.1.4	Cellulase	*Aspergillus niger, A. oryzae, Rhizopus delemar, R. oryzae, Sporotrichum dimorphosporum, Thielavia terrestris, Trichoderma reesei*	9, 10, 18
3.2.1.6	Endo-1,3(4)-β-D-glucanase	*Bacillus circulans, B. subtilis, Aspergillus niger, A. oryzae, Penicillum emersonii, Rhizopus delemar, R. oryzae*	10
3.2.1.7	Inulinase	*Kluyveromyces fragilis*	12
3.2.1.11	Dextranase	*Klebsiella aerogenes, Penicillium funicolosum, P. lilacinum*	12
3.2.1.15	Polygalacturonase	*Aspergillus niger, Penicillium simplicissimum, Trichoderma reesei*	3, 9, 10, 17
		Aspergillus oryzae, Rhizopus oryzae	3, 9, 10
		Aspergillus niger	9, 10, 17

Table 2.20. (continued)

EC Number	Enzyme[a]	Biological Origin	Application[b]
3.2.1.20	α-D-Glucosidase	*Aspergillus niger, A. oryzae, Rhizopus oryzae*	8
3.2.1.21	β-D-Glucosidase	*Aspergillus niger, Trichoderma reesei*	9
3.2.1.22	α-D-Galactosidase	*Aspergillus niger, Mortierella vinacea sp., Saccharomyces carlsbergensis*	12
3.2.1.23	β-D-Galactosidase	*Aspergillus niger, A. oryzae, Kluyveromyces fragilis, K. lactis*	1, 2, 4, 18
3.2.1.26	β-D-Fructofuranosidase	*Aspergillus niger, Saccharomyces carlsbergensis, S. cerevisiae*	14
3.2.1.32	Xylan endo-1,3-β-D-xylosidase	*Streptomyces sp., Aspergillus niger, Sporotrichum dimorphosporum*	8, 10, 13
3.2.1.41	α-Dextrin endo-1,6-α-glucosidase (pullunanase)	*Bacillus acidopullulyticus*	8, 10, 12, 14, 15
		Klebsiella aerogenes	8, 10, 12
3.2.1.55	α-L-Arabinofuranosidase	*Aspergillus niger*	9, 10, 17
3.2.1.58	Glucan-1,3-β-D-glucosidase	*Trichoderma harzianum*	10
3.2.1.68	Isoamylase	*Bacillus cereus*	8, 10
3.2.1.78	Mannan endo-1,4-β-D-mannanase	*Bacillus subtilis, Aspergillus oryzae, Rhizopus delemar, R. oryzae, Sporotrichum dimor-phosporum, Trichoderma reesei*	13
		Aspergillus niger	13, 17
3.5.1.2	Glutaminase	*Bacillus subtilis*	5
3.4.21.14	Serine endopeptidase[c]	*Bacillus licheniformis*	5, 6, 10, 11
3.4.23.6	Aspartic acid endopeptidase	*Aspergillus melleus, Endothia parasitica, Mucor miehei, M. pusillus*	2
		Aspergillus oryzae	2, 5, 6, 8, 9, 10, 11, 15, 18
3.4.24.4	Metalloendopeptidase	*Bacillus cereus, B. subtilis*	10, 15
Lyases			
4.2.2.10	Pectin lyase	*Aspergillus niger*	9, 10, 17
Isomerases			
5.3.1.5	Xylose isomerase[d]	*Streptomyces murinus S. olivaceus, S. olivochromogenes, S. rubiginosus*	8, 9, 10, 12

[a] Principal activity.
[b] 1) Milk, 2) Cheese, 3) Fats and oils, 4) Ice cream, 5) Meat, 6) Fish, 7) Egg, 8) Cereal and starch, 9) Fruit and vegetables, 10) Beverages (soft drinks, beer, wine), 11) Soups and broths, 12) Sugar and honey, 13) Cacao, chocolate, coffee, tea, 14) Confectionery, 15) Bakery, 16) Salads, 17) Spices and flavors, 18) Diet food.
[c] Similar to Subtilisin.
[d] Some enzymes also convert D-glucose to D-fructose, cf. 2.7.2.3.

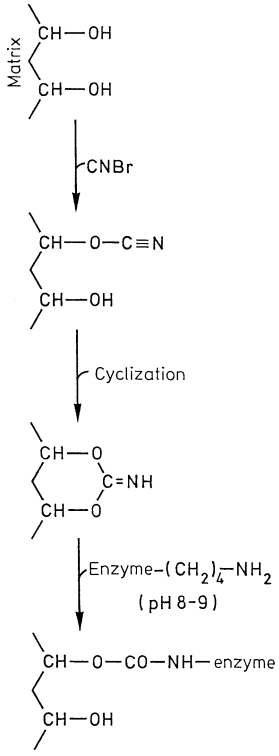


Fig. 2.47. Enzyme immobilization by covalent binding to a cellulose matrix

suitable processes can bring about enzyme encapsulation in a semipermeable membrane (microencapsulation) or confinement in hollow fiber bundles.

2.7.1.2.3 Cross-Linked Enzymes

Derivatization of enzymes using a bifunctional reagent, e. g. glutaraldehyde, can result in cross-linking of the enzyme and, thus, formation of large, still catalytically active insoluble complexes. Such enzyme preparations are relatively unstable for handling and, therefore, are used mostly for analytical work.

2.7.1.2.4 Properties

The properties of an immobilized enzyme are often affected by the matrix and the methods used for immobilization.

Kinetics. As a rule, higher substrate concentrations are required for saturation of an entrapped enzyme than for a free, native enzyme. This is due to a decrease in the concentration gradient which takes place in the pores of the polymer network. Also, there is an increase in the "apparent" *Michaelis* constant for an enzyme bound covalently to a matrix carrying an electrostatic charge. This is also true when the substrate and the functional groups of the matrix carry the same charge. On the other hand, opposite charges bring about an increase of substrate affinity for the matrix. Consequently, this decreases the "apparent" K_m.

pH Optimum. Negatively charged groups on a carrier matrix shift the pH optimum of the covalently bound enzyme to the alkaline region, whereas positive charges shift the pH optimum towards lower pH values. The change in pH optimum of an immobilized enzyme can amount to one to two pH units in comparison to that of a free, native enzyme.

Thermal Inactivation. Unlike native enzymes, the immobilized forms are often more heat stable (cf. example for β-D-glucosidase, Fig. 2.48). Heat stability and pH optima changes induced by immobilization are of great interest in the industrial utilization of enzymes.

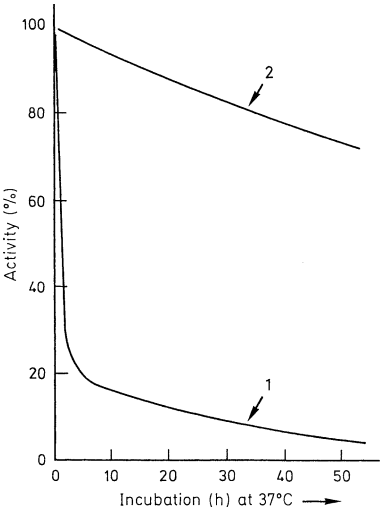


Fig. 2.48. Thermal stabilities of free and immobilized enzymes (according to *Zaborsky*, 1973). 1 β-D-glucosidase, free, 2 β-D-glucosidase, immobilized

2.7.2 Individual Enzymes

2.7.2.1 Oxidoreductases

Broader applications for the processing industry, besides the familiar use of glucose oxidase, are found primarily for catalase and lipoxygenase, among the many enzymes of this group. A number of oxidoreductases have been suggested or are in the experimental stage of utilization, particularly for aroma improvement (examples under 2.7.2.1.4 and 2.7.2.1.5).

2.7.2.1.1 Glucose Oxidase

The enzyme produced by fungi such as *Aspergillus niger* and *Penicillium notatum* catalyzes glucose oxidation by consuming oxygen from the air. Hence, it is used for the removal of either glucose or oxygen (Table 2.20). The H_2O_2 formed in the reaction is occasionally used as an oxidizing agent (cf. 10.1.2.7.2), but it is usually degraded by catalase.

Removal of glucose during the production of egg powder using glucose oxidase (cf. 11.4.3) prevents the *Maillard* reaction responsible for discoloration of the product and deterioration of its whippability. Similar use of glucose oxidase for some meat and protein products would enhance the golden-yellow color rather than the brown color of potato chips or French fries which is obtained in the presence of excess glucose.

Removal of oxygen from a sealed package system results in suppression of fat oxidation and oxidative degradation of natural pigments. For example, the color change of crabs and shrimp from pink to yellow is hindered by dipping them into a glucose oxidase/catalase solution. The shelf life of citrus fruit juices, beer and wine can be prolonged with such enzyme combinations since the oxidative reactions which lead to aroma deterioration are retarded.

2.7.2.1.2 Catalase

The enzyme isolated from microorganisms is important as an auxiliary enzyme for the decomposition of H_2O_2:

$$2\,H_2O_2 \rightleftharpoons 2\,H_2O + O_2 \qquad (2.108)$$

Hydrogen peroxide is a by-product in the treatment of food with glucose oxidase. It is added to food in some specific canning procedures. An example is the pasteurization of milk with H_2O_2, which is important when the thermal process is shut down by technical problems. Milk thus stabilized is also suitable for cheesemaking since the sensitive casein system is spared from heat damage. The excess H_2O_2 is then eliminated by catalase.

2.7.2.1.3 Lipoxygenase

The properties of this enzyme are described under section 3.7.2.2 and its utilization in the bleaching of flour and the improvement of the rheological properties of dough is covered under section 15.4.1.4.3.

2.7.2.1.4 Aldehyde Dehydrogenase

During soya processing, volatile degradation compounds (hexanal, etc.) with a "bean-like" aroma defect are formed because of the enzymatic oxidation of unsaturated fatty acids. These defects can be eliminated by the enzymatic oxidation of the resultant aldehydes to carboxylic acids. Since the flavor threshold values of these acids are high, the acids generated do not interfere with the aroma improvement process.

$$\text{n-Hexanal} + NAD^{\oplus} \rightleftharpoons \text{Caproic acid} + NADH + H^{\oplus} \qquad (2.109)$$

Of the various aldehyde dehydrogenases, the enzyme from beef liver mitochondria has a particularly high affinity for n-hexanal (Table 2.21). Hence its utilization in the production of soya milk is recommended.

2.7.2.1.5 Butanediol Dehydrogenase

Diacetyl formed during the fermentation of beer can be a cause of a flavor defect. The enzyme from *Aerobacter aerogenes*, for example, is able to correct this defect by reducing the diketone to the flavorless 2,3-butanediol:

$$CH_3-CO-CO-CH_3 + NADH + H^{\oplus} \rightleftharpoons CH_3-\underset{OH}{CH}-\underset{OH}{CH}-CH_3 + NAD^{\oplus} \qquad (2.110)$$

Table 2.21. *Michaelis* constants for aldehyde dehydrogenase (ALD) from various sources

Substrate	K_m (μmol/l)			
	ALD (bovine liver)			ALD
	Mitochondria	Cytosol	Microsomes	Yeast
Ethanal	0.05	440	1500	30
n-Propanal	–	110	1400	–
n-Butanal	0.1	< 1	–	–
n-Hexanal	0.075	< 1	< 1	6
n-Octanal	0.06	< 1	< 1	–
n-Decanal	0.05	–	–	–

Such a process is improved by the utilization of yeast cells which, in addition to the enzyme and NADH, contain a system able to regenerate the cosubstrate. In order to prevent contamination of beer with undesirable cell constituents, the yeast cells are encapsulated with gelatin.

2.7.2.2 Hydrolases

Most of the enzymes used in the food industry belong to the class of hydrolase enzymes (cf. Table 2.20).

2.7.2.2.1 Peptidases

The mixture of proteolytic enzymes used in the food industry contains primarily endopeptidases (specificity and classification under section 1.4.5.2). These enzymes are isolated from animal organs, higher plants or microorganisms, i. e. from their fermentation media (Table 2.22). Examples of their utilization are as follows. Proteinases are added to wheat flour in the production of some bakery products to modify rheological properties of dough and, thus, the firmness of the endproduct. During such dough treatment, the firm or hard wheat gluten is partially hydrolyzed to a soft-type gluten (cf. 15.4.1.4.5).

In the dairy industry the formation of casein curd is achieved with chymosin or rennin (cf. Table 2.20) by a reaction mechanism described under section 10.1.2.1.1. Casein is also precipitated through the action of other proteinases by a mechanism which involves secondary proteolytic activity resulting in diminished curd yields and lower curd strength. Rennin is essentially free of other undesirable proteinases and is, therefore, especially suitable for cheesemaking. However, there is a shortage of rennin since it has to be isolated from the stomach of a suckling calf. However, it is now possible to produce this enzyme using genetically engineered microorganism. Proteinases from *Mucor miehei, M. pusillus* and *Endothia parasitica* are a suitable replacement for rennin.

Plant proteinases (cf. Table 2.22) and also those of microorganisms are utilized for ripening and tenderizing meat. The practical problem to be solved is how to achieve uniform distribution of the enzymes in muscle tissue. An optional method appears to be injection of the proteinase into the blood stream immediately before slaughter, or rehydration of the freeze-dried meat in enzyme solutions.

Cold turbidity in beer is associated with protein sedimentation. This can be eliminated by hydrolysis of protein using plant proteinases (cf. Table 2.22). Utilization of papain was suggested by *Wallerstein* in 1911. Production of complete or partial protein hydrolysates by enzymatic methods is another example of an industrial use of proteinases. This is used in the liquefaction of fish proteins to make products with good flavors.

One of the concerns in the enzymatic hydrolysis of proteins is to avoid the release of bitter-tasting peptides and/or amino acids (cf. 1.2.6 and 1.3.3). Their occurrence in the majority of proteins treated (an exception is collagen) must be expected when the molecular weight of the peptide fragments falls below 6000. Bitter-tasting peptides, e. g., those which are formed in the ripening of cheese, can be converted to a hydrolyzate which is no longer bitter by adding a mixture of endo- and exopeptidases from Latobacilli.

2.7.2.2.2 α- and β-Amylases

Amylases are either produced by bacteria or yeasts (Table 2.20) or they belong to the components of malt preparations. The high temperature-resistant bacterial amylases, particularly those of *Bac. licheniformis* (Fig. 2.49) are of interest for the hydrolysis of corn starch (gelatinization at 105–110 °C). The hydrolysis rate of these enzymes can be enhanced further

Table 2.22. Peptidases (proteinases) utilized in food processing

Name	Source	pH optimum	Optimal stability pH range
	A. Peptidases of animal origin		
Pancreatic proteinase[a]	Pancreas	9.0[b]	3–5
Pepsin	Gastric lining of swine or bovine	2	
Chymosin	Stomach lining of calves or genetically engineered microorganisms *B. Peptidases of plant origin*	6–7	5.5–6.0
Papain	Tropical melon tree (*Carica papaya*)	7–8	4.5–6.5
Bromelain	Pineapple (fruit and stalk)	7–8	
Ficin	Figs (*Ficus carica*)	7–8	
	C. Bacterial peptidases		
Alkaline proteinases e. g. subtilisin	*Bacillus subtilis*	7–11	7.5–9.5
Neutral proteinases e. g. thermo-lysin	*Bacillus thermoproteolyticus*	6–9	6–8
Pronase	*Streptomyces griseus*		
	D. Fungal peptidases		
Acid proteinase	*Aspergillus oryzae*	3.0–4.0[d]	5
Neutral proteinase	*Aspergillus oryzae*	5.5–7.5[d]	7.0
Alkaline proteinase	*Aspergillus oryzae*	6.0–9.5[d]	7–8
Proteinase	*Mucor pussillus*	3.5–4.5[d]	3–6
Proteinase	*Rhizopus chinensis*	5.0	3.8–6.5

[a] A mixture of trypsin, chymotrypsin, and various peptidases with amylase and lipase as accompanying enzymes.
[b] With casein as a substrate.
[c] A mixture of various endo- and exopeptidases including amino- and carboxypeptidases.
[d] With hemoglobin as as substrate.

by adding $Ca^{2\oplus}$ ions. α-Amylases added to the wort in the beer production process accelerate starch degradation. These enzymes are also used in the baking industry (cf. 15.4.1.4.8).

2.7.2.2.3 Glucan-1,4-α-D-Glucosidase (Glucoamylase)

Glucoamylase cleaves β-D-glucose units from the non-reducing end of an 1,4-α-D-glucan. The α-1,6-branching bond present in amylo-pectin is cleaved at a rate about 30 times slower than the α-1,4-linkages occurring in straight chains. The enzyme preparation is produced from bacterial and fungal cultures. The removal of transglucosidase enzymes which catalyze, for example, the transfer of glucose to maltose, thus lowerung the yield of glucose in the starch saccharification process, is important in the production of glucoamy-lase. The starch saccharification process is illustrated in Fig. 2.50. In a purely enzymatic process (left side of the figure), the swelling and gelatinization and liquefaction of starch can occur in a single step using heat-stable bacterial α-amylase (cf. 2.7.2.2.2). The action of amylases

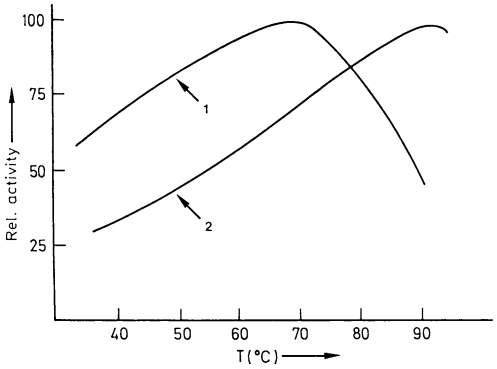


Fig. 2.49. The activity of α-amylase as influenced by temperature. 1 α-amylase from *Bacillus subtilis*, 2 from *Bacillus licheniformis*

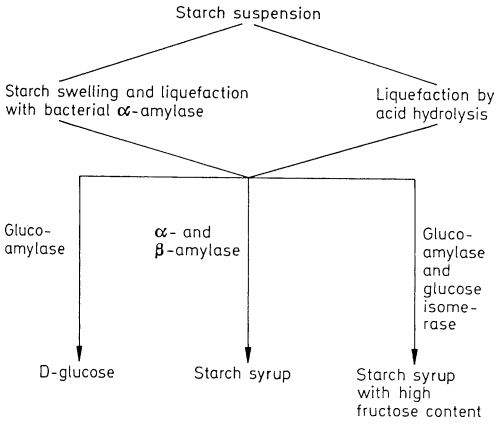


Fig. 2.50. Enzymatic starch degradation

yields starch syrup which is a mixture of glucose, maltose and dextrins (cf. 19.1.4.3.2).

2.7.2.2.4 Pullulanase (Isoamylase)

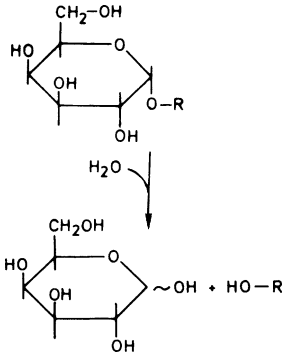
Pullulanase (cf. 4.4.5.1.4) is utilized in the brewing process and in starch hydrolysis. In combination with β-amylase, it is possible to produce a starch sirup with a high maltose content.

2.7.2.2.5 Endo-1,3(4)-β-D-Glucanase

In the brewing process, β-glucans from barley increase wort viscosity and impede filtration. Enzymatic endo-hydrolysis reduces viscosity.

2.7.2.2.6 α-D-Galactosidase

This and the following enzymes (up to and including section 2.7.2.2.9) attack the non-reducing ends of di-, oligo- and polysaccharides with release of the terminal monosaccharide. The substrate specificity is revealed by the name of the enzyme, e. g., α-D-galactosidase:



(2.111)

In the production of sucrose from sugar beets (cf. 19.1.4.1.2), the enzymatic preparation from *Mortiella vinacea* hydrolyzes raffinose and, thus, improves the yield of granular sugar in the crystallization step. Raffinose in amounts >8% effectively prevents crystallization of sucrose.
Gas production (flatulence) in the stomach or intestines produced by legumes originates from the sugar stachyose (cf. 16.2.5). When this tetrasaccharide is cleaved by α-D-galactosidase, flatulency from this source is eliminated.

2.7.2.2.7 β-D-Galactosidase (Lactase)

Enzyme preparations from fungi (*Aspergillus niger*) or from yeast are used in the dairy industry to hydrolyze lactose. Immobilized enzymes are applied to produce milk suitable for people suffering from lactose malabsorption. Milk treated in this way can also be used to make products like skim milk concentrate or ice cream, thus avoiding interference by lactose due to its low solubility.

2.7.2.2.8 β-D-Fructofuranosidase (Invertase)

Enzyme preparations isolated from special yeast strains are used for saccharose (sucrose) inver-

sion in the confectionery or candy industry. Invert sugar is more soluble and, because of the presence of free fructose, is sweeter than saccharose.

2.7.2.2.9 α-L-Rhamnosidase

Some citrus fruit juices and purées (especially those of grapes) contain naringin, a dihydrochalcone with a very bitter taste. Treatment of naringin with combined preparations of α-L-rhamnosidase and β-D-glucosidase yields the nonbitter aglycone compound naringenin (cf. 18.1.2.5.4).

2.7.2.2.10 Cellulases and Hemicellulases

The baking quality of rye flour and the shelf life of rye bread can be improved by partial hydrolysis of the rye pentosans. Technical pentosanase preparations are mixtures of β-glycosidases (1,3- and 1,4-β-D-xylanases, etc.).
Solubilization of plant constituents by soaking in an enzyme preparation (maceration) is a mild and sparing process. Such preparations usually contain exo- and endo-cellulases, α- and β-mannosidases and pectolytic enzymes (cf. 2.7.2.2.13). Examples of the utilization are: production of fruit and vegetable purées (mashed products), disintegration of tea leaves, or production of dehydrated mashed potatoes. Some of these enzymes are used to prevent mechanical damage to cell walls during mashing and, thus, to prevent excessive leaching of gelatinized starch from the cells, which would make the purée too sticky.
Glycosidases (cellulases and amylases from *Aspergillus niger*) in combination with proteinases are recommended for removal of shells from shrimp. The shells are loosened and then washed off in a stream of water.

2.7.2.2.11 Lysozyme

The cell walls of gram-positive bacteria are formed from peptidoglycan (synonymous with murein). Peptidoglycan consists of repeating units of the disaccharide N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) connected by β-1,4-glycosidic linkages, a tetrapeptide and a pentaglycine peptide bridge. The NAG and NAM residues in peptidoglycan alternate and form the linear polysaccharide chain.
Lysozyme (cf. 11.2.3.1.4) solubilizes peptidoglycan by cleaving the 1,4-β-linkage between NAG and NAM. Combination preparations containing both lysozyme and nisin (cf. 1.3.4.3) are recommended for the preservation of meat preparations, salad dressings and cheese preparations. They are more effective than the components.

2.7.2.2.12 Thioglucosidase

Proteins from seeds of the mustard family (*Brassicaceae*), such as turnip, rapeseed or brown or black mustard, contain glucosinolates which can be enzymatically decomposed into pungent mustard oils (esters of isothiocyanic acid, R−N=C=S). The oils are usually isolated by steam distillation. The reactions of thioglycosidase and a few glucosinolates occurring in *Brassicaceae* are covered in section 17.1.2.6.5.

2.7.2.2.13 Pectolytic Enzymes

Pectolytic enzymes are described in section 4.4.5.2. Pectic acid which is liberated by pectin methylesterases flocculates in the presence of Ca^{2+} ions. This reaction is responsible for the undesired "cloud" flocculation in citrus juices. After thermal inactivation of the enzyme at about 90 °C, this reaction is not observable. However, such treatment brings about deterioration of the aroma of the juice. Investigations of the pectin esterase of orange peel have shown that the enzyme activity is affected by competitive inhibitors: oligogalacturonic acid and pectic acid (cf. Fig. 2.51). Thus, the increase in turbidity of citrus juice can be prevented by the addition of such compounds.
Pectinolytic enzymes are used for the clarification of fruit and vegetable juices. The mechanism of clarification is as follows: the core of the turbidity causing particles consists of carbohydrates and proteins (35%). The prototropic groups of these proteins have a positive charge at the pH of fruit

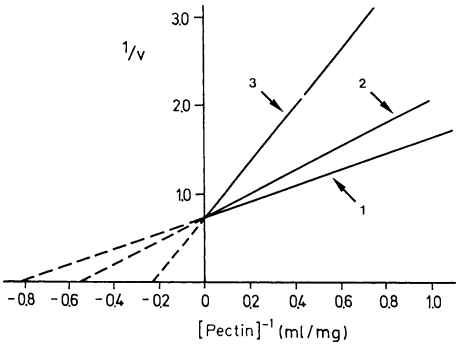


Fig. 2.51. Pectin esterase (orange) activity as affected by inhibitors (according to *Termote*, 1977). 1 Without inhibitor, 2 hepta- and octagalacturonic acids, 3 pectic acid

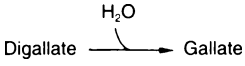
juice (3.5). Negatively charged pectin molecules form the outer shell of the particle. Partial pectinolysis exposes the positive core. Aggregation of the polycations and the polyanions then follows, resulting in flocculation. Clarification of juice by gelatin (at pH 3.5 gelatin is positively charged) and the inhibition of clarification by alginates which are polyanions at pH 3.5 support this suggested model.
In addition, pectinolytic enzymes play an important role in food processing, increasing the yield of fruit and vegetable juices and the yield of oil from olive fruits.

2.7.2.2.14 Lipases

The mechanism of lipase activity is described under section 3.7.1.1. Lipase from microbial sources (e. g. *Candida lipolytica*) is utilized for enhancement of aromas in cheesemaking.
Limited hydrolysis of milk fat is also of interest in the production of chocolate milk. It enhances the "milk character" of the flavor.
The utilization of lipase for this commodity is also possible.
Staling of bakery products is retarded by lipase, presumably through the release of mono- and diacylglycerols (cf. 15.4.4). The defatting of bones, which has to be carried out under mild conditions in the production of gelatin, is facilitated by using lipase-catalyzed hydrolysis.

2.7.2.2.15 Tannases

Tannases hydrolyze polyphenolic compounds (tannins):

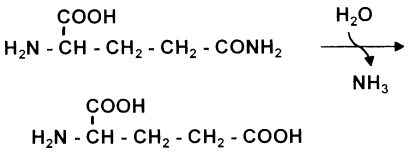


(2.112)

For example, preparations from *Aspergillus niger* prevent the development of turbidity in cold tea extracts.

2.7.2.2.16 Glutaminase

This enzyme catalyzes the hydrolysis of glutamine (Formula 2.113). For meat preparations, the addition of an enzyme preparation from *Bacillus subtilis* is under discussion. It increases the concentration of glutamic acid, which substantially contributes to the taste of meat.



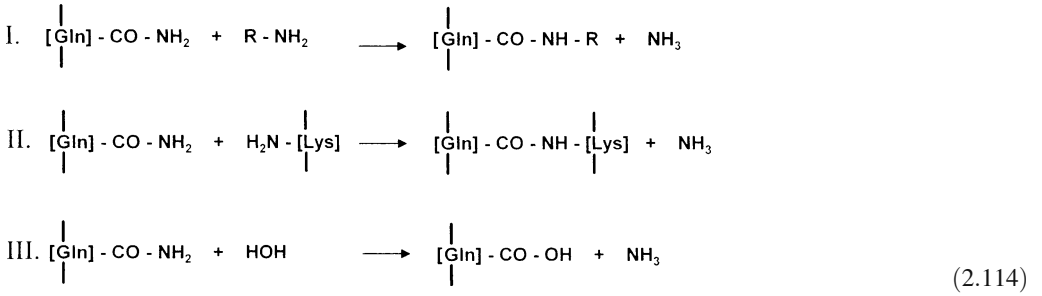
(2.113)

2.7.2.3 Isomerases

Of this group of enzymes, glucose isomerse, which is used in the production of starch syrup with a high content of fructose (cf. 19.1.4.3.5), is very important. The enzyme used industrially is of microbial origin. Since its activity for xylose isomerization is higher than for glucose, the enzyme is classified under the name "xylose isomerase" (cf. Table 2.4).

2.7.2.4 Transferases

Protein glutamine-γ-glutamyl transferase (transglutaminase, TGase) catalyzes the acyl transfer between the γ-carboxyamide group of peptide-bound glutamine (acyl donor) and primary amines (acyl acceptor, I in Formula 2.114), e. g., peptide-bound lysine (II in Formula 2.114). Free



(2.114)

acid amides and amino acids also react. Proteins or peptides are cross linked in this way. If amines are absent, TGase can catalyze the deamination of glutamine residues in proteins with H_2O as the acyl acceptor (III in Formula 2.114).

TGases play an important role in the metabolism of animals and plants. For the production of protein gels (cf. 1.4.6.3.3), the TGase from the actinomycete *Streptoverticillum mobaraense* is of special interest. In contrast to the TGases from mammals, the activity of this enzyme, which is released in large amounts by the microorganisms into the nutrient medium, does not depend on $Ca^{2\oplus}$. This enzyme consists of 331 amino acids (M_r: 37,842) of known sequence. A cysteine residue is probably at the active center. The pH optimum of TGase activity is between 5 and 8. This enzyme can also be used at low temperatures and is rapidly denatured at 70 °C. Proteins are cross linked by the formation of ε-(γ-glutamyl)lysine isopeptide bonds. However, the biological availability of lysine is not appreciably reduced. The viscoelastic properties of the resulting protein gels depend not only on the type of proteins and the catalytic conditions (TGase concentration, pH, temperature, time), but also on the pretreatment of the protein, e. g., heat denaturation.

Possible applications of TGase in the production of food are shown in Table 2.23.

Table 2.23. Possible applications of transglutaminase

Raw material	Application
Meat	Restructured meat from small pieces Partial replacement of cutter aids in the production of boiling sausage ("Brühwurst").
Fish	Production of fish gel (surimi, cf. 13.1.6.11) Reducing water loss in the thawing of frozen fish.
Milk	Texture control of low-fat yoghurt to produce the palate feeling of a whole-fat product Increasing the solubility of casein in the presence of $Ca^{2\oplus}$ ions or at a lower pH, e. g., for beverages. Cross linking of casein with whey proteins to increase the protein yield in cheese making.
Wheat	"Hardening" of soft wheat flour for the production of pasta.

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