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Faculty of Nature and Life Sciences
Department of Molecular and Cellular Biology

Advanced Enzymology

Course handout

Third-year Bachelor's degree in Biochemistry

UEF 3.1.1

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Ref……

Preface

This handout provides advanced enzymology teaching material for third-year undergraduate biochemistry students. This module aims to consolidate and deepen knowledge in general biochemistry, while introducing advanced concepts related to the function, regulation, and applications of enzymes in various fields.

The aim of this course is to provide students with a detailed understanding of enzymes at the structural, kinetic, and functional levels, with a particular emphasis on protein-protein and protein-ligand interactions, as well as on different kinetic models (Michaelian, multi-substrate, and allosteric enzymes). It also aims to introduce students to experimental approaches and the applications of enzyme engineering in various industrial and biotechnological sectors.

This handout is prepared in accordance with the official syllabus recommended by the Ministry of Higher Education and Scientific Research. It is structured into several chapters organized progressively:

The first chapter presents general information about enzymes.

The second chapter deals with the structure and properties of enzymes, including monomeric enzymes, oligomeric enzymes, isoenzymes and multi-enzyme complexes.

The third chapter is devoted to protein-ligand interactions, detailing the different modes of association.

The fourth chapter develops the basics of enzyme kinetics, including Michaelis-Menten kinetics and multi-substrate systems.

The fifth chapter addresses the functioning and regulation of allosteric enzymes as well as methods for determining kinetic constants.

The sixth chapter describes the mechanisms of enzymatic catalysis, including the study of active centers, coenzymes, and certain specific enzymatic mechanisms.

The seventh chapter presents the techniques for isolating and purifying enzymes, as well as their methods of study.

Finally, the last chapter is devoted to enzyme engineering, in particular to methods of enzyme immobilization and their numerous applications in biotechnology, including biosensors and artificial enzymes.

This document aims to support the student in acquiring in-depth knowledge of enzymology and developing their scientific analytical skills, while highlighting current issues and application prospects of enzymes.

Table of contents

Chapter V: Functioning and regulation of allosteric enzymes

Chapter VI: Mechanism of enzyme catalysis

Chapter VII: Isolation and purification of enzymes

Chapter VIII: Enzyme engineering

List of figures

List of tables

Chapter I: General overview of enzymes

1. Definitions

1.1. Enzyme

An enzyme is a protein that exhibits specific catalytic properties of a chemical reaction in the metabolism of the living organism that produces it.

Enzymatic proteins are catalysts, meaning that by acting at very low concentrations, they increase the rate of chemical reactions without altering the outcome. At the end of the reaction, the enzyme's structure remains unchanged.

Enzymatic proteins are synthesized by living organisms. This synthesis is genetically determined: its conservation in the genome is favored by the need that this living organism has to carry out this reaction.

In nature, there are non-protein biocatalysts such as Ribozymes (catalysts made up of RNA) and DNAzymes (DNA-based catalysts). They are also called catalytic nucleic acids.

1.2. Substrate

A molecule that enters a reaction to be transformed through the catalytic action of an enzyme.

1.3. Product

A molecule that appears during a reaction catalyzed by an enzyme.

1.4. Ligand

A chemical compound that has a specific bond with an enzyme.

All molecules that bind specifically to a protein are called ligands. For each ligand, there is at least one binding site on the protein that receives it.

1.5. Cofactor

Chemical body necessarily involved in an enzymatic reaction to transport or complete a substrate, to accept a product or as a participant in the structure of the enzyme.

1.6. Coenzymes

They are biological molecule acting as an essential cofactor in the enzymatic catalysis of a reaction. We distinguish:

-Free coenzymes: participate in the reaction stoichiometrically. They are called free coenzymes because they dissociate from the enzyme at each catalyzed reaction.

-Bound coenzymes: participate in the reaction in a catalytic manner. They are called bound coenzymes because they do not dissociate from the enzyme.

2. Enzyme Nomenclature

2.1. The code number

The old nomenclature consisted of adding the suffix "ase" to the name of the enzyme's substrate of the enzyme. However, this nomenclature quickly became outdated due to the large number of enzyme (general and imprecise). For these reasons, the International Union of Biochemistry and Molecular Biology (IUBMB) proposed a classification open enough to include enzymes recognized subsequently (*http://www.chem.qmul.ac.uk/iubmb/enzyme/index.html*. This classification was established based on criteria of specificity (catalyzed reaction).

All currently known enzymes are classified under a number consisting of four digits separated by periods and preceded by E.C. "Enzyme Commission" written as (E.C. X1.X2.X3.X4). The meaning of the numbers is as follows:

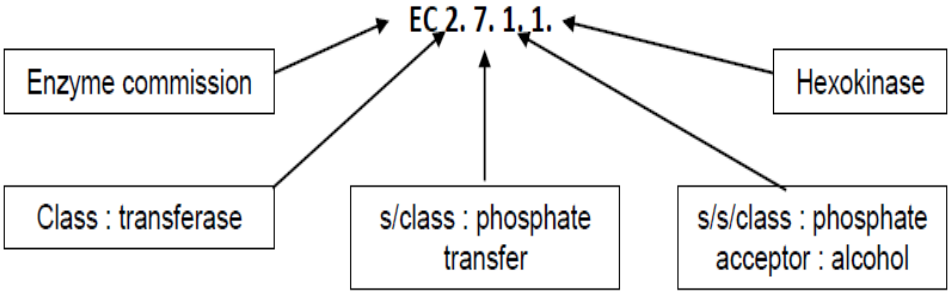
1: The type of reaction (class)

2: The type of function of the metabolized substrate (subclass)

3: The type of acceptor (the sub-subclass)

4: The order number (in the sub-subclass).

Example :



2.2. Systematic name

He states: -the nature of the donor

-the type of catalyzed reaction

2.3. Recommended common name

It is a simple name established by usage.

Example:

Code number: E.C.2.7.1.2.

2: It's a transferase

7: The transferred group contains phosphorus.

1: The acceptor group has alcohol reactivity

2: This is the number of the glucokinase in the series defined by the first three numbers.

Systematic name: ATP-D-Glucose-6-PhosphoTransferasse

Common name: Glucokinase.

- **The 7 classes of enzymes**

1. Oxidoreductases

They catalyze reactions where electrons are transferred, with or without protons. These enzymes work with various coenzymes (NAD+, FAD, …) which behave as intermediate and transient electron acceptors.

Examples:

-alcohol dehydrogenase: ethanol + NAD+ → ethanol + NADH,H+

-lactate dehydrogenase: L-lactate + NAD+ → pyruvate + NADH,H+

2. Transferases

They catalyze the transfer of groups from one molecule to another.

Examples:

-Phosphofructokinase: D-fructose-6-P + ATP →D-fructose-1,6-di-P + ADP

-Alanine aminotransferase: L-alanine + 2-oxo-glutarate →pyruvate + L-glutamate

3. Hydrolases

They catalyze the hydrolysis of various bonds (esters, glycosidic compounds, etc.)

Example:

-amylase: starch →dextrins →maltose

-Trypsin: hydrolyzes peptide bonds of the Arg-Y or Lys-Y type with Y ≠ Pro

4. Lyases

They catalyze the movement of a group from a substrate with the appearance of a double bond on that substrate.

Example:

-Fumarase: L-malate →fumarate + H2O

5. Isomerases

They catalyze configuration reversals.

Examples:

-glucose-6-phosphate isomerase: D-glucose-6-P →D-fructose-6-P

-glucose epimerase: β-D-glucose ↔α-D-glucose

6. Ligases or synthetases

They catalyze synthesis reactions where energy is generally supplied by ATP:

Examples:

-butyryl-CoA ligase: butyrate + HS—CoA + ATP →butyryl-CoA + AMP + PPi

-Glutamine Ammonium ligase: L-glutamate + ATP + NH4+ →L-glutamine + ADP + Pi

7. Translocases

This class was added in September 2018. These enzymes catalyze the translocation of protons (H^+), inorganic cations and anions, amino acids and peptides, carbohydrates and their derivatives. These enzymes catalyze the movement of molecules or ions across cellular membranes in general.

Key Points

- Enzymes are biological catalysts that accelerate biochemical reactions without being consumed.

- The substrate is the molecule transformed by the enzyme, whereas the product is the molecule formed during the reaction.
- A ligand is any molecule that binds specifically to an enzyme.
- Many enzymes require cofactors, including free or tightly bound coenzymes, to exert their catalytic activity.
- The IUBMB enzyme nomenclature assigns each enzyme a four-level EC number that precisely describes the reaction catalyzed.
- Enzymes are classified into seven major classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, and translocases.
- This classification reflects the diversity of catalytic mechanisms and the essential role of enzymes in cellular metabolism.

Chapter II: Structure and properties of enzymes

1. Structure of enzymes

1.1. Structure and conformation of enzymes

Enzymes are globular proteins, composed of the 20 standard amino acids and consist of one or more units. Moreover, their catalytic activity depends directly on their three-dimensional structure. Several structural levels of organization can be distinguished:

-**Primary structure:** a chain of amino acids following a defined sequence

-**Secondary structure:** conformation preferentially adopted by the polypeptide chain (e.g., α helix, β-sheet…)

-**Tertiary structure:** organization in space, through the various foldings of the chain, into a compact structure that gives the molecule its biological properties

-**Quaternary structure:** proteins are said to have a quaternary structure if they contain two or more polypeptide chains. Such proteins are called oligomers, and the individual chains that constitute them are called monomers or subunits. The different subunits of an oligomeric protein may be identical or different. The quaternary structure is stabilized by weak bonds (hydrogen, hydrophobic, electrostatic) and, more rarely, by disulfide bridges (Figure 01).

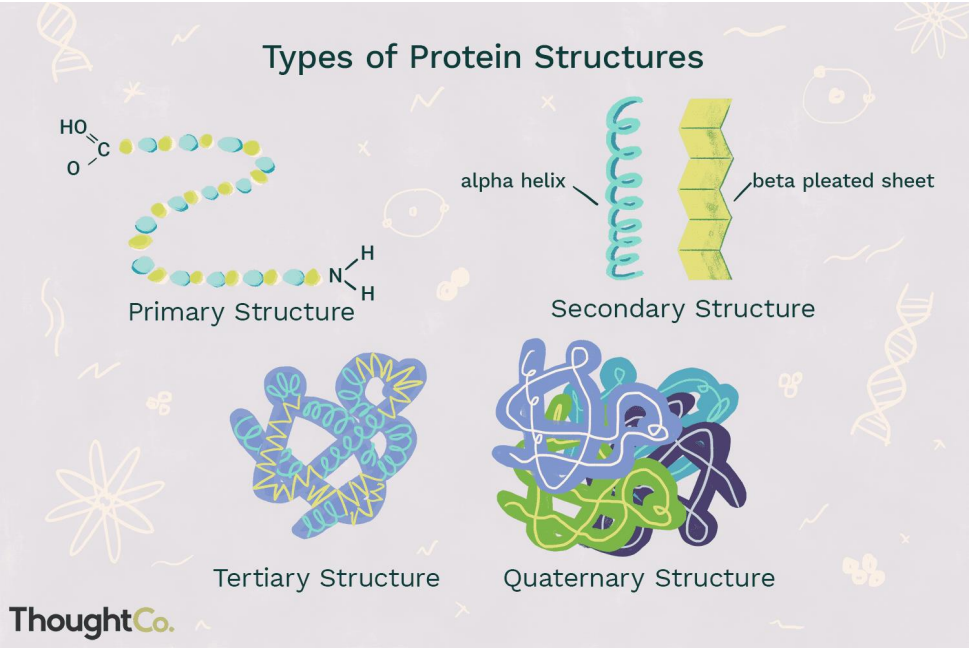


Figure 01: Levels of protein structure (Nelson & Cox, 2021).

Some enzymes are monomeric holoproteins (e.g., ribonuclease with 124 amino acid residues, M = 13700 Da, Trypsin, Chymotrypsin, urease…) or oligomeric (e.g., lactate dehydrogenase with 4 protomers, M = 150,000 Da).

Others are heteroproteins; the protein part (apoenzyme) is not sufficient to ensure catalysis alone; they require the presence of a cofactor (such as a metal ion) or a coenzyme (a small non-protein molecule ± attached to the apoenzyme).

Note

An active enzymatic protein is therefore the association of two elements: apoenzyme (inactive) and cofactor.

The catalytically active enzyme cofactor complex is callede: Holoenzyme.

1.2. Monomeric enzymes

These are enzymes made up of a single polypeptide chain, meaning they consist of a single functional unit arranged in a tertiary structure (they do not have a quaternary structure).

Example: Chymotrypsin (E.C.3.4.4.5.)

It is a serine protease whose structure has been known since 1969. It is synthesized by the pancreas. The bovine form contains 245 amino acids. It hydrolyzes peptide bonds whose carboxyl group (COOH) belongs to aromatic amino acids (tyrosine and phenylalanine). It is stereo-selective, meaning it preferentially acts on the L-form. Its main characteristic is its kinetic pattern. It exhibits Michaelis-Menten kinetics.

1.3. Oligomeric enzymes

An oligomeric enzyme is a protein formed by the association of several polypeptide chains (functional units). Each subunit corresponds to a single polypeptide and forms a protomer. The quaternary structure results from the association of protomers into an oligomer. They have a high molecular weight, the number of units is even, the subunits are linked together by ionic or hydrogen bonds, and they exhibit allosteric kinetics.

Example: *E. coli* β-galactosidase

It hydrolyzes lactose, and it is a medical diagnostic enzyme used to detect coliform bacteria capable of hydrolyzing lactose. *E. coli* β-galactosidase comprises four identical subunits with a molecular weight of approximately 105 kDa (over 900 amino acids). In other

species, the subunits range from 80 to 100 kDa; when these subunits are separated, they become inactive.

1.4. Active site

The active site (or active center) of an enzyme is a small, preferred region of the enzyme protein, whose geometry has considerable importance on the specificity, it is located in a hydrophobic zone in the internal part of the enzyme structure.

It performs two functions: substrate binding (binding site) and substrate transformation (catalytic site). Only a few amino acids are directly involved in catalysis (contact amino acids). The other amino acids are classified as helpers, collaborators, or non-collaborators depending on their degree of involvement in the catalytic process.

The amino acids that make up the active site are most often those with functional groups (OH, SH, NH_2, COOH).

Examples: cysteine, histidine, serine, lysine and tyrosine.

1.5. Isoenzymes (LDH)

Isoenzymes are enzymes that catalyze the same reaction on the same substrate, but have different structures, distinct kinetic properties and a specific tissue distribution.

- ***Lactate dehydrogenase*** **(E.C.1.1.1.27.)**

It is the best-known and most studied example. It is an enzyme that reversibly transforms pyruvic acid into lactic acid.

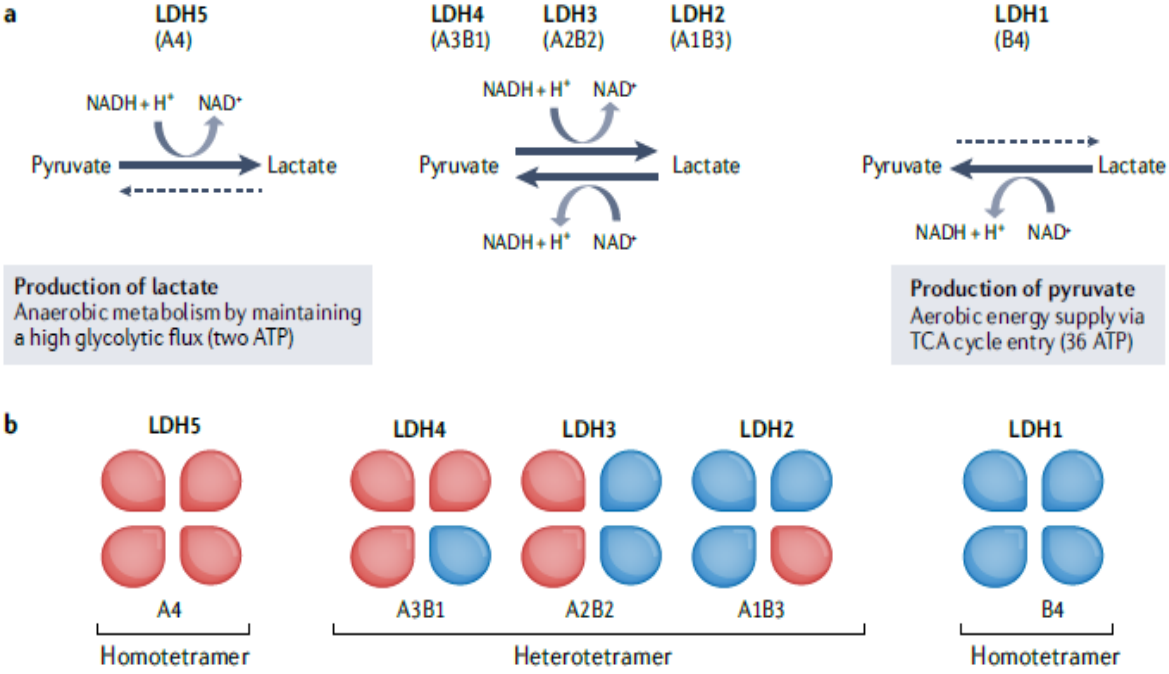
Tetrameric LDH has two types of protomers (H and M: M4, M3H, M2H2, MH3, and H4), resulting in five isoenzymes that can be separated by electrophoresis. The distribution varies depending on the organ (H predominates in the heart, M in muscle).

LDH isoenzymes are used in clinical biochemistry. In pathology, during cell lysis, intracellular enzymes are released and deposited into the bloodstream at abnormal levels.

The nature of LDH isoenzymes can provide interesting information about the origin of this cell lysis (Figure 02).

- Liver: M4
- Muscles: M4+++ and M3H
- Red blood cells: M2H2

- Heart: MH3 and H4
- Rein: the 5 meet at roughly the same rate.



c

	LDH5	LDH4	LDH3	LDH2	LDH1
Expressed in	• Liver • Skeletal muscle • Kidney (medulla)	• Liver • Skeletal muscle • Kidney (medulla)	• Lymphoid tissue • Lung • Platelets • Brain	• Heart • Red blood cells • Kidney (cortex)	• Heart • Red blood cells • Germ cells
Non-malignant serum (% of activity)	8–20%	9–15%	18–25%	27–37%	17–27%
Related diseases	• Liver diseases • Skeletal muscle diseases • Lung cancer • Advanced-stage CRC	• Breast cancer • Advanced-stage melanoma • Advanced-stage CRC	• Leukaemia • Breast cancer • Advanced-stage melanoma	• Haemolytic diseases • Non-Hodgkin lymphoma • Lymphocytic leukaemia • Myeloproliferative syndrome	• Myocardial infarction • Haemolytic anaemia • Ovarian cancer • Testicular cancer

Figure 02: Structure and function of human lactate dehydrogenase isoenzymes (Claps et al., 2022).

1.6. Multienzyme complexes (FAS)

Multi-enzyme complexes are organized assemblies of several enzymes that catalyze successive steps of the same metabolic pathway.

The classic example is the fatty acid synthase (FAS) complex, a multifunctional structure where several enzymatic activities are carried by a single protein (in eukaryotes) or by a set of subunits (in prokaryotes).

This system enables the sequential catalysis of fatty acid elongation reactions from acetyl-CoA and malonyl-CoA, while maintaining the intermediates covalently bound to an acyl carrier protein (ACP). The complex organization promotes increased catalytic efficiency through the phenomenon of "substrate channeling," limiting the diffusion of intermediates and protecting them from side reactions (Figure 03).

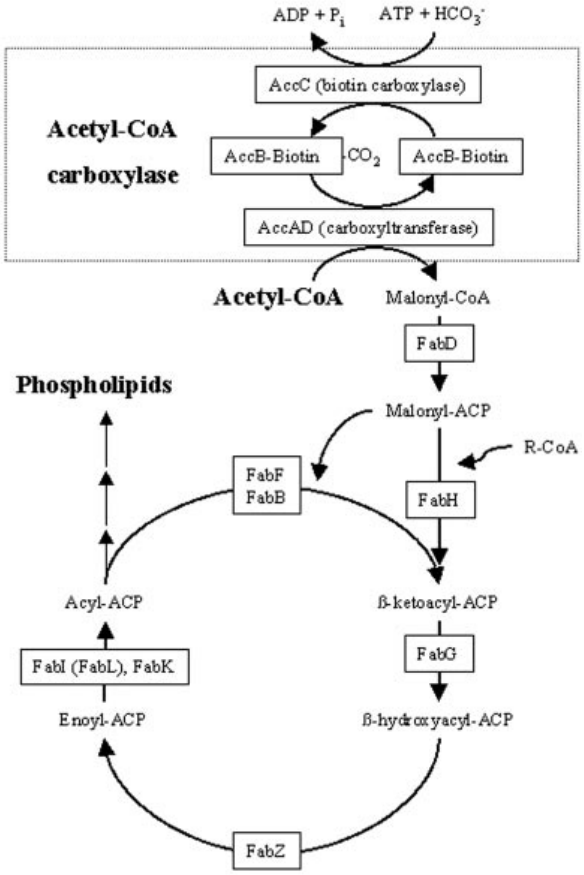


Figure 03: Bacterial fatty acid synthesis pathway (FAS) (Freiberg et al., 2004).

2. General properties of enzymes

2.1. Enzyme efficiency

Enzyme efficiency suggests close contact between the enzyme and its substrate. The efficiency is quantitatively expressed by the molecular enzymatic activity, defined as this is the number of moles of substrate transformed per minute per mole of enzyme. Enzymatic

activity can be assessed colorimetrically by monitoring, as a function of time, either the decrease in substrate concentration or the formation of the reaction product.

International Unit (IU) or unit of enzyme activity: this is the amount of enzyme capable of transforming one micromole of the substrate per minute at 25ºC.

The specific activity: it is the number of units of enzymatic activity per milligram of enzyme protein. It measures the purity of the enzyme and increases with the degree of purification.

The katale: it is the amount of enzyme that transforms one mole of substrate per second.

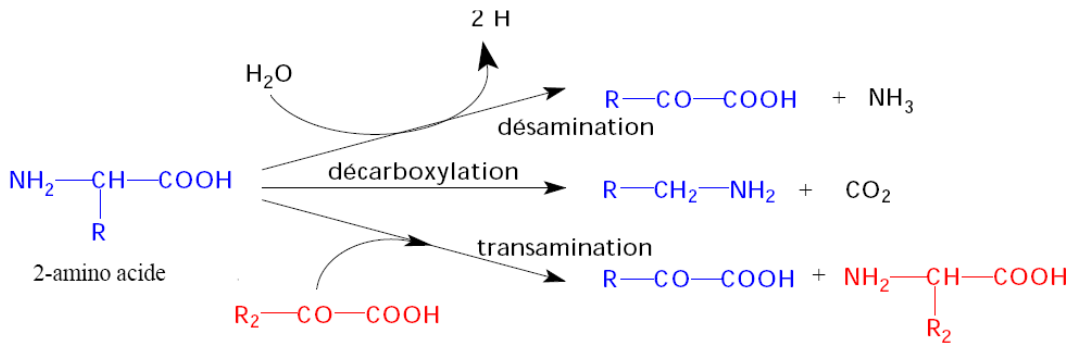
The enormity of this unit leads to the use of microkatal, or nanokatal: **1 IU = 16.67 nkat.**

2.2. Specificity

- **Specificity with respect to the type of reaction**

An enzyme can only perform one type of reaction: hydrolysis, methyl transfer…

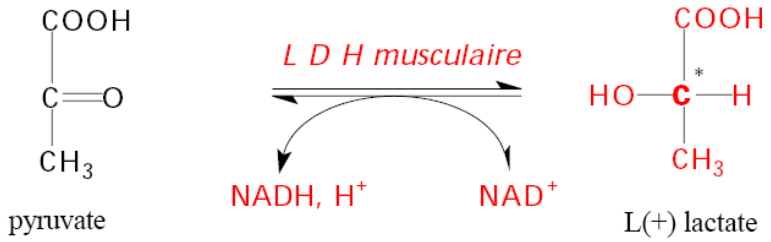
Based on the specificity of action, seven categories of enzymes are distinguished.



- **Specificity with respect to the substrate**

An enzyme can only act on a given substrate (but a substrate can have several enzymes).

Specificity depends on the spatial configuration and optical activity of the substrate (α or β anomer, D or L series), this is referred to as stereospecificity.



2.3. Denaturation

It consists of breaking all intra-chain bonds (labile bonds) of the protein without disturbing the peptide bonds. The different structures of the enzyme (helix, sheet, etc.) are stabilized by non-covalent bonds such as hydrogen bonds, hydrophobic bonds, etc. These bonds can be broken by various factors or denaturing agents (Figure 04).

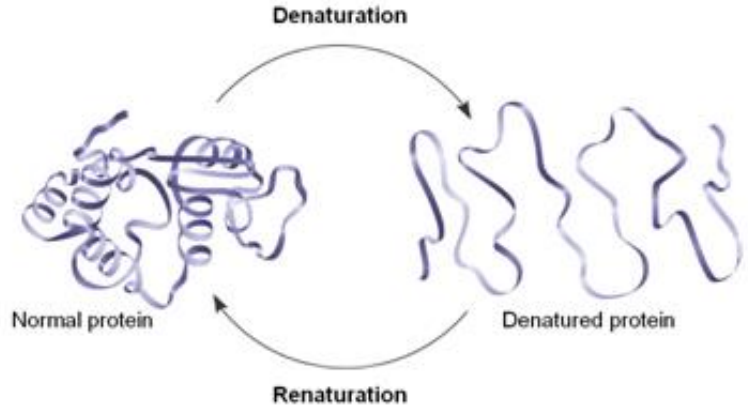


Figure 04: Protein denaturation-renaturation (Reece et al., 2012).

2.4. Reversibility and irreversibility of enzymatic reactions

A reaction is said to be reversible (equilibrium) when it can occur in both directions at the same rate. An equilibrium reaction is characterized by an equilibrium constant (Keq) calculated as follows:

The following reaction is possible:

$$A + B \underset{K_2}{\overset{k1}{\rightleftharpoons}} C+D$$

$$V1 = K1[A][B]$$

V2 = K2[C][D]

At equilibrium: V1 = V2, therefore, K1[A][B] = K2[C][D]

$$\frac{K1}{K2} = \frac{[C][D]}{[A][B]} = Keq$$

Maintaining equilibrium depends on the variation of free energy.

$$\Delta G = \Delta G'0 + RTLn(\frac{[C][D]}{[A][B]})$$

ΔG: change in free energy

ΔG'0: variation of free energy under standard conditions (1M, Tº=25º)

R: ideal gas constant (8.314 J/mol/K), (we have 1 cal = 4.18 J)

T: absolute temperature (T (Cº) + 273)

Ln: natural logarithm

When equilibrium is reached: ΔG = 0.

2.5. Activation energy

Activation energy (Ea) is the energy that reactant molecules must absorb in order to react; therefore, it represents the potential energy barrier that must overcome for the reaction to take place.

Thus, the enzyme increases the rate of a reaction by decreasing the activation energy (Ea).

It lowers the energy barrier that the substrate must overcome; therefore it facilitates the formation of the transition state (activated complex).

Therefore, the enzyme provides an alternative reaction pathway that is more accessible than the one in its absence (Figure 05).

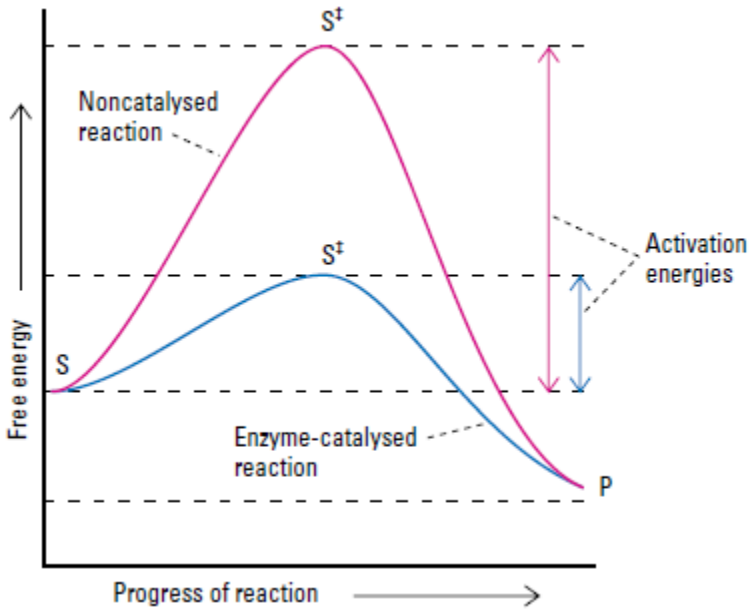


Figure 05: Energy profiles of non-catalysed and enzyme-catalysed reactions **(**Papachristodoulou et al., 2018).

2.6. Physical and chemical effectors of enzymatic activity

The rate of an enzymatic reaction depends on the concentration of the enzyme, the concentration of the substrate present in the reaction medium, and the presence or absence of effectors.

A. Temperature

Thermal agitation facilitates overcoming of the activation energy barrier, thereby explaining the increase in enzymatic reaction rates with rising temperature. However, temperature variations influence enzymatic systems through two distinct mechanisms: an effect on the kinetic constants of the reaction, which enhances reaction rates, and a denaturing effect on the enzymatic protein that can lead to its inactivation.

Each enzyme is characterized by an optimal temperature at which its catalytic activity is maximal, typically around 37 °C in mesophilic organisms, but higher in thermophilic bacteria. Beyond this optimum, particularly above 40 °C, the three-dimensional structure of the enzyme undergoes significant perturbations. Destabilization of the tertiary structure results in thermal denaturation, generally irreversible, leading to loss of function and a marked decline in enzymatic activity (Figure 06).

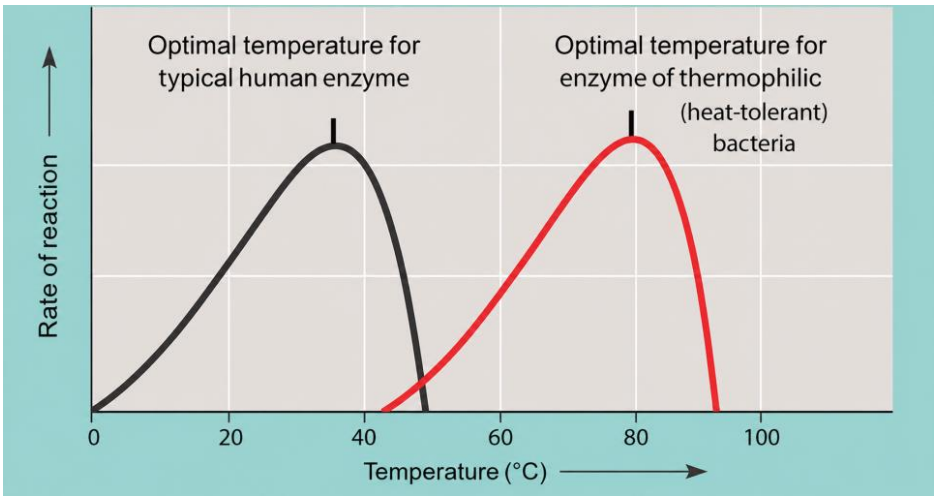


Figure 06: Effect of temperature on enzyme activity (Urry et al., 2021).

B. pH

Enzymes are proteins composed of amino acids bearing chemical groups that are sensitive to pH variations. These functional groups can undergo protonation or deprotonation depending on the pH of the surrounding medium. Consequently, pH affects several factors, including the ionization state of enzyme residues as well as that of the substrate and/or product, and the integrity of the protein's tertiary structure, thereby influencing enzyme stability (Figure 07).

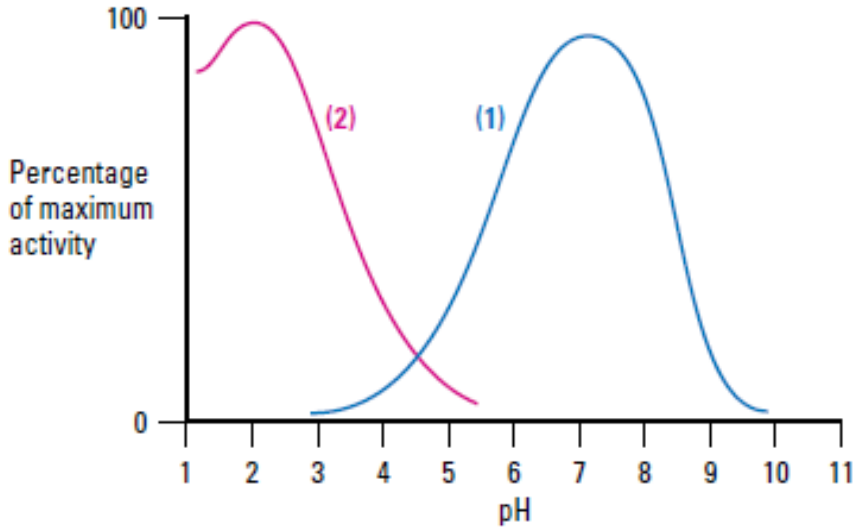


Figure 07: Effect of pH on enzyme activity **(**Papachristodoulou et al., 2018).

Curve 1 is typical of the majority of enzymes with maximal activity near physiological pH. Curve 2 represents pepsin, an exceptional case, since the enzyme functions in the acidic stomach contents.

C. Inhibitions

An inhibitor reduces enzyme activity by binding to the enzyme in a manner that alters substrate and/or catalytic turnover rate. From an experimental perspective, enzyme inhibition can provide valuable informations on enzyme mechanism of action as well as their substrate specificity.

D. Activators

An activator increases the reaction rate of the enzyme. They are later recycled in metabolism.

Key Points

- Enzymes are globular proteins whose activity depends on their three-dimensional structure (primary, secondary, tertiary, and sometimes quaternary structure).
- An active enzyme (holoenzyme) consists of an apoenzyme associated, when required, with a cofactor or coenzyme.
- Enzymes may be monomeric or oligomeric; oligomeric enzymes possess a quaternary structure and often exhibit allosteric regulation.
- The active site comprises a substrate-binding site and a catalytic site; only a few amino acid residues participate directly in catalysis.
- Isoenzymes catalyze the same reaction but differ in structure, kinetic properties, and tissue distribution.
- Multienzyme complexes catalyze consecutive reactions with high efficiency through substrate channeling.
- Enzymatic activity is expressed in international units (IU), specific activity, or katal.
- Enzymes exhibit a dual specificity: for the type of reaction catalyzed and for the substrate.
- Denaturation disrupts the weak interactions responsible for the enzyme's conformation and generally results in loss of activity.
- Enzymes lower the activation energy without altering the thermodynamic equilibrium of the reaction.
- Temperature, pH, inhibitors, and activators modulate enzymatic activity; each enzyme has its own optimal operating conditions.

Chapter III: *Protein-ligand interactions*

Quantifying the ligand binding sites on a protein generally requires measuring the concentration of ligand bound to the protein at equilibrium:

1. Association on a single site

Either :

$$P + L \underset{K_2}{\overset{k1}{\rightleftharpoons}} PL$$

P: Protein

L: Ligand

PL: Protein-Ligand complex

k1: Association rate constant

k2: Rate constant of dissociation

We have:

- Association speed: v1 = k1 . [P] . [L]
- Dissociation rate: v2 = k2. [PL]

At equilibrium, the velocities are equal, we obtain:

v1 = v2 therefore, k1 . [P] . [L]=k2. [PL]

We obtain:

$$\frac{[P][L]}{[PL]} = \frac{K2}{K1} = \text{KD (Dissociation equilibrium constant)}$$

$$\frac{[PL]}{[P][L]} = \frac{K1}{K2} = \text{KA (Association equilibrium constant)}$$

We have: [P]total = [P] + [PL] therefore [P] = [P]total - [PL]

$[P]_{total}$: molar concentration of total protein.

Substituting into K_D's equation, we obtain:

$$KD = \frac{[P][L]}{[PL]} = \frac{K2}{K1} = \frac{([P]t - [PL])[L]}{[PL]}$$

K_D.[PL] = [P]t.[L] - [PL].[L]

[PL].(K_D + [L]) = = [P]t.[L]

$[\mathrm{PL}] = \frac{[\mathrm{P}]t.[\mathrm{L}]}{\mathrm{KD}+[\mathrm{L}]}$ $\frac{[\mathrm{L}]}{\mathrm{KD}+[\mathrm{L}]}$= saturation function

(Michaelis saturation is named by analogy with the Michaelis–Menten equation, which describes the rate of an enzymatic reaction).

2. Association across n equivalent and independent sites

A protein may have several binding sites for a ligand; these sites are:

- **Independent:** binding of the ligand to one site is independent of the occupancy of the other sites.
- **Equivalent:** each site has the same dissociation constant (K_D) for the ligand.

If a protein has n binding sites, we obtain:

$$nP + nL \underset{k2}{\overset{k1}{\rightleftharpoons}} nPL$$

The reaction rate: at equilibrium V1 = V2. Therefore V=0:

V = k1[P]n[L]n – k2[PL]n

[P]t = [P] + [PL] = nPt

[P] = n Pt – [PL]

Substituting into the K_D equation, and after calculation, we obtain:

$$[\mathrm{PL}] = \frac{\mathrm{n}[\mathrm{P}]t.[\mathrm{L}]}{\mathrm{KD}+[\mathrm{L}]}.$$

3. Graphical representations

Linearization methods can be used to determine the parameters that characterize the binding of the ligand to the receptor.

Indeed, there are two most commonly used representations:

3.1. Scatchard's representation

A transformation of the saturation equation resulting in the equation:

$$KD = \frac{(n[P]t - [PL])[L]}{[PL]}$$

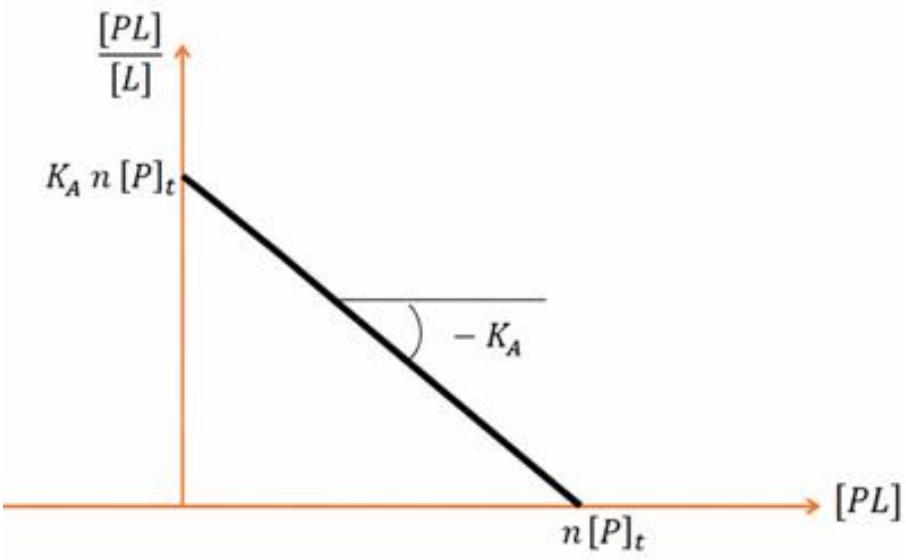
$$\frac{[L]}{[PL]} = \frac{KD}{n[P]t - [PL]}$$

$$\frac{[PL]}{[L]} = \frac{n[P]t - [PL]}{KD}$$

So,

$$\frac{[PL]}{[L]} = \frac{-[PL]}{KD} + \frac{n[P]t}{KD}$$

The graphical representation of [PL]/[L] = f([PL]) is a linear regression:



Therefore, K_D is determined by the slope of the linear regression: **slope** = $-\frac{\mathbf{1}}{\boldsymbol{KD}} = -\boldsymbol{K}\mathbf{A}$

The point of intersection of the line with the x-axis: y = 0, therefore x = n[P]t

The point of intersection of the line with the y-axis: x= 0 therefore $y = \frac{\text{n[P]t}}{\text{KD}}$

3.2. Representation of Klötz

In order to accurately calculate the parameters K_D and n, a linear representation was proposed by Klötz; in this case, it involves transforming the saturation equation into double inverses:

We have:

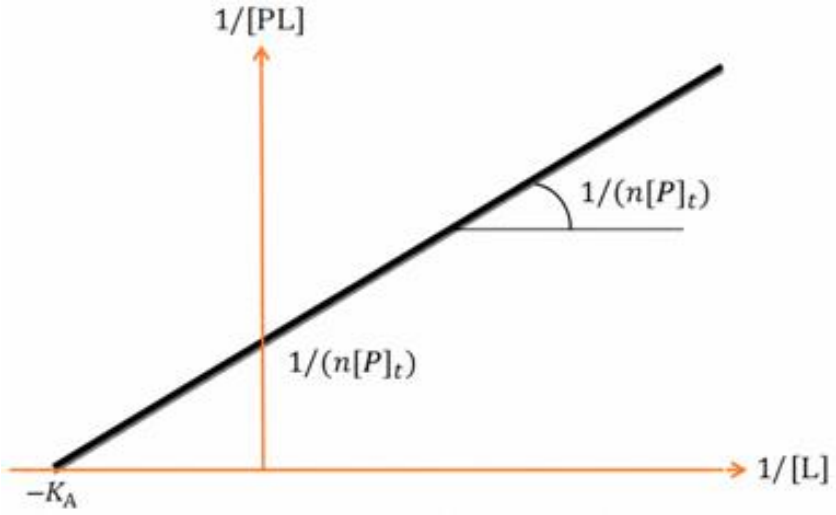
$$[\mathrm{PL}] = \frac{\mathrm{n[P]t\,[L]}}{\mathrm{KD} + [\mathrm{L}]}$$

$$\frac{1}{[PL]} = \frac{\mathrm{KD}}{\mathrm{n[P]t\,[L]}}\frac{1}{\mathrm{n[P]t}}$$

$$\frac{1}{[PL]} = \frac{KD}{n[P]t} * \frac{1}{[L]} + \frac{1}{n[P]t}$$

$$\frac{1}{[PL]} = \frac{KD}{n[P]t} * \frac{1}{[L]} + \frac{1}{n[P]t}$$

The graphical representation of 1/[PL] = f(1/[L]) is a linear regression:



X = 0 $\Longleftrightarrow$ $y = \frac{1}{\mathrm{n[P]t}}$

Y=0 $\Longleftrightarrow$ $x = -\frac{1}{\mathrm{KD}}$

4. Association of a ligand on two different sites

In some enzymatic proteins, the same ligand can bind to two different types of sites with distinct (non-equivalent) affinities. These are called heterogeneous sites, generally with one site of high affinity, occupied even at low ligand concentrations, and a site of low affinity, occupied as the concentration increases.

These sites are considered independent, so ligand binding to one does not affect the other. Total binding is simply the sum of bindings to each type of site. A Scatchard plot reveals a

progressive binding pattern, with an initial phase dominated by high-affinity sites, followed by a second phase involving lower-affinity sites.

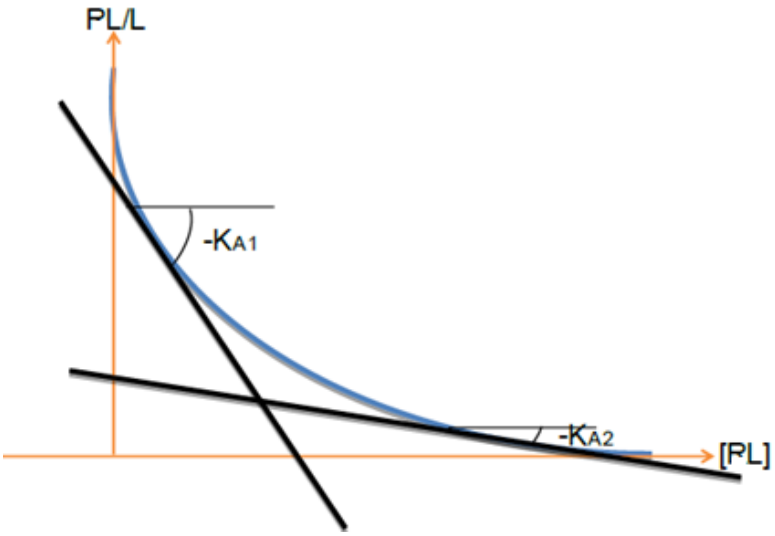


Figure 08: Scatchard plot for association of a ligand on two different sites (Bobrovnik, 2002).

This model explains the behavior of many enzymes and proteins, particularly those involved in biological regulation. It is important to distinguish it from cooperativity, because here the sites do not interact with each other, despite the apparent complexity of the binding.

Key Points

- For a single binding site: $KD = \frac{=[P][L]}{[PL]}$ and $KA = \frac{1}{KD}$. A low K_D value indicates high affinity.
- The degree of saturation follows a hyperbolic relationship: $[PL] = \frac{[P]t.[L]}{KD+[L]}$
- For n independent and equivalent binding sites: $[PL] = \frac{n[P]t.[L]}{KD+[L]}$.
- The Scatchard plot allows estimation of K_D (from the slope) and the number of binding sites (from the intercepts).
- The Klötz double-reciprocal plot also provides estimates of K_D and n by linear regression.
- Some proteins possess heterogeneous binding sites with different affinities; in such cases, the total binding is the sum of the binding to each site type, without necessarily implying cooperativity.

Chapter IV: *Enzyme kinetics*

1. Michaelis-Menten kinetics with a single substrate (reminder)

1.1. Definition of enzyme kinetics

It is the study of reaction rates and their modifications in response to changes in experimental conditions.

1.2. The different phases of the enzymatic reaction

The different phases of the enzymatic reaction are represented in the figure below (Figure 09).

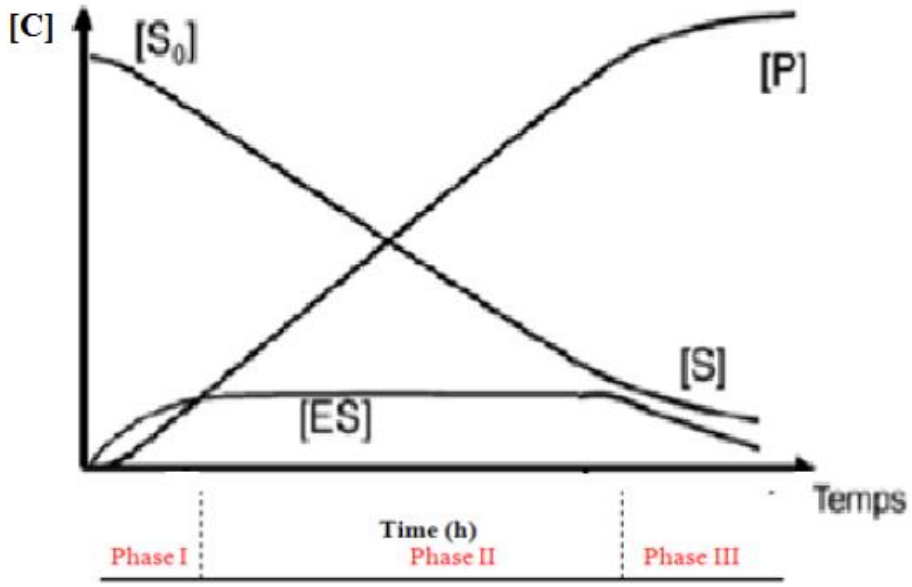


Figure 09: Variation of enzyme (E), substrate (S), product (P) and enzyme-substrate complex (ES) concentrations in relation to reaction time **(**Vitolo, 2020).

Different phases	Pre-stationary	Stationary	Stationary Post
Features	Enzyme in the presence of excess substrate Very fast ES combination	Enzyme saturated by the substrate Combination ES is at maximum concentration Constant The reaction rate is constant: Initial rate (Remains constant as long as the substrate is at a saturating concentration of the enzyme)	Significant decrease in S after a period of time that varies depending on the enzyme

1.3. Definition of the rate of an enzymatic reaction

The speed (V) of an enzymatic reaction is defined by:

- The quantity of substrate metabolized per unit time $V = \frac{-d[S]}{dt}$
- Or by the quantity of product formed per unit time $V = \frac{d[P]}{dt}$

1.4. Concept of initial velocity

The rate of the enzymatic reaction increases with substrate concentration until reaching the maximum rate Vmax (enzyme saturation by S).

The initial velocity is defined as the reaction rate measured at the very beginning of the reaction. Because the pre-stationary phase is very brief; this initial rate is generally taken to be equal to the stationary phase rate, during which the concentration of the enzyme-substratee complex remains essentially constant.

- **Effect of substrate concentration**

Enzyme activity varies greatly depending on the substrate concentration. For a constant enzyme concentration [E], the product concentration [P] can be measured in function of time t for increasing values of substrate concentration [S].

At low substrate concentrations, product formation is slow because the enzyme rarely encounters substrate molecules, and the reaction rate is proportional to substrate concentration.

At high substrate concentrations, enzyme-substrate encounters become more frequent, resulting in a higher reaction rate than at low substrate levels. Under these conditions, the reaction rate becomes independent of substrate concentration and approaches a constant value, the maximal velocity (Vmax). Further increases in substrate concentration do not increase Vmax, indicating that the enzyme is saturated with substrate.

Accordingly, the plot of enzymatic activity as a function of substrate concentration exhibits a hyperbolic shape (Figure 10).

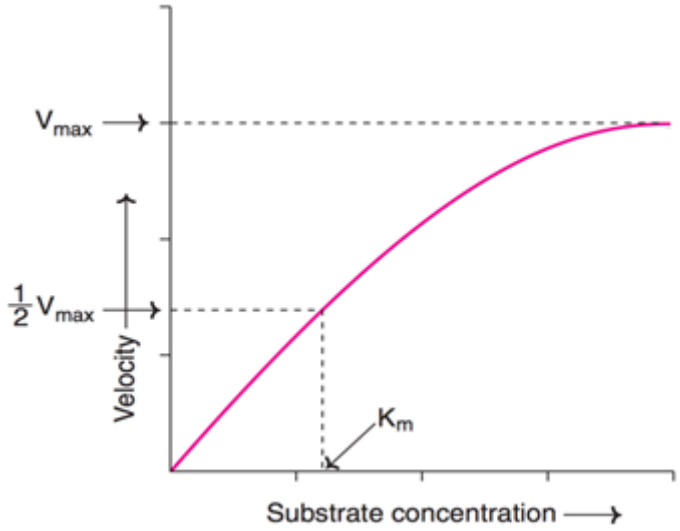


Figure 10: Effect of substrate concentration on the rate enzymatic reaction (Mehler, 2014).

- **Effect of enzyme concentration**

The higher the enzyme cocentration [E], the faster the reaction; therefore, the initial velocity (Vi) is directly proportional to [E].

However, if the amount of the substrate is limited, there will be more enzyme than substrate so adding more enzymes will have no impact on the rate of reaction (Figure 11).

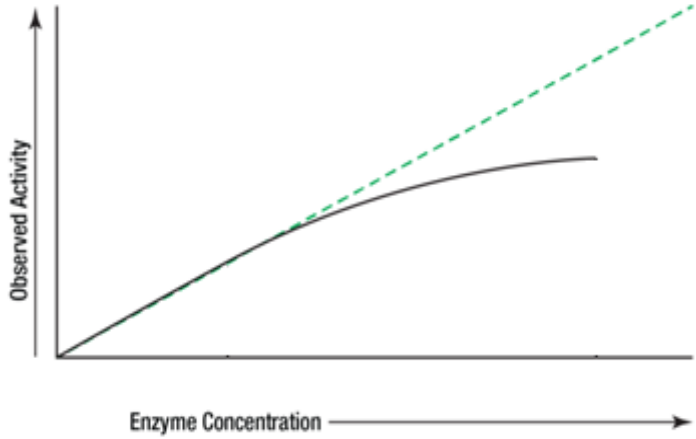


Figure 11: The variation of the reaction rate with enzyme concentration (Yassin, 2024).

1.5. Michaelis-Menten Hypothesis

Consider the following reaction:

$$E + S \underset{k2}{\overset{k1}{\rightleftarrows}} ES \xrightarrow{k3} E + P$$

Formation of the ES complex: *(Rapid and reversible step)*

$$E + S \underset{v2}{\overset{v1}{\leftrightarrows}} ES$$

Dissociation of the ES complex: (Disappearance of S, appearance of P and regeneration of E)

$$ES \xrightarrow[v3]{} E + P$$

1.5.1. Michaelis' constant

According to the law of mass action:

V1 = k1 [E][S] and V2 = k2 [ES]

Rate of appearance of products: $\frac{d[P]}{dt}$ = V3 = k3.[ES]

The rate of disappearance of the substrates is equal to the rate of appearance of the product:

$$\frac{-d[S]}{dt} = \frac{d[P]}{dt}$$

The rate of substrate disappearance: $\frac{-d[S]}{dt}$ = V1 - V2

V1-V2= V3

k1. [E].[S]= (k2 + k3).[ES]

$$\frac{k2 + k3}{k1} = \frac{[E].[S]}{[ES]} = Km$$

Km: Dissociation constant of the ES complex Michaelis constant

The value of the km is higher when the:

- dissociation of the ES complex is strong
- And therefore, the enzyme's affinity for its substrate is low.

1.5.2. The Michaelis equation

[Et]: Concentration of the enzyme present in the system [E]=[Et]-[ES]

$$Km = \frac{[E].[S]}{[ES]} = \frac{([Et] - [ES])\ [S]}{[ES]}$$

$$Km + [S] = \frac{[Et][S]}{[ES]}$$

$$[ES] = \frac{[Et][S]}{Km + [S]}$$

V = rate of the enzymatic reaction = V3 therefore, V = V3 = k3 [ES]

$$V = \frac{k3.[Et]\ .[S]}{Km + [S]}$$

The speed is at its maximum when [ES] is at its greatest possible value:
When the entire enzyme is combined with the substrate [Et] = [ES], Vm = K3 . [Et]

$$v = \frac{Vm.[S]}{Km + [S]}$$

1.5.3. Interpretation and interests

If $v = \frac{Vm}{2}$ we obtain Km= [S].

(When the reaction rate is equal to half the maximum rate, **Km= [S]**).

Km: reflect of the stability of the ES complex
High Km: weak binding.
Low Km: strong binding.

1.6. Meaning of the Vmax and Km constants

Km (Michaelis constant): affinity indicator

Km represents the dissociation constant of the [ES] complex, that is, the inverse of the enzyme's affinity for the substrate. The smaller Km is, the greater the enzyme's affinity for the substrate.

Vmax (maximum speed): indicator of catalytic concentration

Vmax corresponds to the maximum initial rate of the reaction, reached when all enzymes (total E) is present in the form [ES]. In this situation, the substrate is in large excess relative to the enzyme, and no active sites remain unoccupied.

It is a quantity proportional to the catalytic capacity of the enzyme and reflects its ability to catalyse the reaction. Thus, it can be used to express the catalytic activity of the enzyme.
Vmax also allows the calculationof the enzyme's catalytic constant (kcat) according to the relationship:

$$Vmax = kcat \times [E_{total}].$$

The specificity constant (Ksp)

This constant reflects the overall specificity of an enzyme for a substrate. It is given by the following formula:

$$Ksp = \frac{Kcat}{\mathrm{Km}}$$

This constant allows the selection of the best substrate(s) for an enzyme; in this case, the highest value should be sought.

1.7. Graphic representations

Experimentally, it is not possible to determine Vmax exactly; only an approximate can be obtained. However, both Vmax and Km can be recovered using various mathematical methods.

The most commonly used method to linearize the Michaelis equation is the Lineweaver-Burk representation, expressed as 1/v = f(1/[S]). This transformation converts the hyperbolic relationship into a straight line (Figure 12).

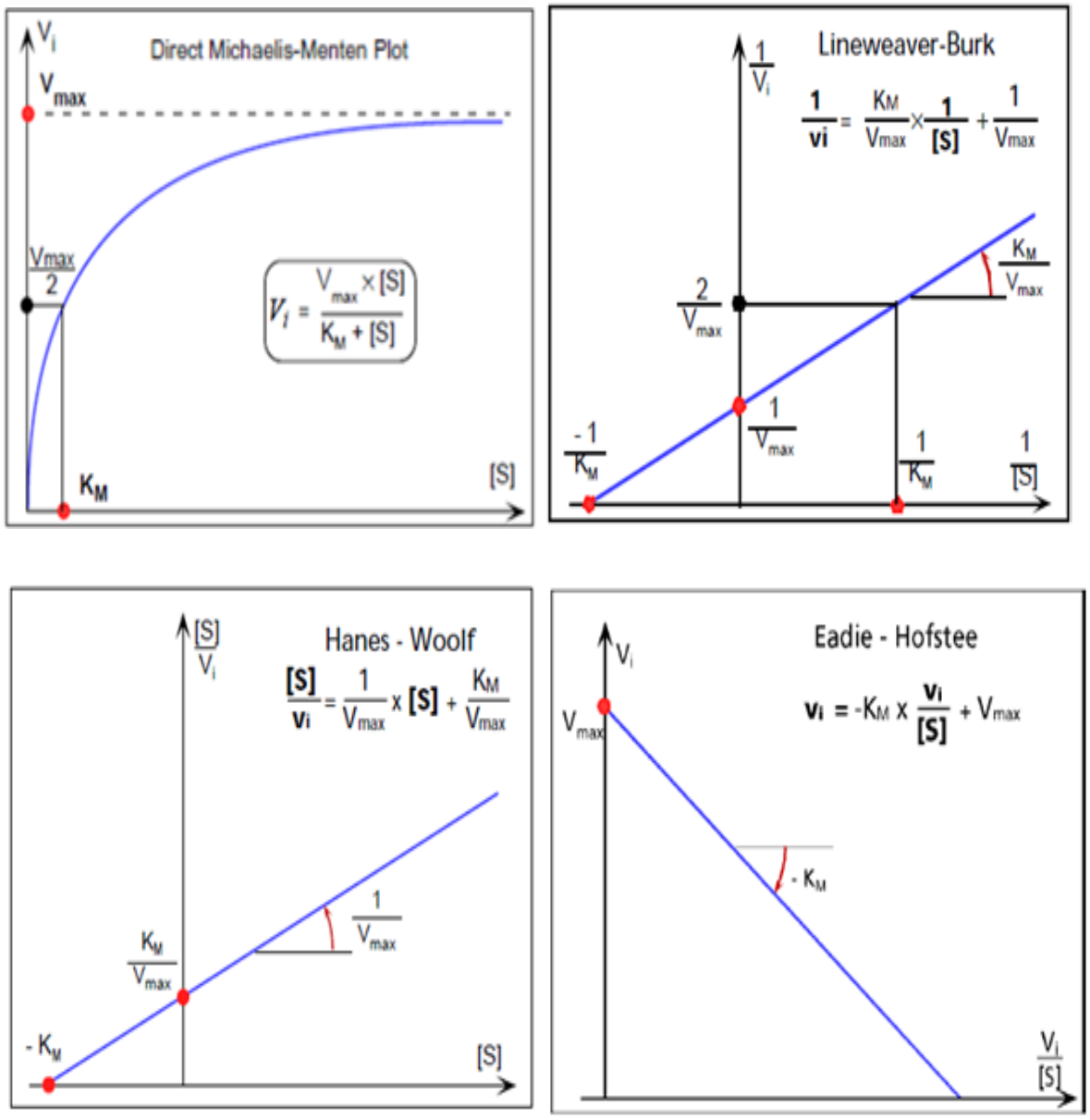


Figure 12: Graphical representations of the Michaelis-Menten equation (Bhagavan & Ha, 2015).

1.8. Enzyme inhibitors

1.8.1. Definition

An enzyme inhibitor is any effector which, by binding to the enzyme, modifies the behavior of that enzyme.

They represent an essential control mechanism of biological systems and also constitute a source of information to better understand the mechanism of action of enzymes.

Enzyme inhibitors can be classified into two main categories according to their mode of action.

Irreversible inhibitors: They bind permanently to the enzyme and act rapidly by causing its denaturation, resulting in a permanent loss of its activity.

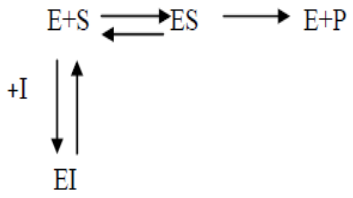
Reversible inhibitors: They disrupt enzyme kinetics and can slow down or stop the reaction, but their effect can be reversed under certain reaction conditions, which makes them of great interest for the detailed study of the molecular mechanisms of catalysis.

Among reversible inhibitions, three main types are distinguished: competitive, non-competitive and uncompetitive inhibition.

1.8.2. Competitive inhibitors

Competitive inhibitors have a structural similarity to the substrate and compete with it to bind to the enzyme's active site.

They decrease the reaction rate by reducing the proportion of enzymes bound to the substrate. However, at high substrate concentrations, the substrate can compete with inhibitory molecules for binding to the active site, displacing them from the catalytic centers.



The velocity equation is as follows:

$$v = \frac{Vm.[\mathrm{S}]}{\mathrm{Km}(1+\frac{[\mathrm{I}]}{\mathrm{Ki}})+[\mathrm{S}]}$$

And:

$$\frac{1}{v} = \frac{\mathrm{Km}}{\mathrm{Vm}}\left(1+\frac{[\mathrm{I}]}{\mathrm{Ki}}\right).\frac{1}{[\mathrm{S}]}+\frac{1}{\mathrm{Vm}}$$

The graphical representations give (Figure 13):

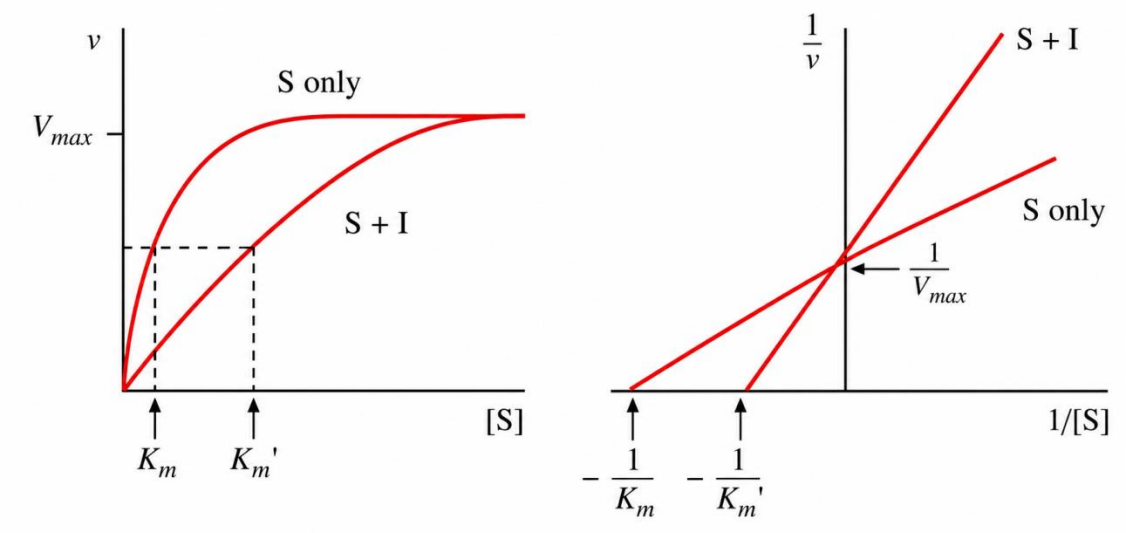


Figure 13: Graphical representations of competitive inhibition (Bhagavan & Ha, 2015).

We observe that the maximum velocity does not change. On the other hand, Km increases by a factor equal to $\left(1+\frac{[I]}{Ki}\right)$.

Example of competitive inhibition

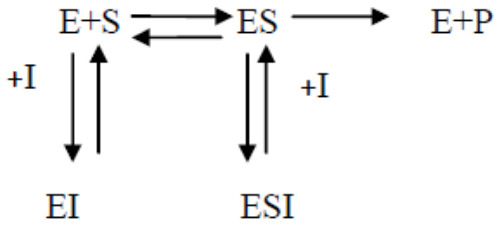
Succinate dehydrogenase catalyzes the dehydrogenation of succinic acid to fumaric acid. This enzyme is competitively inhibited by malonic acid, which structurally resembles succinic acid. Due to this similarity, the enzyme cannot effectively distinguish between succinate and malonate.

In this case, both the substrate (succinate) and the inhibitor (malonate) compete for binding at the active site, resulting in a reduction of enzymatic activity.

This type of inhibition can be reversed by increasing the substrate concentration, thereby allowing substrate molecules to outcompete the inhibitor for access to the active site.

1.8.3. Non-competitive inhibitors

The non-competitive inhibitor binds reversibly to a site different from the enzyme's active site. Thus, the enzyme can bind to the inhibitor, the substrate, or both. However, once the inhibitor is bound, the enzyme becomes inactive, regardless of the substrate's presence. The effect of this type of inhibition cannot be reversed by increasing the substrate concentration, as the inhibitor reduces the amount of active enzyme, resulting in a decrease in the maximum reaction velocity.



The velocity equation is as follows:

$$v = \frac{Vm}{(1 + \frac{[I]}{Ki})} \cdot \frac{[S]}{Km + [S]}$$

And:

$$\frac{1}{v} = \frac{Km}{Vm}\left(1 + \frac{[I]}{Ki}\right).\frac{1}{[S]} + \frac{1}{Vm}\left(1 + \frac{[I]}{Ki}\right)$$

The graphical representations give (Figure 14):

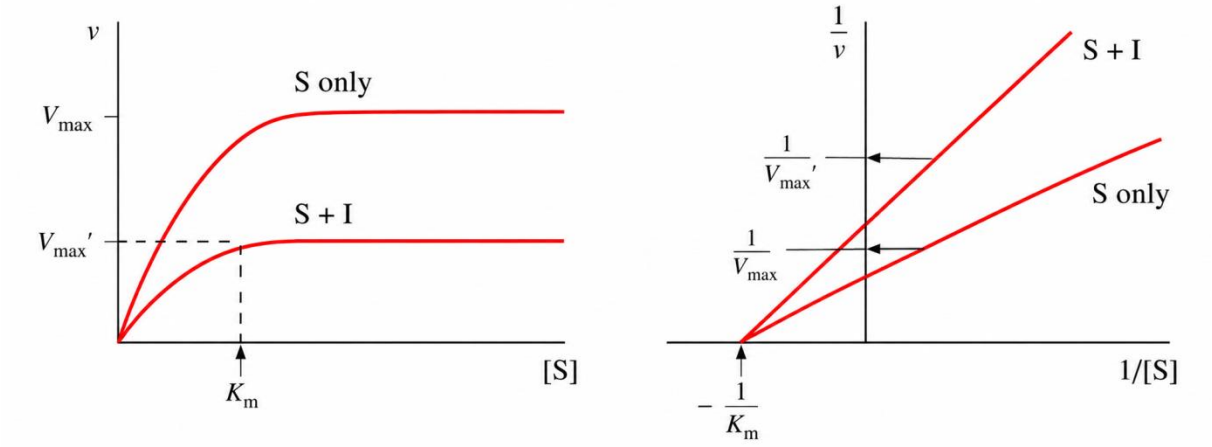
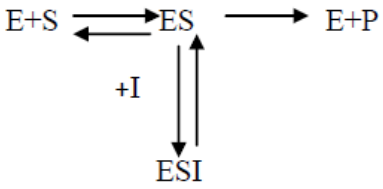


Figure 14: Graphical representations of non-competitive inhibition (Bhagavan & Ha, 2015).

We observe that the Michaelis constant is not modified while the maximum speed decreases by a factor $\left(1 + \frac{[I]}{Ki}\right)$.

1.8.4. Uncompetitive inhibitors

The inhibitor does not bind to the free enzyme, but only to the ES complex.



The velocity equation is as follows:

$$v = \frac{Vm}{(1 + \frac{[I]}{Ki})} \cdot \frac{[S]}{\frac{Km}{(1 + \frac{[I]}{Ki})} + [S]}$$

And:

$$\frac{1}{v} = \frac{Km}{Vm} \cdot \frac{1}{[S]} + \frac{1}{Vm}\left(1 + \frac{[I]}{Ki}\right)$$

The graphical representations give (Figure 15):

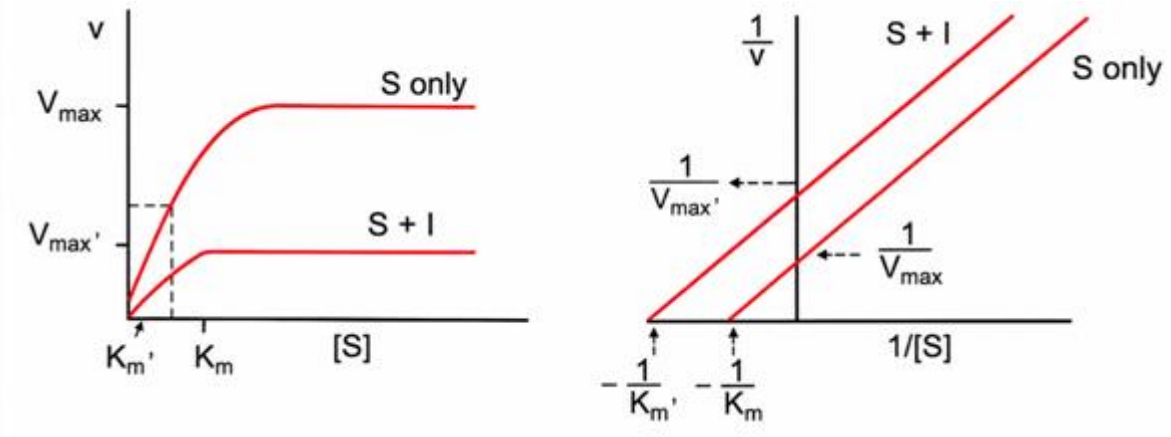


Figure 15: Graphical representations of uncompetitive inhibition (Bhagavan & Ha, 2015).

Modifier Kmapp par Km' et Vmax app par Vmax'

We observe that the two kinetic constants (Km and Vm) decrease by a factor of (1 + [I] /Ki).

The effect of different inhibition types on Michaelis–Menten kinetic parameters is summarized in Table 1.

Table1: Effect of inhibition type on Michaelis-Menten kinetic parameters (Bhagavan & Ha, 2015).

Inhibitor Type	V_{max}	K_m
Competitive	None ($V_{max}' = V_{max}$)	$K_m' = K_m\left(1 + \frac{[I]}{K_i}\right)$
Noncompetitive	$V_{max}' = \frac{V_{max}}{\left(1 + \frac{[I]}{K_i}\right)}$	None ($K_m' = K_m$)
Uncompetitive	$V_{max}' = \frac{V_{max}}{\left(1 + \frac{[I]}{K_i}\right)}$	$K_m' = \frac{K_m}{\left(1 + \frac{[I]}{K_i}\right)}$

2. Two-substrates kinetics

Unlike the simple Michaelis-Menten model, most enzymatic reactions involve two or more substrates. Studying two-substrate enzyme kinetics allows us to understand the mechanism of action of enzymes, including the order of substrate binding and product formation.

It relies on specific experimental approaches and allows us to distinguish different mechanisms, in particular sequential mechanisms (ordered or random) and ping-pong. This study is essential for a better understanding of the actual metabolic reactions.

2.1. Cleland's notation (1963)

To facilitate the understanding of two-substrate enzymatic mechanisms, representations called Cleland diagrams are used, which allow the different stages of the reaction process to be clearly visualized.

In these diagrams, the enzyme is represented by a horizontal line illustrating its evolution during the reaction, while arrows pointing towards this line indicate the order of substrate binding. Conversely, arrows leaving the line correspond to the release of products.

2.2. Nomenclature

The nomenclature of enzymatic mechanisms is based on the number of substrates and products involved in the reaction.

Uni Bi: a substrate and two products;
Bi Bi: two substrates and two products;
Ter Bi: three substrates and two products;
Ter Quad: three substrates and four products.

2.3. Sequential mechanisms

A sequential mechanism is one in which the enzymatic reaction only occurs after the formation of a complex between the enzyme and the two substrates. The binding of the substrates can itself be ordered or occur randomly.

2.3.1. Random fixation

In this case, one or the other substrate binds first. The enzyme has two binding sites, one for each substrate, and the reaction can only occur when both substrates are present.

Two cases must be considered:

- The associations of A and B with the enzyme are independent: the association of one of the substrates occurs in the same way in the absence of the second.
- The associations are dependent; that is to say, the binding of A modifies the affinity of the enzyme for B and vice versa.

Cleland's Representation

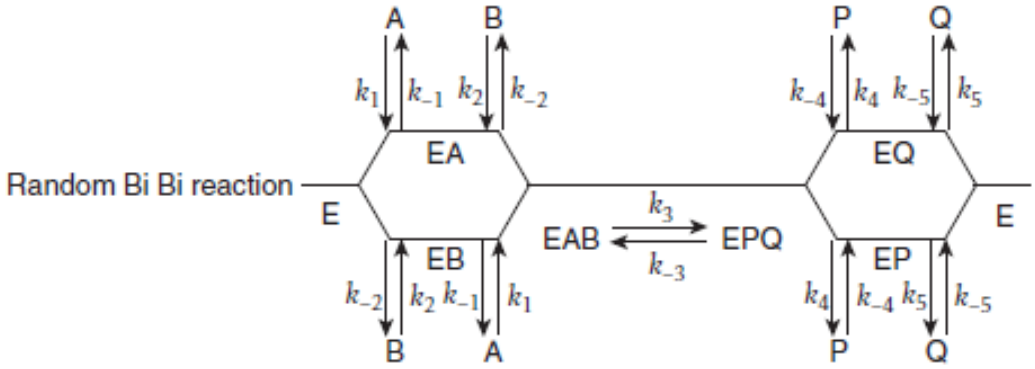


Figure 16: Cleland's Representation of random reaction (Abedi, 2011).

a- Independent association

The attachment of one of the two substrates does not influence the attachment of the other.

The velocity equation is of the form:

$$v = \frac{Vm}{1 + \frac{Ka}{[A]} + \frac{Kb}{[B]} + \frac{Ka.Kb}{[A].[B]}}$$

Graphical representations

Graphical representations can be made in order to calculate the kinetic parameters Ka, Kia, Kb, Kib and Vm or Kcat.

Primary representations

1/ [B] / 1/ [A]	1/[B]1	1/[B]2	1/[B]3
1/ [A]1	1/V1	1/V2	1/V3
1/ [A]2	1/V1'	1/V2'	1/V3'
1/ [A]3	1/V1"	1/V2"	1/V3"

The Lineweaver-Burk representations of 1/V as a function of the inverse of one of the substrates are linear.

In this case: Kia = Ka and Kib = Kb

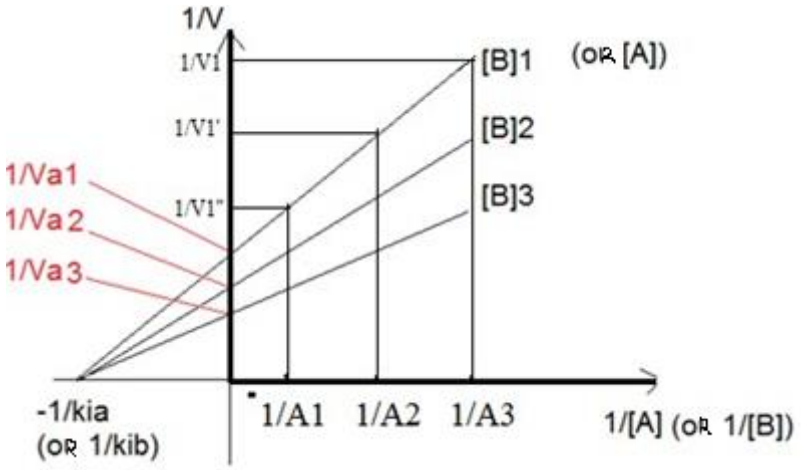
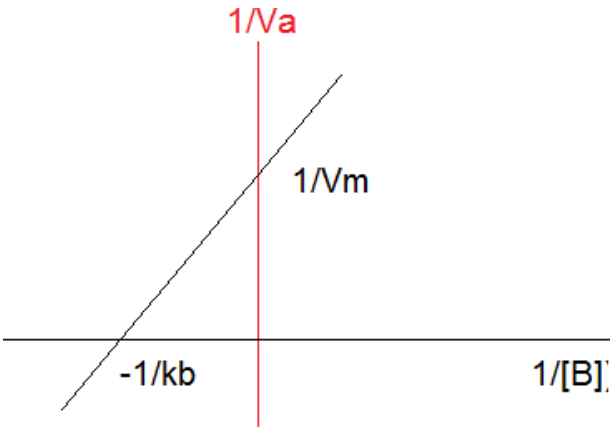


Figure 17: Graphical representations of independent association (Voet & Voet, 2011).

Secondary representations

From the primary representation, the points of intersection of the lines converging with the y-axis have coordinates (1/V). For this, the coordinates are taken

1/VA	1/Va1	1/Va2	1/Va3
1/[B]	1/[B]1	1/[B]2	1/[B]3



These coordinates allow for the creation of a secondary graph using double inverses.
(1/VappA = f (1/[B])

This allows us to calculate Kb and Vm

b- Dependent association

The attachment of one of the two substrates can influence the attachment of the other substrate by either facilitating or hindering it.

The velocity equation is of the form:

$$v = \frac{Vm}{1 + \frac{Ka}{[A]} + \frac{Kb}{[B]} + \frac{Kia.Kb}{[A].[B]}}$$

Two scenarios must be considered:

- ***Facilitated Dependent Association (Kia > Ka)***

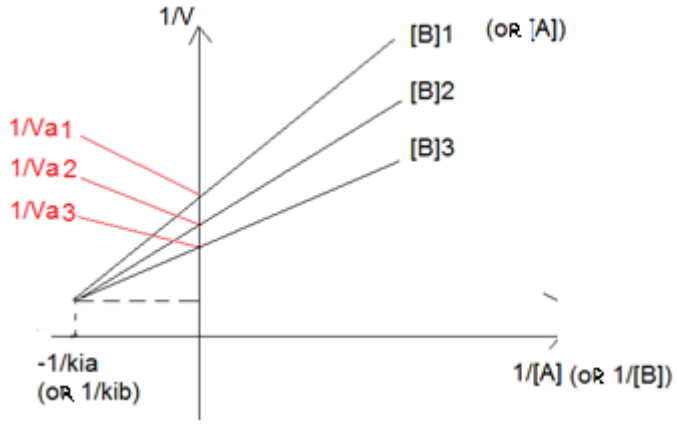


Figure 18: Graphical representations of facilitated dependent association (Voet & Voet, 2011).

❖ ***Hindered Dependent Association Kia < Ka***

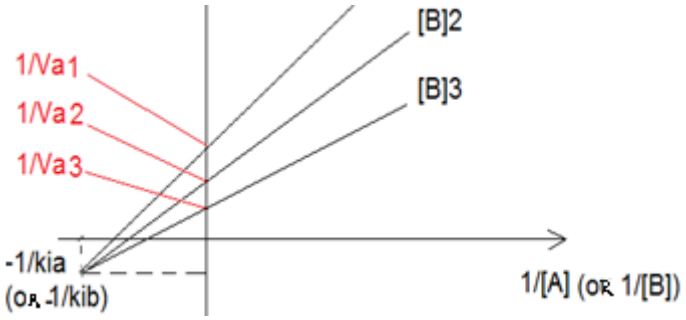


Figure 19: Graphical representations of hindered dependent association (Voet & Voet, 2011).

The kinetic parameters Vm and Km are determined using the secondary representations.

Example of a Random Bi–Bi Mechanism: Creatine PhosphoKinase (CPK)

Creatine phosphokinase (CPK) catalyzes the transfer of a phosphoryl group from the substrate, phosphocreatine, to the coenzyme ADP. Because the enzyme exhibits similar affinity for both substrates, binding can occur in a random order, depending primarily on their respective concentrations.

2.3.2. Ordered fixation

In this case, the order of binding is mandatory: substrate A must bind to free enzyme E before substrate B.

Cleland's Representation

If A is the substrate that binds first, the constant KiB is infinite.

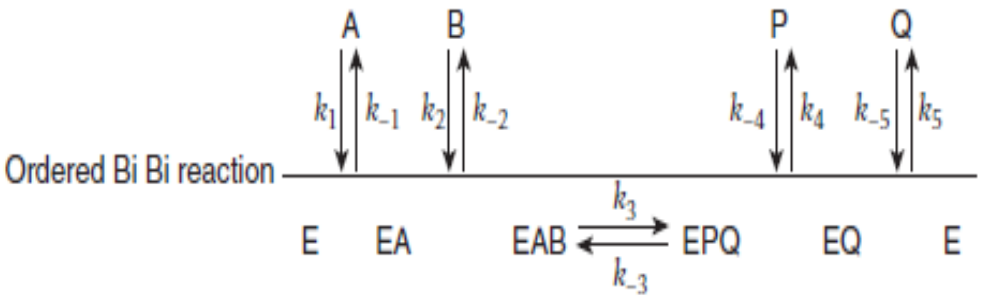


Figure 20: Cleland's Representation of ordered reaction (Abedi, 2011).

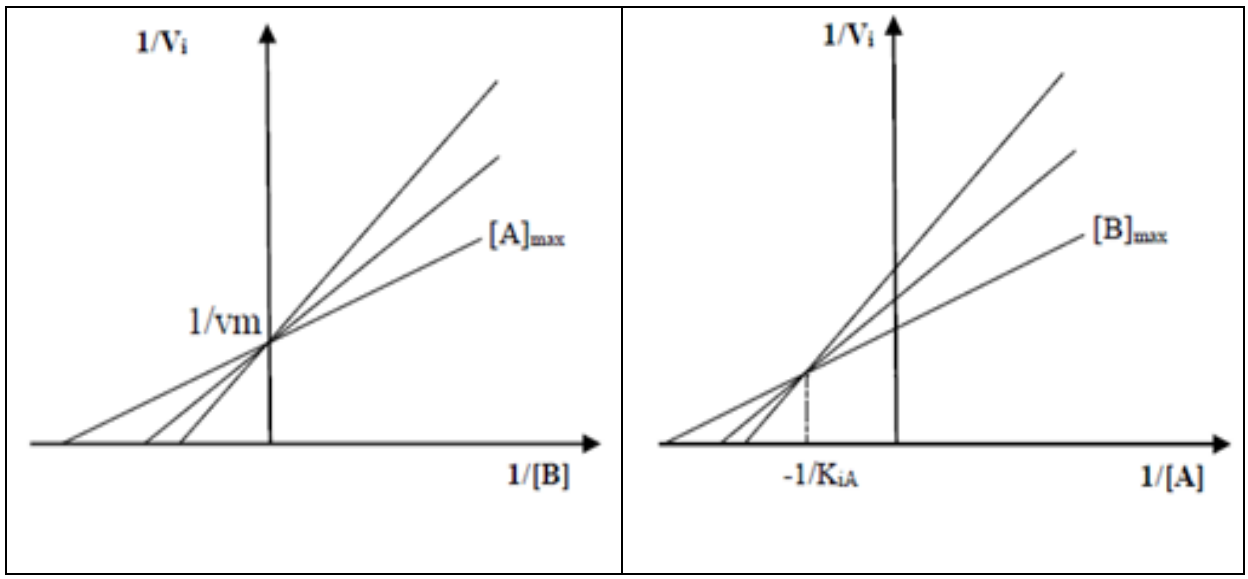
(B has no affinity for the free enzyme) and Ka = 0 (A cannot dissociate from the EAB complex, the dissociation equilibrium constant is zero).

The velocity equation simplifies:

$$v = \frac{Vm}{1 + \frac{Kb}{[B]} + \frac{Kia.Kb}{[A].[B]}}$$

With (Ka=0)

Primary representations



Secondary representations

The kinetic parameters Vm and Km are determined using the secondary representations.

Example of an Ordered Bi–Bi Mechanism: Malate Dehydrogenase

Malate dehydrogenase is an enzyme that catalyzes the oxidation of malate to oxaloacetate, coupled with the reduction of the coenzyme NAD^+ to NADH. This reaction follows an ordered Bi-Bi mechanism: the enzyme exhibits no affinity for malate unless it is first bound to NAD^+, forming an initial enzyme-NAD^+ complex. Subsequently, a ternary complex (enzyme-NAD^+-malate) is formed, which is then converted into an enzyme-NADH-oxaloacetate complex. This complex finally dissociates, releasing oxaloacetate first, followed by the reduced coenzyme NADH.

2.4. Ping-pong mechanism

If the enzymatic reaction does not require the formation of a ternary complex, the enzyme, after binding one of the substrates, transforms it and releases the corresponding product. The enzyme then binds the other substrate, transforms it, and releases the second product.

Cleland's Representation

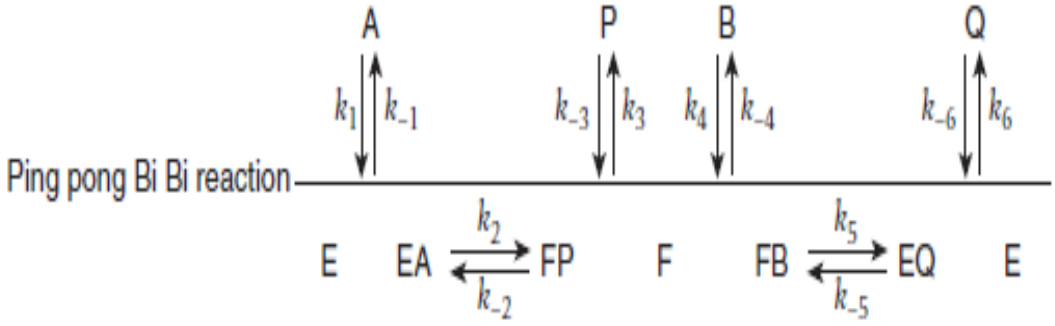


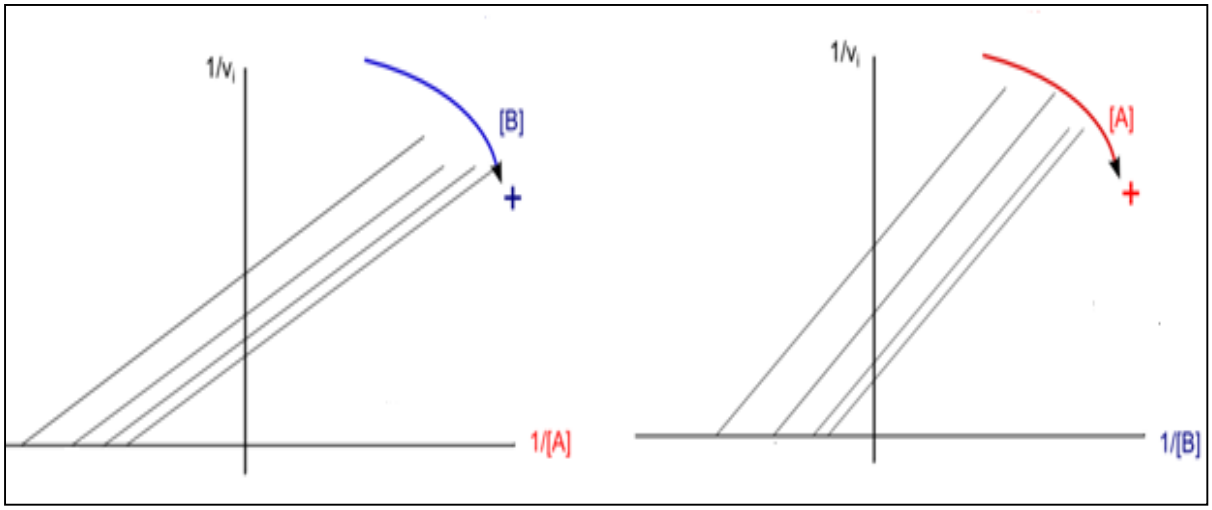
Figure 21: Cleland's Representation of ping pong reaction (Abedi, 2011).

The equation for velocity is of the form:

$$v = \frac{Vm}{1 + \frac{Ka}{[A]} + \frac{Kb}{[B]}}$$

Primary representations

The primary representations of 1/V = f(1/[A]) or f(1/[B]) are parallel lines.



Secondary representations

The kinetic parameters Vm and Km are determined using the secondary representations.

Example of a Ping-Pong Mechanism: Aspartate Aminotransferase (ASAT)

Aspartate aminotransferase (ASAT) catalyzes the transfer of an amino group from aspartate to α-ketoglutarate, producing glutamate. This reaction proceeds via a ping-pong mechanism involving the coenzyme pyridoxal phosphate (PLP).

In the first step, ASAT binds aspartate and transfers its amino group to the enzyme-bound PLP, converting it into pyridoxamine phosphate (PMP). During this process, oxaloacetate is released, and the enzyme remains temporarily modified by the bound PMP.

In the second step, the enzyme-PMP complex interacts with α-ketoglutarate. The amino group is then transferred from PMP to α-ketoglutarate, regenerating PLP and producing glutamate.

Finally, the enzyme-glutamate complex dissociates, restoring the enzyme and its coenzyme to their original states, ready for another catalytic cycle.

3. Multi-substrate kinetics

Some enzymes utilize more than two substrates, particularly within complex metabolic pathways. These enzyme systems are characterized by more elaborate reaction mechanisms, involving multiple interactions between the substrates and the enzyme.

Such reactions generally involve a combination of mechanisms, including both sequential mechanisms (with the formation of multiple complexes) and ping-pong type mechanisms (with transient modification of the enzyme). This type of functioning is frequently encountered in biosynthetic reactions as well as in multi-enzyme complexes.

Analyzing these systems is more difficult and often requires the use of advanced mathematical models as well as specific experimental approaches, in order to better understand the organization and dynamics of enzymatic reactions involving multiple substrates.

Key Points

- The initial velocity increases with substrate concentration until it reaches Vmax, when the enzyme is saturated.
- The Michaelis-Menten equation is $v = \frac{Vm.[S]}{Km+[S]}$. When v=Vmax/2, $[S] = Km$
- A low Km indicates high enzyme-substrate affinity, whereas Vmax=kcat.[E]t reflects the enzyme's catalytic capacity. The ratio k_{cat}/K_m is a measure of overall catalytic efficiency.
- In competitive inhibition, K_m increases while V_{max} remains unchanged; in noncompetitive inhibition, V_{max} decreases with no change in K_m; in uncompetitive inhibition, both K_m and V_{max} decrease simultaneously.
- Reactions involving two substrates may follow a sequential mechanism (ordered or random), requiring a ternary complex, or a ping-pong mechanism, characterized by a transient modification of the enzyme and the absence of a ternary complex.

Chapter V: Functioning and regulation of allosteric enzymes

1. Structural properties

Allosteric enzymes are characterized by a quaternary (oligomeric), structure composed of multiple protomers associate through non-covalent interactions.In addition to the catalytic (active), these enzymes possess a distinct regulatory site known as the allosteric site, to which specific molecules called effectors (also called a modulator) can be attached.

An allosteric enzyme can generally have two interconvertilbe states: an active form R (relaxed), and an inactive form T (tense). Binding of an effector to the allosteric site stabilize one of these conformations, thereby modulating enzymatic activity.

- **Inhibitory effector (negative effector):** stabilize the enzyme in its inactive (T) form. By binding to the allosteric site, the inhibitory effector induces a conformational change that reduces or abolishes catalytic activity.

- **Activating effector (positive effector):** stabilizes the enzyme in its active (R) form, thereby enhancing catalytic activity (Figure 22).

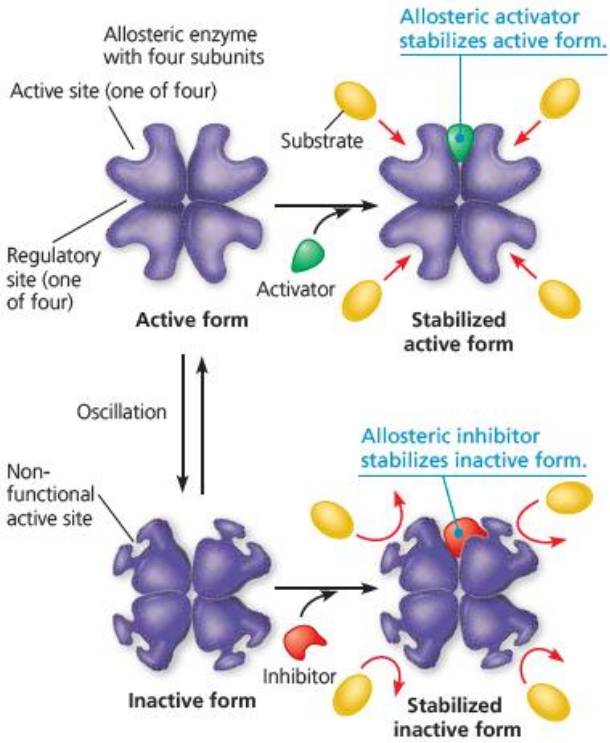


Figure 22: Allosteric regulation of enzyme activity (Urry et al., 2017).

Effectors play an important role in regulating enzymatic activity within the cell. A common regulatory mechanism is feedback inhibition, in which the final product of a metabolic pathway acts as an inhibitory effector of an enzyme catalyzing the first step in the pathway. As the concentration of the end product increases, the activity of the enzyme decreases, thereby reducing further product synthesis.

- **Example of allostery: feedback inhibition in isoleucine biosynthesis**

Isoleucine is an essential amino acid required for protein synthesis. In certain organisms (e.g., bacteria), it can be synthesized from threonine through a metabolic pathway.

When isoleucine accumulates in the cell, it acts as a negative allosteric effector of threonine deaminase (the first enzyme in the pathway), leading to its inactivation. As a result, isoleucine production decreases (Figure 23).

Conversely, when intracellular isoleucine levels are low (the cell constantly uses this amino acid in the synthesis of its proteins), the effector dissociates from the enzyme, restoring its activity. This reactivation promotes increased synthesis of isoleucine.

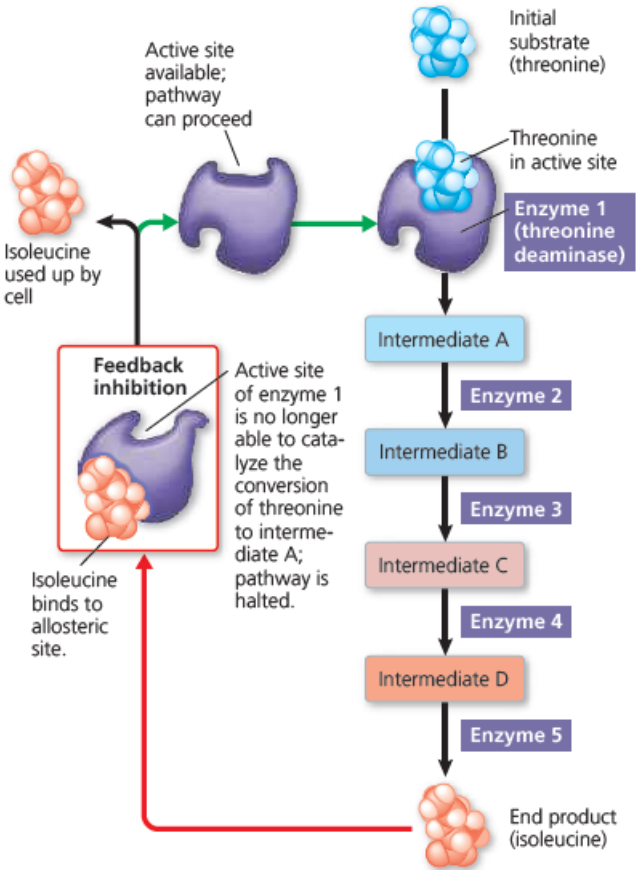


Figure 23: Feedback inhibition in isoleucine synthesis (Urry et al., 2017).

2. Functional properties

Functionally, allosteric enzymes are distinguished by their sigmoid kinetics (S-shaped), reflecting a non-Michaelian behavior due to cooperative interactions between subunits.

2.1. Cooperative effect

Cooperativity reflects the fact that the binding of one molecule of an allosteric effector to the enzyme influences the binding of subsequent molecules. In the case of cooperativity, if the allosteric effector is the substrate itself, the process is termed homotropic modulation. If the effector is different from the substrate, it is referred to as heterotropic modulation. In positive cooperativity, a molecule of an effector leads to an increase in affinity for the same molecules, and vice versa in negative cooperativity.

This behavior explains the sigmoidal shape of the velocity versus substrate concentration curve (v = f([S])).

2.2. Desensitization effect

Treating an allosteric enzyme with physical agents (e.g., heating) or chemical agents (urea, mercuric derivatives) can result in desensitization, defined as the loss of responsiveness to allosteric effectors. In this case, the enzyme retains catalytic activity. Only the allosteric site is destroyed. This leads to a loss of cooperativity, and the kinetic behavior become hyperbolic.

3. Kinetic behavior of allosteric enzymes

For allosteric enzymes, plotting reaction velocity (v) as a function of substrate concentration ([S]) yields a sigmoid curve. Saturation is still achieved at sufficient substrate concentrations. Although there is a substrate concentration on the sigmoid saturation curve at which the velocity is equal to half the maximum velocity, it is incorrect to call this Km, since the enzyme is not Michaelis-Menten enzyme. Instead, we will use the expressions $S_{o.5}$ or $K_{o.5}$, $K_{1/2}$ or Kh. This parameter reflects the apparent affinity of the enzyme for its substrate under cooperative conditions (Figure 24).

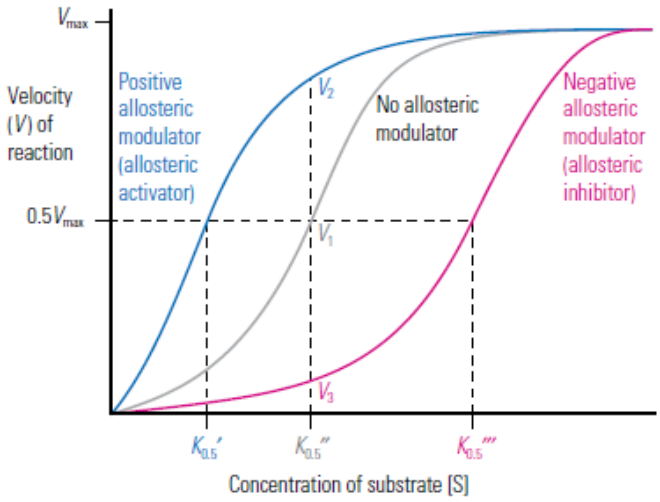


Figure 24: Effect of substrate concentration on a typical allosterically regulated enzyme (Papachristodoulou et al., 2018).

4. Determining kinetic constants from graphical representation

The kinetic study of allosteric enzymes cannot be described by the Michaelis-Menten equation. Specific approaches such as the Hill representation are then used to analyze cooperativity.

4.1. Hill's equation

For an allosteric enzyme, we have:

E + nS <===> ESn

$$v = \frac{\text{Vmax.}\,[\text{S}]^{\text{n}}}{\text{Kh} + [\text{S}]^{\text{n}}}$$

n: cooperativeness coefficient (Hill coefficient)

Kh: Hill constant (substrate concentration at half-saturation)

Rearranging: v.Kh+v.[S]n=Vmax.[S]n therefore,

$$\frac{\text{v}}{(\text{Vm} - \text{v})} = \frac{[\text{S}]^{\text{n}}}{\text{Kh}}$$

Taking the logarithm:

$$\text{Log}\left[\frac{\text{v}}{(\text{Vm} - \text{v})}\right] = \text{Log}\,(\frac{[\text{S}]^{\text{n}}}{\text{Kh}})$$

Therefore,

$$\boldsymbol{Log\left[\frac{v}{(Vm - v)}\right] = n.Log[S] - LogKh}$$

This linear form allows construction of the Hill plot, where:

- y-axis: $\text{Log}\left[\frac{\text{v}}{(\text{Vm}-\text{v})}\right]$
- x-axis: log [S]

The slope of this line corresponds to the Hill coefficient (n), which indicates the degree of cooperativity (Figure 25).

- n = 1: absence of cooperativity
- n > 1: positive cooperativity
- n < 1: negative cooperativity.

This representation allows us to determine parameters analogous to Km, such as $K_{0.5}$, corresponding to the substrate concentration for which the velocity is equal to half the maximum velocity.

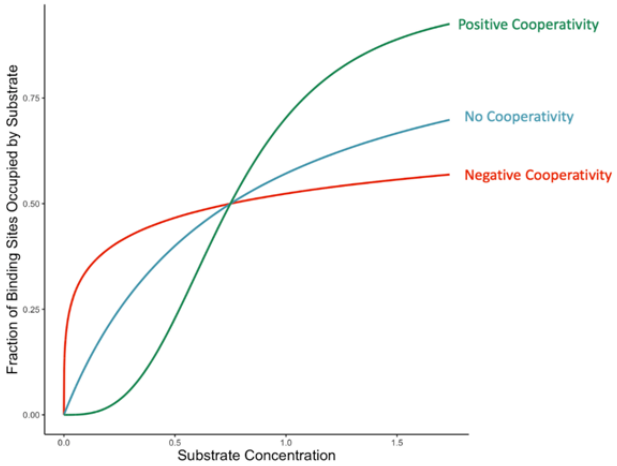


Figure 25: Graph showing velocity as a function of the Hill number

4.2. Study models

4.2.1. Monod-Wyman-Changeux (MWC) concerted model, the all-or-nothing or symmetrical model (1965)

The Monod-Wyman-Changeux model describes allosteric enzyme as oligomeric protein that exists in equilibrium between two conformational states: R (relaxed active state) and T (tense inactivate state). These two states pre-exist in the absence of a ligand (Figure 26), and all subunits within a given conformartion are symmetrical arranged. The transition between T and R occurs in a concerted manner, meaning that all subunits switch conformation simultaneously. A ligand that shifts the equilibrium between these two states is called an allosteric effector.

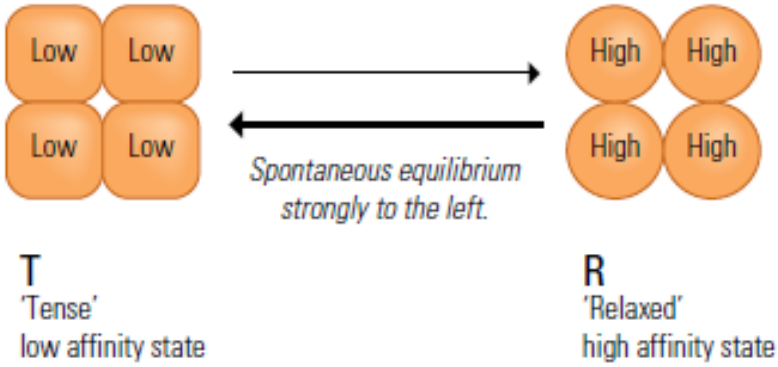


Figure 26: Concerted model of cooperative binding of substrate (Papachristodoulou, et al., 2018).

If a ligand has a greater affinity for one of the forms, for example the R form, it binds preferentially to it and decreases the value of R_0 ([R] which has not bound the ligand, R1 [R] which has bound a ligand molecule…etc); this decrease in R0 leads to a transformation of the protein from the T form to the R form, until the equilibrium between R0 and T0 is maintained. This transition leads to an increase in [Rt] and a decrease in [Tt]. When the equilibrium is almost entirely shifted towards the R forms, the binding sites are equivalent and independent, and the enzyme behaves in a Michaelis-Menten kinetic manner (Figure 27).

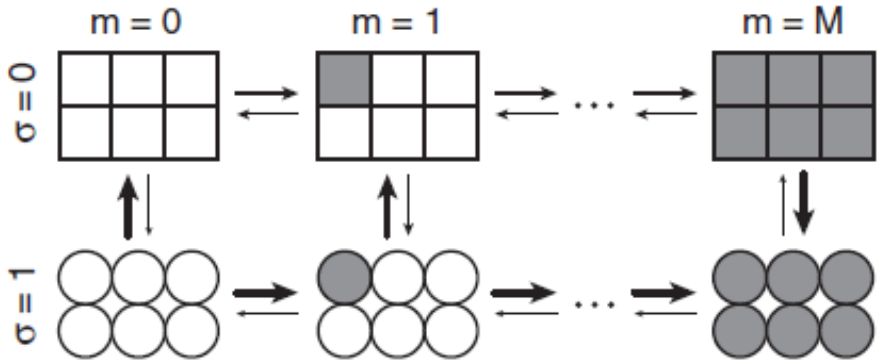


Figure 27: Schematics of the enzymatic MWC model (Hatakeyama & Kaneko, 2020).

Two main classes of allosteric enzymes are distinguished based on the effect of modulators:

a. K-system enzymes (most common)

In these enzymes, effectors modify substrate affinity without affecting the maximum velocity (Vmax). The substrate and effectors have different affinities for the R and T forms of the enzyme (the Km of R is lower than the Km of T). This results in consistently sigmoidal binding. The R and T forms have the same maximum velocity. Kinetically, these are enzymes where the effector can only modify the apparent affinity (relative to Km) of the enzyme for the substrate. The affinity for the substrate (S) decreases in the presence of an allosteric inhibitor (I). It increases in the presence of an allosteric activator (A).

b. V- system enzyme (less common)

In these enzymes, effectors modify the maximum velocity (Vmax) without significantly affecting substrate affinity. The substrate (S) has the same affinity for both enzyme forms R and T. It is as if there was only one enzyme form. This result in hyperbolic binding of S. Homotropic cooperativity is absent. However, the effectors A and I have different affinities for each form, R and T. This results in sigmoid binding, indicating homotropic cooperativity for A and I.

The R and T forms differ in their catalytic activities. By binding to one of the forms, an effector will increase or decrease Vmax simply by shifting the equilibrium (R ⇌ T). If R is catalytically more active, the binding of an activator to this form shifts the equilibrium towards it, resulting in an increase in Vmax. The reverse reasoning holds true for an allosteric inhibitor binding to the T form.

4.2.2. Sequential model or Koshland-Nemethy-Filmer (KNF) model (1966)

The sequential model or KNF model (Koshland, Nemethy, Filmer, 1966) is based on the notion that a protein is sufficiently flexible so that ligand binding at one site directly modifies the conformation of another site of the protein molecule and affects the affinity of this latter site for its ligand.

In the absence of the substrate, all molecules of enzymes are in the T conformation. The binding of the first substrate molecule induces the (T → R) transition of the first subunit, which facilitates the (T → R) transition of the neighboring subunit and the binding of a second substrate molecule, and so on for the enzyme molecule (subunit by subunit). These changes and their effects on site affinity appear sequentially, as ligands bind (Figure 28).

Thus, cooperativity arises from induced conformational changes transmitted between subunits, rather than a concerted global transition.

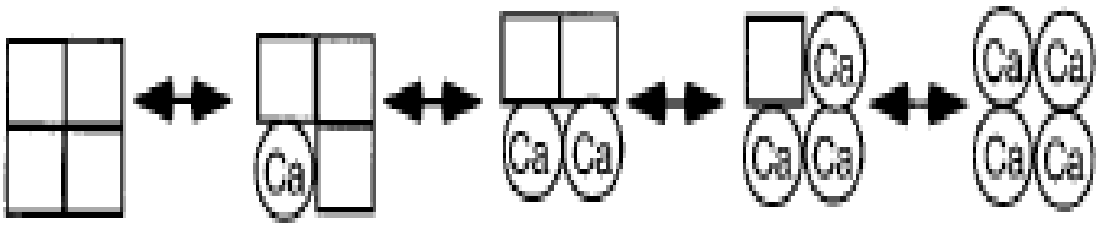


Figure 28: Schematics of the enzymatic Koshland-Nemethy-Filmer (KNF) model (Talukder & Aldrich, 2000).

4.3. Action of allosteric effectors

- **Allosteric activators**

An allosteric activator shifts the equilibrium from the T (tense) state to the R (relaxed) state. As a result, the enzyme's affinity for its substrate increases, the $K_{0.5}$ decreases, and the v = f([S]) curve is shifted to the left.

- **Allosteric inhibitors**

An allosteric inhibitor shifts the equilibrium from the R state to the T state. Consequently, the enzyme's affinity for its substrate decreases, the $K_{0.5}$ increases, and the v = f([S]) curve is shifted to the right.

Allosteric inhibition is a form of negative heterotropic regulation.

Example: *Aspartatet ranscarbamoylase* (ATCase)

ATCase is is a well-studied allosteric enzyme from *Escherichia* coli. This enzyme catalyzes the transfer of the carbamoyl group from carbamoyl phosphate to the α-amino group of aspartate. ATCase is inhibited by CTP (cytidine triphosphate) and specifically activated by ATP.

This regulation ensures a balance between purine and pyrimidine nucleotide synthesis (Figure 29).

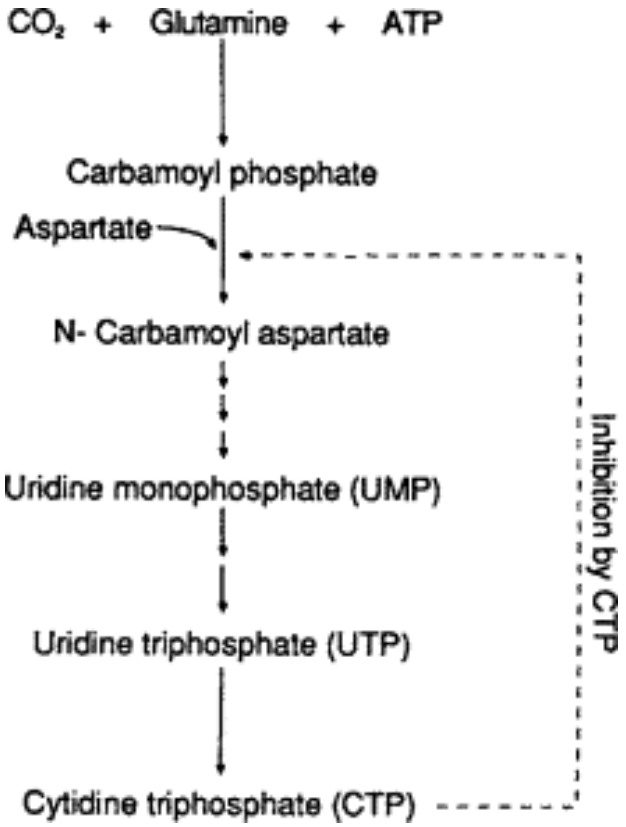


Figure 29: Regulation of pyrimidine nucleotide biosynthesis in *E. coli* (Bhagavan, 2002).

4.4. Study of allosteric enzymes

The study of allosteric enzymes that obey the MWC model aims to define the corresponding model (K or V) and to determine the different parameters that characterize it.

In the presence of a substrate:

$$\frac{[R]t}{[R]t} = \frac{\bar{R}}{\bar{T}} = \frac{1}{L_0}\left(\frac{[S]}{Km}\right)^n$$

The representation of $\sqrt[n]{\frac{\bar{R}}{\bar{T}}}$ as a function of [S] is a straight line with ordinate at the origin $\sqrt[n]{\frac{1}{[L0]}}$ and the intersection with the x-axis gives -Km.

Key Points

- Allosteric enzymes are oligomeric and, in addition to the active site, possess allosteric sites that bind effectors stabilizing either the active R state or the inactive T state.
- They exhibit sigmoidal kinetics due to cooperativity among subunits; the characteristic parameter is $K_{0.5}$ (or $S_{0.5}$), rather than Km.
- The Hill coefficient (n) quantifies cooperativity: n=1 indicates no cooperativity, n>1 positive cooperativity, and n<1 negative cooperativity.
- Two major models account for allostery: the concerted model of Monod-Wyman-Changeux (a global T↔R transition) and the sequential model of Koshland-Nemethy-Filmer (successive induced conformational changes).
- Allosteric activators shift the equilibrium toward the R state and decrease $K_{0.5}$, whereas inhibitors favor the T state and increase $K_{0.5}K$. Feedback inhibition is a fundamental mechanism of metabolic regulation.

Chapter VI: Mechanism of enzyme catalysis

1. Topology and identification of active centers

1.1. Definition

The term topology derives from the Greek words topos (place) and logia (study), literally meaning "study of a place or space." It refers to the study of spaces, the properties that characterize them, and the laws that govern their organization.

In the context of biochemistry and enzymology, topology refers to the spatial organization of molecular structures, particularly the three-dimensional arrangement of the elements constituting the active center of an enzyme.

1.2. Concept of the active center

The active site is a specific three-dimensional region of the enzyme where the substrate binds and the catalytic reaction takes place. It constitutes both a structural and functional entity, ensuring substrate recognition and its chemical transformation.

The active center consists of:

- The binding site consists of enzyme residues that establish non-covalent interactions with the substrate.
- The catalytic site consists of the residues that directly participate in the chemical process.

The amino acids involved in the active center can be classified according to their role:

a. The contact amino acids that provide the function of fixation and catalysis.

b. Conformational amino acids (collaborators) that ensure the integrity of the enzymatic conformation.

c. The auxiliary amino acids that ensure the flexibility of the enzyme molecule (promotes substrate binding).

d. Indifferent (non-collaborating) amino acids are present as a result of the sequential chain. They play no role, and their modification or replacement does not change the enzyme's activity.

1.3. Enzyme-substrate interaction

There are two models explaining enzyme-substrate interaction (Figure 30):

Fisher Model: key-lock model

This model assumes that the active site is rigid and complementary to the substrate, which binds to it like a key in a lock. It explains enzyme specificity but does not take into account the enzyme's flexibility.

Koshland Model: model of induced adjustment.

The enzyme is not rigid, but flexible; the enzyme and the substrate mutually adapt their respective shapes, which are complementary only within the enzyme-substrate complex.

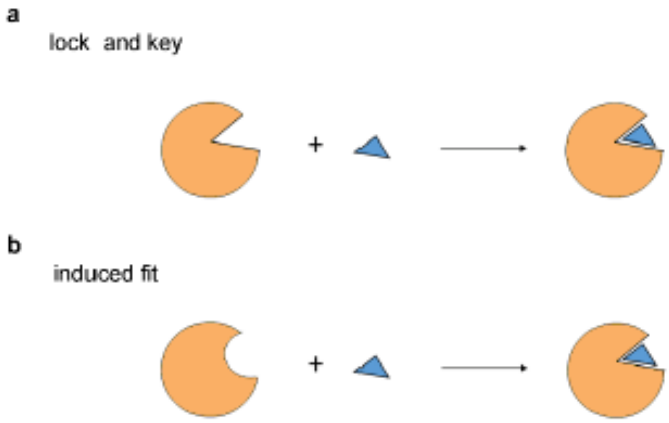


Figure 30: Models of protein-ligand interaction. (a) The lock and key model. (b) The induced fit model (Spassov, 2024).

1.4. Methods for studying (identifying) the topology of the active center

Determining the precise topology of the active center is not easy. Several methods have been used for this purpose. They are complementary and provide information that must be analyzed sequentially. These include:

1.4.1. Kinetic approach

This approach involves analyzing the influence of pH on enzymatic reactions (pH profile). In fact, the kinetic parameters of the enzyme vary considerably depending on the pH, as pH determines the ionization of enzyme residues important for activity. Therefore, the existence of an optimum pH results from the stability of the conformation and the efficiency of the active site. Experimentally, it is necessary to distinguish the various effects of pH:

- *Study of the effect of pH on the conformational state of the protein*
- *Study of the ionization state of the substrate*

- *Study of the effect of pH on enzyme-substrate associations*
- *Study of the effect of pH on the ionization of catalytic groups*

1.4.2. Chemical approach

The kinetic approach allows us to determine the role of ionizable groups, but not their nature. Therefore, other experimental approaches are needed to obtain more information.

Principle of chemical labeling

Chemical labeling involves modifying the side chains of a protein (which are involved in catalysis) using nucleophilic or electrophilic reagents. The choice of reagent depends on the amino acid residues targeted for modification.

The chemical nature and secondary-tertiary structure of the protein make the enzyme a heterogeneous environment; with a polar surface and an internal nonpolar portion, creating a local microenvironment within the enzyme's active center. This microenvironment can significantly alter the reactivity of a group within a side chain.

Chemical modification strategy

There are different strategies used to selectively label the active site of the enzyme:

- *Labeling by a substrate, quasi-substrate or coenzyme*
- *Affinity marking*
- *Photoaffinity labeling*

1.4.3. Genetic methods

Site-directed mutagenesis techniques allow any amino acid in a protein to be replaced by another and are perfectly suited for determining the amino acids involved in enzymatic catalysis.

1.4.4. Structural studies using physical methods

The physical methods used are X-ray crystallography and nuclear magnetic resonance.

1.5. Catalytic Mechanisms

The remarkable efficiency of enzymes as catalysts is explained by their high substrate specificity and the optimal arrangement of their catalytic groups.

The principal enzymatic mechanisms can be classified as follows:

• **Acid-base catalysis**: the enzyme provides functional groups that act as proton donors or acceptors during the reaction. This can involve general acid-base catalysis, where amino acid side chains (such as histidine, aspartate, or glutamate) facilitate proton transfer, thereby stabilizing charged intermediates and promoting bond cleavage or formation.

• **Covalent catalysis**: the enzyme forms a transient covalent bond with the substrate, producing a short-lived enzyme-substrate intermediate. This intermediate follows an alternative reaction pathway with lower activation energy. Amino acid residues such as serine, cysteine, or lysine often participate in this mechanism.

• **Metal-dependent catalysis**: the enzyme employs metal ions (e.g., Zn^{2+}, Mg^{2+}, Fe^{2+}/Fe^{3+}) as cofactors. These ions can stabilize negative charges in intermediates, facilitate redox reactions, activate water molecules by increasing their nucleophilicity, and help properly orient the substrate within the active site.

• **Electrostatic catalysis**: the enzyme stabilizes the transition state through electrostatic interactions between charged or polar groups in the active site and the substrate. These interactions reduce the energy barrier and contribute significantly to catalytic efficiency.

• **Proximity and orientation effects**: the enzyme increases the effective concentration of reactants by bringing them into close proximity and aligning them in an optimal geometric arrangement. This reduces the entropic cost of the reaction and enhances the probability of productive collisions.

• **Strain (distortion) catalysis**: the enzyme induces structural distortion or strain in the substrate upon binding, particularly at the bond that will be cleaved. This destabilization makes the bond more reactive and easier to break. In doing so, the enzyme preferentially stabilizes a transition-state-like conformation, thereby accelerating the reaction.

2. Functioning of coenzymes

2.1. Definition

A coenzyme is a small, non-protein organic molecule with a low molecular weight and is generally heat-stable. Essential for enzymatic activity, it acts at low concentrations by participating in the catalyzed reaction, and is then regenerated at the end of the reaction.

Coenzymes are of biological origin, often derived from vitamins or synthesized from metabolic intermediates (notably nucleotides or their derivatives). In mammals, they are generally provided by the diet in the form of vitamins or growth factors.

A single coenzyme can associate with several enzymes and participate in different types of reactions on various substrates. When the coenzyme binds to the protein part of the enzyme, called the apoenzyme, it forms an active complex called a holoenzyme.

Thus, the coenzyme participates directly in the catalytic reaction, while the specificity of the enzyme towards the substrate remains determined by the apoenzyme.

2.2. Types

The distinction between coenzymes is based primarily on the nature and stability of their interaction with the enzyme, which determines their mode of action during catalysis. Two main categories can be identified:

2.2.1. Bound coenzymes or prosthetic groups

These coenzymes are tightly bound to the enzyme, most often by covalent bonds, and do not dissociate during the reaction. Their separation generally leads to protein denaturation. Their concentration is therefore equivalent to that of the enzyme, which corresponds to a very low catalytic quantity.

Examples: FAD, cytochromes, coenzyme Q.

2.2.2. Free coenzymes or co-substrates (true coenzymes)

These coenzymes are weakly bound (non-covalent bonds) and easily dissociate from the apoenzyme after the reaction. Their association with the enzyme is transient and is renewed with each catalytic cycle. They act as carriers of chemical groups (electrons, protons, etc.) and are regenerated after their involvement.

Example: NAD^+.

2.3. Functions of coenzymes

Coenzymes play a crucial role as acceptors and transporters of chemical groups released during enzymatic catalysis. Their primary functions are:

- the transfer of electrons and protons (H^+**)** during redox reactions;
- the transfer of chemical groups other than H^+(various radicals) in other types of reactions.

Thus, enzymes requiring coenzymes catalyze different types of enzymatic reactions, including:

- isomerization;
- redox reaction;
- group transfer;
- of covalent bond formation.

2.4. Classification of coenzymes

Coenzymes can be classified according to the nature of the groups they carry and the type of reactions in which they participate.

2.4.1. Redox Coenzymes

They are involved in electron and proton transfer reactions, catalyzed by dehydrogenases or reductases.

- **Nicotinic coenzymes $(NAD)^+$ $NADP^+$)**: these are derivatives of niacin (vitamin PP), they are free coenzymes that transport electrons and hydrogens.
  -NADH: involved in catabolic reactions and ATP production (respiratory chain).
  -NADPH: is involved in anabolic reactions (biosynthesis).

- **Flavin coenzymes (FAD, FMN)**: these are derivatives of riboflavin (vitamin B2), generally bound to the enzyme, transporting hydrogens.
- **Quinonic coenzymes (ubiquinone or CoQ)**: these are lipid-soluble electron carriers in the mitochondrial respiratory chain.
- **Metalloporphyrins (cytochromes, catalases, peroxidases)**: these are prosthetic groups containing a metal (often iron) ensuring the transfer of electrons by change of valence.

2.4.2. One-carbon radical transport coenzymes

They ensure the transfer of groups to a carbon atom (CO_2, CH_3, CHO…). Examples include:

- **Biotin (vitamin H)**: bound coenzyme, CO2 transporter in carboxylation reactions.
- **Vitamin K**: involved in post-translational carboxylation reactions.
- **S-adenosylmethionine (SAM)**: universal donor of methyl groups ($–CH_3$).

- ♣ **Tetrahydrofolate (THF)**: transports various one-carbon groups (methyl, formyl) essential for the synthesis of nucleic acids.

2.4.3. Coenzymes for transporting radicals with two or more carbon atoms

They carry acyl, aldehyde or carboxyl groups.These include:

- ♣ **Thiamine pyrophosphate (TPP)**: derived from vitamin B1, involved in the decarboxylation of α-keto acids and transketolation reactions.
- ♣ **Lipoic acid (lipoamide)**: bound coenzyme involved in oxidative decarboxylations and the transfer of acyl groups.
- ♣ **Coenzyme A (CoA)**: major transporter of acyl groups, forming high-energy compounds (acyl-CoA) involved in energy metabolism.

2.4.4. Coenzymes of aminotransferases: pyridoxal phosphate (PLP)

It is derived from vitamin B6, an essential coenzyme involved in amino acid metabolism. It plays a role in various reactions such as transamination, decarboxylation, racemization, and certain cleavage reactions.

2.4.5. Phosphate transfer coenzymes and other groups

These coenzymes, often phosphorylated nucleotides, are involved in the transfer of energy or specific groups:

- ♣ **ATP**: main transporter of energy and phosphate groups.
- ♣ **UDP**: carrier of sugars (glucose, galactose…).
- ♣ **CDP**: transporter of compounds such as choline or ethanolamine.

3. Activation of zymogens

3.1. Definition

A zymogen (or proenzyme) is a specific conformational structure of an enzyme that is inactive and therefore requires activation to perform its catalytic function. Proenzyme activation occurs through the cleavage of one or more peptides of varying sizes. This cleavage results in a complete modification or rearrangement of the three-dimensional structure, or gives rise to a conformation possessing enzymatic activity.

Most proteolytic enzymes are usually synthesized as longer, inactive precursors.

The benefit for the body is the ability to synthesize an enzyme in an inactive form and activate it later when needed. The enzyme can thus be stored in an inactive, and therefore

harmless, state (as in the case of trypsin). This mechanism prevents cellular autodigestion and ensures controlled activation of enzymatic activity.

3.2. Activation mechanisms

3.2.1. Activation by conformational change

A number of enzymes, such as digestive proteases, are synthesized as inactive precursors. Their transformation into active enzymes occurs through limited proteolysis (local cleavage of one or more peptide bonds). This reaction is itself catalyzed by an enzyme. The enzymatic cleavage results in a change in the enzyme's spatial conformation, which then presents its active site.

Example: **Activation of chymotrypsinogen**

This enzyme is synthesized in the pancreas as an inactive precursor known as chymotrypsinogen (pre-chymotrypsin). It initially consists of a single polypeptide chain of 245 amino acid residues, stabilized by five intrachain disulfide bonds. Upon reaching the intestine, where proteolytic activity is required for dietary protein digestion, chymotrypsinogen is activated by trypsin. Trypsin cleaves the peptide bond between arginine 15 and isoleucine 16, yielding π-chymotrypsin. Although this form already exhibits enzymatic activity, it undergoes further processing: a dipeptide is removed from positions 14 and 15 through the action of another π-chymotrypsin molecule, followed by additional cleavage that removes a dipeptide from positions 147 and 148, ultimately producing the fully active α-chymotrypsin.

The resulting chymotrypsin consists of three polypeptide chains linked by disulfide bonds and is therefore not strictly a monomeric enzyme, although the original residue numbering of chymotrypsinogen is generally retained. In contrast, trypsin is a truly monomeric enzyme.

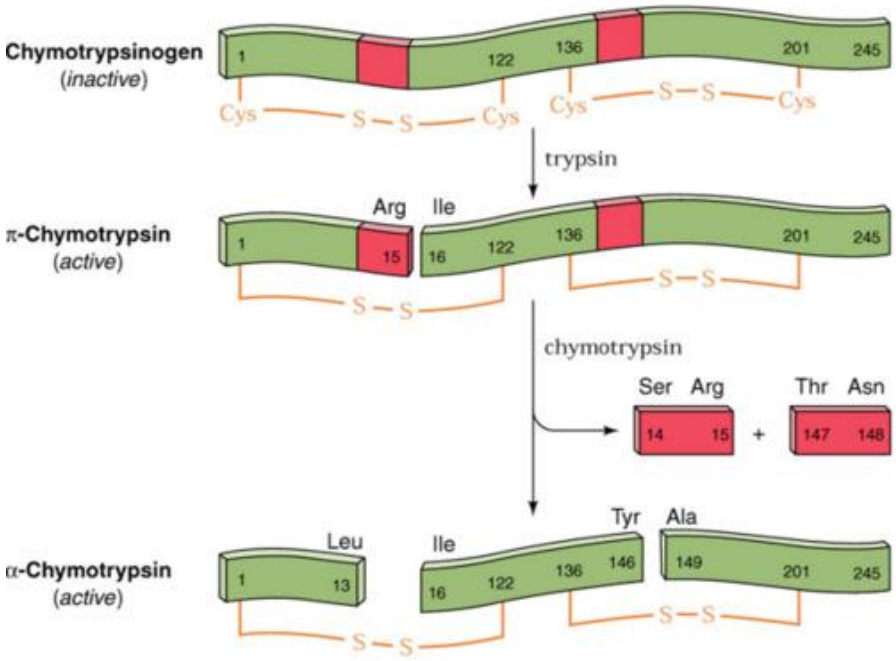


Figure 31: Activation of chymotrypsinogen **(**Voet & Voet, 2011).

3.2.2. Activation by exposure of the active site

Trypsinogen, secreted in pancreatic juice, reaches the duodenum where it is activated into trypsin by intestinal enterokinase.

Trypsinogen is inactive because access to its active site is blocked by a propeptide corresponding to the first six amino acids. This propeptide is held in place by electrostatic interactions between the aspartate residues of the propeptide and cationic charges located on an α-helix of the carboxy-terminal domain.

Enterokinase, which reconized this propeptide sequence, hydrolyzes the peptide bond between lysine at position 6 and isoleucine at position 7. This cleavage leads to the release of the propeptide and exposes the active site, thus making the enzyme fully active.

4. Specific markers of catalytic centers

Specific markers are molecules capable of selectively binding to the active site of an enzyme. This binding can be reversible or irreversible. In some cases, these compounds mimic the substrate or the transition state, allowing them to interact precisely with catalytic residues.

When the fixing is covalent, it generally leads to a loss of enzymatic activity. This property is used to identify essential functional groups for catalysis. These markers often exhibit high specificity towards certain amino acid residues.

Examples:

- **Diisopropyl fluorophosphate (DIFP)**: an organophosphorus reagent that selectively reacts with the hydroxyl group of the active-site serine in serine proteases. It forms a stable covalent phosphoester bond, irreversibly inactivating the enzyme and thereby demonstrating the essential role of this serine residue in catalysis.

- **TLCK (tosyl-L-lysyl chloromethyl ketone)**: a substrate analog and specific inhibitor of certain proteases (notably trypsin-like enzymes). It binds within the active site and reacts covalently with the imidazole group of a histidine residue, leading to irreversible inhibition and highlighting the functional importance of histidine in the catalytic mechanism.

- **Iodoacetate**: an alkylating agent that reacts specifically with thiol (–SH) groups of cysteine residues. By forming a covalent modification, it inactivates enzymes that depend on an active-site cysteine, thus revealing the involvement of this residue in catalysis.

- **N-ethylmaleimide (NEM)**: a sulfhydryl-reactive compound that selectively modifies cysteine residues. Its inhibitory effect on enzyme activity helps identify cysteine residues that participate directly in catalysis or are crucial for maintaining the structure of the active site.

5. Mechanisms of action of serine proteases

Serine proteases are proteolytic enzymes characterized by the presence of a highly reactive serine residue at their active site, associated with a histidine and an aspartate, forming the catalytic triad. They are widely distributed in viruses, bacteria, and eukaryotes.

Their catalytic mechanism, which takes place in several stages, relies on the intervention of this activated serine to hydrolyze peptide bonds.

For example, chymotrypsin catalyzes the cleavage of peptide bonds on the C-terminal side of aromatic or hydrophobic side-chain amino acids (Phe, Trp, Tyr).

At the active site of chymotrypsin, serine 195 reacts directly with the substrate, while histidine 57 and aspartic acid 102 increase its reactivity.

The catalytic reaction proceeds as follows (Figure 32):

1-Serine 195 performs a nucleophilic attack the carbonyl carbon of the peptide bond, leading to the formation of a covalent tetrahedral intermediate.

2- This intermediate collapses following proton transfer mediated by histidine 57; resulting in the formation of an acyl-enzyme intermediate through general acid-base catalysus involving histidine, whose reactivity is modulated by aspartate 102.

3- The amine product is then released and replaced by a water molecule.

4-The activated water molecule subsequently attacks the carbonyl carbon of the acyl–enzyme intermediate, leading to the formation of a second tetrahedral intermediate.

5-The decomposition of this intermediate (in the reverse direction of reaction 1) gives the carboxylic product and regenerates the active enzyme.

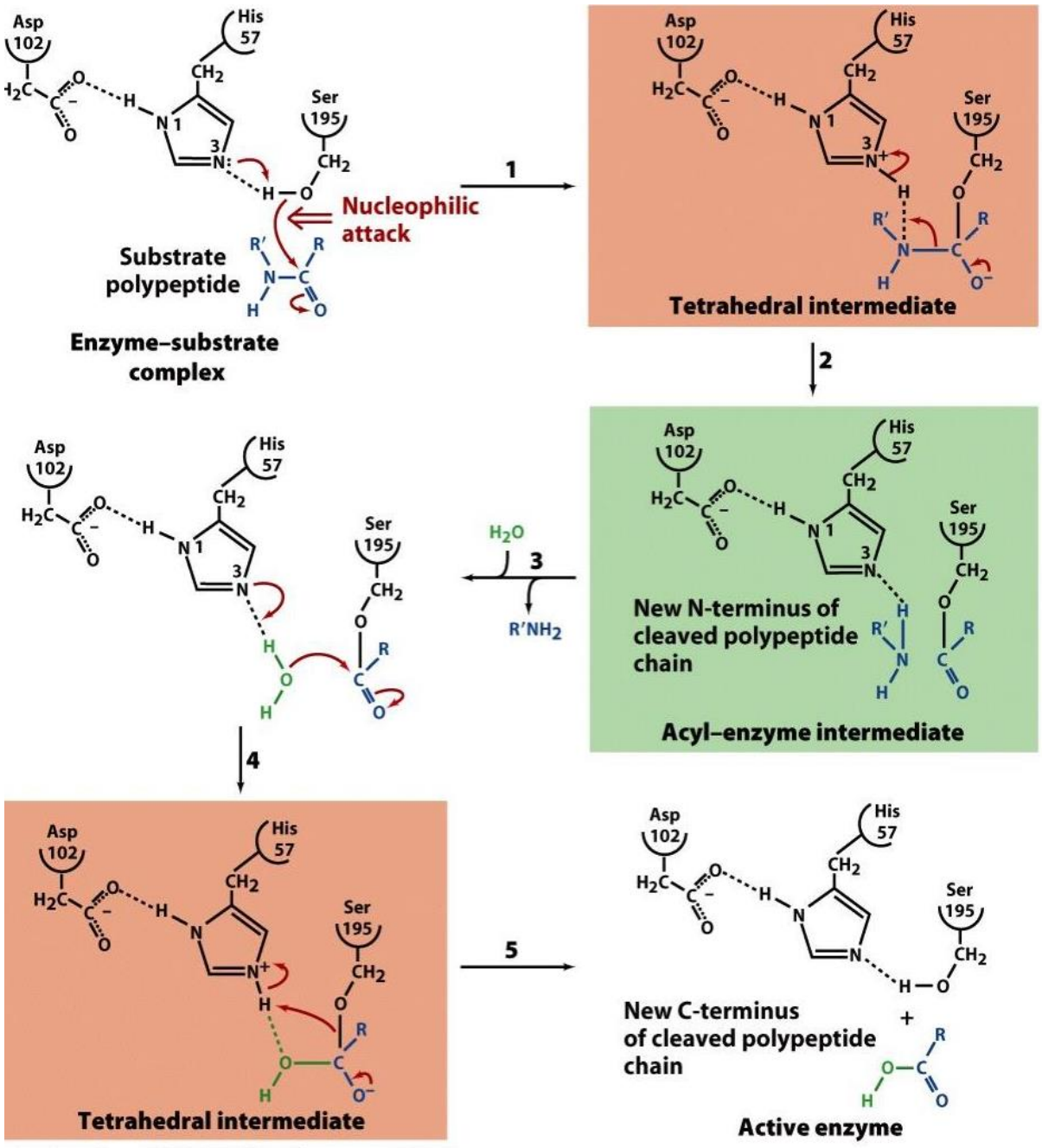


Figure 32: The catalytic mechanism of serine protease **(**Voet & Voet, 2011).

6. Mechanism of action of pyridoxal transferases

Pyridoxal transferases, or aminotransferases, are enzymes dependent on pyridoxal phosphate, a derivative of vitamin B6 (or pyridoxine). They primarily catalyze transamination reactions, which are essential for amino acid metabolism.

Pyridoxal phosphate (PLP) acts as a coenzyme, covalently linked to the enzyme (prosthetic group) by a schiff-type base bond with a lysine residue in the active site. Upon substrate

binding, this lysine is displaced, and the pyridoxal phosphate forms a new schiff base with the amine group of the amino acid.

This interaction generates a conjugated system that weakens the bonds around the α carbon (Cα), notably the Cα–N and Cα–C bonds. Depending on the nature of the apoenzyme, the selective breaking of one or the other of these bonds leads either to a transamination (formation of an α-keto acid), or to a decarboxylation (release of an amine).

Key Points

- The active site consists of a substrate-binding site and a catalytic site; its topology can be investigated using kinetic, chemical, genetic, and structural approaches. The key-lock and the induced-fit models explain substrate recognition.
- The principal mechanisms of enzymatic catalysis include acid-base catalysis, covalent catalysis, metal ion catalysis, electrostatic catalysis, proximity and orientation effects, and substrate strain.
- Coenzymes are organic molecules essential for enzymatic activity; they may be tightly bound (prosthetic groups) or freely diffusible (co-substrates) and mediate the transfer of electrons, chemical groups, or energy.
- Zymogens are inactive enzyme precursors activated by limited proteolysis, allowing spatial and temporal control of enzymatic activity.
- Serine proteases use a catalytic triad (Ser-His-Asp) and proceed through covalent acyl-enzyme intermediates, whereas pyridoxal phosphate (PLP) forms a transient Schiff base that is essential for transamination and related reactions.

Chapter VII: Isolation and purification of enzymes

1. Origins of enzymes

Biologically active enzymes can be isolated from all living organisms. On an industrial scale, a wide variety of sources are used for their production. Of the enzymes used industrially, more than 50% come from fungi and yeasts, about a third from bacteria, while animal and plant sources account for approximately 8% and 4%, respectively.

Microorganisms are the main source of enzymes due to several advantages:

- production cost is generally low;
- high growth rate;
- ease of optimizing growing conditions;
- reproducible and controllable enzymatic production;
- lower presence of undesirable compounds, unlike plant tissues (rich in phenolic compounds) and animal tissues (containing enzyme inhibitors and proteases).

1.1. Plant-derived enzymes

Plant-derived enzymes include amylase, invertase, papain, bromelain, and ficin. They play an important role in the food industry, particularly in the production of syrups, bread, fermented beverages, and dairy products.

Furthermore, plants can also be used as substrates or sources of activating compounds to improve the activity of microbial enzymes used in industry.

1.2. Animal-derived enzymes

Animal-derived enzymes are primarily extracted from specific tissues or organs:

- **Trypsin**: isolated from the pancreas of pigs or cattle; it plays a role in protein hydrolysis.
- **Catalase**: extracted from the liver of cattle or horses; it catalyzes the dismutation of hydrogen peroxide into water and dioxygen.
- **Lipases**: obtained from the pancreas of pigs, sheep or cattle; they ensure the hydrolysis of lipids.
- **Lysozyme**: extracted from egg white; it has antibacterial activity by hydrolyzing the cell wall of bacteria.

1.3.Microbial enzymes

The microorganisms used for enzyme production include bacteria and fungi generally classified as GRAS (Generally Recognized As Safe), guaranteeing their safety for industrial applications.

The majority of industrial enzymes are of microbial origin: about 50% come from molds and yeasts, 35% from bacteria, and 15% from other sources.

The most commonly used microorganisms include:

- **Bacteria**: *Bacillus subtilis*, *Bacillus licheniformis* and various species of *Streptomyces*.
- **Fungi and yeasts**: *Aspergillus, Mucor, Rhizopus, Kluyveromyces* and *Saccharomyces*.

These microorganisms have the advantage of being cultivable in large quantities over short periods, using established fermentation processes. Microbial enzyme production is therefore particularly suited to large-scale industrial exploitation, due to inexpensive culture media and rapid production cycles.

2. Study methods

2.1. Enzyme extractions

Enzyme extraction encompasses all processes aimed at isolating an enzyme from the biological environment in which it is naturally present. It primarily involves releasing the enzyme from the cell by rupturing the cell wall or plasma membrane, followed by its recovery in a suitable medium.

Depending on the nature of the organism and the characteristics of the enzyme, this release can be achieved by mechanical, physical, chemical, or enzymatic processes.

2.1.1. Choice of extraction techniques

The choice of extraction method depends on several key factors, including the nature of the biological material (cell type and structural complexity), the intracellular or extracellular localization of the target enzyme, and the conditions required to preserve its stability and activity during purification. Additional considerations include the sensitivity of the enzyme to factors such as temperature, pH, and shear forces, as well as the availability of appropriate equipment and the intended scale of the extraction process.

2.1.2. Extraction techniques

a. Mechanical methods

Mechanical extraction methods are based on the physical disruption of cells to release intracellular enzymes. They involve the application of shear forces, pressure, or cavitation, and are particularly useful for breaking robust cell types.

-Homogenizers (Dounce and Potter–Elvehjem): these devices ensure controlled cell disruption using a piston within a glass tube. The generated shear forces gently break cells while preserving subcellular structures such as nuclei or mitochondria, making them suitable for fractionation studies.

-French Press: cells are subjected to very high pressure and then forced through a narrow valve, causing abrupt decompression. This pressure drop leads to efficient cell lysis, especially in bacteria, with relatively uniform disruption.

-Disruption bomb: cells are incubated under high pressure in the presence of a gas (commonly nitrogen). Rapid decompression results in gas expansion within the cells, leading to their rupture. This method is effective for microorganisms and some plant tissues.

-Sonication: ultrasound waves generate cavitation (formation and collapse of microbubbles), producing localized shock waves that disrupt cell membranes. It is widely used for microbial and animal cells but may generate heat, requiring cooling to preserve protein activity.

b. Physical methods

Physical extraction methods exploit changes in the physicochemical environment to permeabilize or rupture cell membranes without the use of chemical reagents.

Osmotic shock: Cell lysis is a physical method that involves placing cells in a hypo-osmotic solution. Water then enters the cell, causing the plasma membrane to rupture, especially in fragile cells, with minimal damage. This method can preserve the nuclei, but their accidental rupture increases viscosity due to the release of chromatin. Its main drawback is the potential for organelle damage.

c. Chemicals methods

Chemical extraction methods employ reagents such as detergents, enzymes, or osmotic modifiers to solubilize cellular membranes and release intracellular contents.

Chemical lysis: This technique relies on the use of a lysis buffer containing salts (Tris-HCl, EDTA) to control pH and osmolarity, and detergents (Triton X-100, SDS) to disrupt cell membranes. It is applicable to animal, plant, and microbial cells. Appropriate detergent dosage allows for membrane disruption while preserving the native structure of the proteins.

d. Enzymatic methods

Enzymatic lysis involves the destruction of the cell wall using specific enzymes. Lysozyme digests bacterial peptidoglycan, especially in Gram-positive bacteria, while in Gram-negative bacteria, prior permeabilization of the outer membrane (by Tris-EDTA) is necessary. Other enzymes, such as lyticase and zymolyase, act on yeast cell walls by hydrolyzing their components.

2.2.Purification of the enzymes

Purification is a step that involves removing impurities from a crude extract in order to isolate the enzyme of interest. It generally relies on the use of several successive or complementary techniques.

2.2.1. Choice of purification techniques

The choice of methods depends on the specific properties of the enzymes, such as their molecular size, solubility, electrical charge, affinity for certain molecules, and adsorption characteristics.

2.2.2. Purification techniques

a. Separation techniques based on solubility properties

-***Differential precipitation with ammonium sulfate***: proteins are selectively precipitated by increasing salt concentration, which reduces their solubility (“salting out”). This step concentrates the enzyme and removes a large proportion of contaminants.

b. Separation techniques based on size properties

-***Dialysis***: small molecules (salts, metabolites) are removed through a semi-permeable membrane, while macromolecules such as proteins are retained.

-***Membrane filtration*** **(ultrafiltration)**: uses membranes with defined molecular weight cut-offs to concentrate proteins or separate them by size.

-***Gel filtration*** **(size-exclusion chromatography)**: separates molecules according to their hydrodynamic volume; larger molecules elute first, followed by smaller ones.

c. Separation techniques based on density

-***Centrifugation and ultracentrifugation***: particles are separated according to their size and density under high centrifugal force. These techniques are particularly useful for clarifying extracts and isolating sub-cellular fractions.

d. Separation techniques based on molecular weight differences

-***Polyacrylamide gel electrophoresis*** (SDS-PAGE): separates proteins according to molecular weight after denaturation, mainly used for analysis and purity assessment.

-***Gel filtration*** (size-exclusion chromatography): also contributes to molecular weight-based separation under native conditions.

e. Separation techniques based on isoelectric point (pHi) / charge

-***Simple electrophoresis***: proteins migrate in an electric field depending on their net charge.

-***Ion-exchange chromatography:*** separates proteins based on their interaction with charged resins (anion or cation exchangers), allowing fine resolution by adjusting pH and ionic strength.

f. Separation techniques based on protein hydrophobicity and hydrophilicity

-***Partition chromatography***: proteins are separated according to the degree of hydrophobic interactions with the stationary phase, often modulated by salt concentration.

g. Separation techniques based on specific interactions with other biomolecules

-***Affinity chromatography***: a highly selective method that exploits the specific binding between the enzyme and a ligand (e.g., substrate analog, inhibitor, cofactor). This technique often yields high purity in a single step.

2.2.3. Characteristic parameters of a purification step

Establishing a purification protocol involves selecting and combining several extraction and isolation techniques. At each purification step, it is necessary to perform a test to monitor the purification process. This test must be specific, reproducible, and quick to carry out.

Two parameters are then determined:

Quantitative criterion (yield): this parameter reflects the preservation of enzymatic activity throughout the purification process. It is expressed as:

- **R** = (enzymatic activity of step n) / (enzymatic activity of step n-1) x 100

Qualitative criterion (degree of purification): this parameter indicates the enrichment of the enzyme relative to total protein content. It is defined as:
Purification Degrees = specific activity of step n / specific activity of step n-1

Because multiple techniques are applied successively, purification requires a careful balance between these two criteria. Excessive purification steps may increase enzyme purity but often lead to a significant loss of activity. Therefore, an optimal compromise must be achieved to maximize both enzyme recovery and enrichment.

Key Points

- Enzymes used in industry are derived primarily from microorganisms, which are favored for their rapid growth, low production cost, and the ease with which fermentation processes can be controlled.

- Enzyme extraction involves releasing the enzyme from its biological source while preserving its activity; mechanical, physical, chemical, or enzymatic methods may be employed depending on the sample and the enzyme's fragility.
- Purification exploits differences in protein solubility, size, density, charge, hydrophobicity, and specific affinity, often through a combination of complementary techniques.
- Affinity chromatography is one of the most selective purification methods and frequently yields highly purified enzymes in a single step.
- The progress of purification is assessed in terms of yield (retention of enzymatic activity) and purification factor (increase in specific activity); thus, a balance must be achieved between purity and activity recovery.

Chapter VIII: Enzyme engineering

1. Nature and origin of enzymes

Enzymes are protein biomolecules (more rarely catalytic RNAs called ribozymes) that play a crucial role as biological catalysts by accelerating chemical reactions within cells. They originate from a wide range of biological sources such as animal and plant tissues, as well as microorganisms such as bacteria, yeasts, and fungi. Their origin and nature influence their physicochemical properties, catalytic activity, and optimal operating conditions.

2. Methods for enzyme immobilizing

2.1. Definition and principles

Immobilized enzymes are enzymes fixed or confined within a solid support while retaining their catalytic activity. They can be bound to the surface of the support or entrapped within a matrix, while still enabling the diffusion of substrates and products.

An immobilized enzyme system consists of three elements: the enzyme, the support, and the method of fixation.

Enzyme immobilization offers several advantages, including enzyme reusability, improved stability, easier reaction control, and simple enzyme/product separation, thus limiting contamination. It also enables for the development of multi-enzyme systems.

Based on the interaction modes between enzymes and support carriers, enzyme-immobilization methods can be classified into physical methods and chemical methods. Adsorption and entrapment could be assigned to physical methods, in which there are no covalent interactions between enzymes and support carriers, while covalent attachment and cross-linking belong to chemical methods. In some cases, multiple enzyme-support interactions are exploited for enzymes immobilization. The various enzyme immobilization methods are illustrated in the figure below.

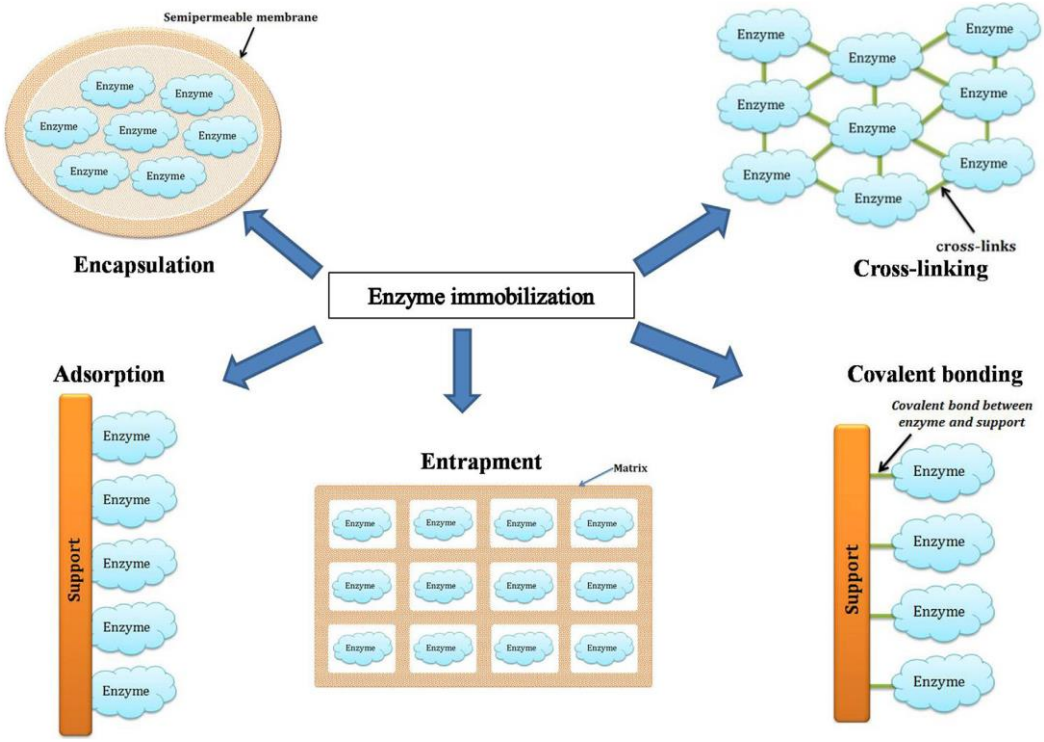


Figure 33: The methods for enzyme immobilization (Sirisha et al., 2016).

2.2. Physical methods

2.2.1. Immobilization by adsorption

Adsorption is a method of enzyme immobilization that involves attaching the enzyme to the surface of an inert solid support through weak interactions, without altering its structure. These interactions include hydrogen bonds, Van der Waals forces, as well as electrostatic and hydrophobic interactions.

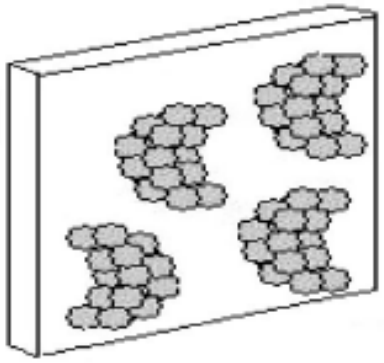


Figure 34: Immobilization by adsorption (Wahab, 2025).

The supports used may be inorganic (clays, porous glass or porous silica) or organic (polysaccharides such as cellulose acetate, cellulose nitrate, dextran, agarose, alginate and polymers such as polystyrene and polyethylene). The enzyme may be adsorbed onto the surface of the support or within the pores of mesoporous materials.

The immobilization procedure involves contacting the substrate with an enzymatic solution, followed by an incubation step to enable fixation. The adsorption efficiency depends on several parameters, including pH, temperature, ionic strength, and enzyme concentration.

Advantages

- **Simplicity of implementation**: the method is easy to perform and does not require chemical reagents or complex activation steps.
- **Low cost**: it is an economical and accessible process.
- **Preservation of enzymatic activity**: the method is generally non-denaturing.
- **Reversibility**: possibility of regenerating the support by desorption and replacement of the enzyme.

Disadvantages

- **Low stability:** due to the weak and reversible interactions.
- **Risk of desorption:** specially under variations in pH, temperature or ionic strength.
- **Reduced accessibility of the active site:** related to the random orientation of the enzyme.

2.2.2. Immobilization by entrapment

Immobilization by entrapment involves retaining enzyme molecules within a three-dimensional network formed by a water-insoluble polymer, or encapsulating them within microcapsules delimited by a semi-permeable membrane. In these systems, the enzyme is retained purely physically, without covalent bonding. The pore size of the network or membrane is adjusted to enable the diffusion of substrates and reaction products, while preventing enzyme leakage.

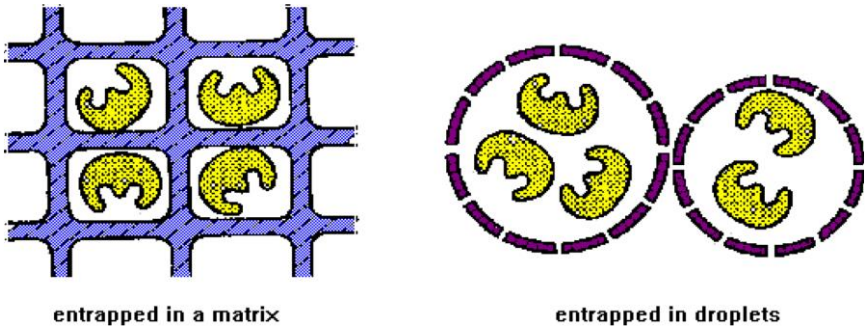


Figure 35: mmobilization by entrapment (Wahab, 2025).

a. Entrapment in a gel

In this approach, the enzyme is incorporated into an insoluble gel made of an organic (polymer) or inorganic matrix. The enzyme is retained within the three-dimensional network, whose porosity ensures both its retention and the diffusion of substrates toward the active site, as well as the removal of byproducts. Commonly used matrices include polyacrylamide, alginate, and starch gels, as well as cellulose polyacetate fibers.

The choice of polymer depends on several parameters:

- mechanical properties of the gel (resistance to compression and shear forces),
- chemical properties (toxicity, stability depending on pH),
- physical properties (permeability and pore size).

b. Encapsulation

Encapsulation involves enclosing the enzyme within microcapsules surrounded by a semi-permeable membrane. The diameter of these capsules typically ranges from a few micrometers to around one hundred micrometers. The membrane ensures the retention of the enzyme within a defined volume, which is particularly well-suited to continuous processes, especially for hydrolases. It enables for the selective diffusion of small molecules (substrates and products), thus facilitating product recovery from the effluent, while preventing the release of macro-enzyme molecules.

The membranes used can be inorganic (silica gels), organic (nafion), polymeric (polyacrylamide, polyurethanes) or composite (carbon pastes).

Advantages

- Well-known polymerization and gelation reaction.
- Absence or very weak chemical interaction with the enzyme, thus preserving its intrinsic properties and conformation.
- Applicable to a wide variety of enzymes, including in mixture form.
- Obtaining supports in various adaptable forms (films, beads, fiber…)
- Possibility of immobilizing the enzyme in a single step with good yield
- Does not involve the active groups of the enzyme.

Disadvantages

- The localization of the enzyme inside the polymer poses steric problems (limited efficiency due to difficult access of the substrate to the enzyme and of the product outside the polymer).
- Polymerization conditions (e.g., high pH) can prove denaturing for the enzyme.
- Risk of enzyme leakage when the pore size is too large.

2.3. Chemical methodss

Immobilization by covalent bonding is one of the most widely used methods in applied enzymology, due to the high stability of the complexes formed between the enzyme and the support.

The methods of immobilizing enzymes by covalent bonding can be divided into two groups:

2.3.1. Immobilization by covalent bonds on support

The principle of this method is based on the formation of irreversible covalent bonds between the functional groups of the enzyme and those of a previously activated support. The functions involved notably include amine groups (-$NH._2$), carboxylics (-COOH), thiols (-SH) and hydroxyls (-OH), as well as other functions such as imidazole, indole or phenolic.

Since these groups are generally unreactive, an activation step is necessary. This can involve either the enzyme or the support; however, activation of the support is most often preferred in order to limit the risk of denaturation and preserve the enzyme's catalytic activity.

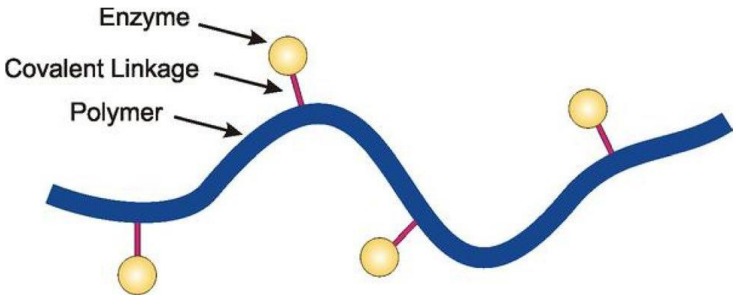


Figure 36: Immobilization by covalent bonding (Wahab, 2025).

Activation Agents

Currently, the most commonly used activating agents for establishing covalent bonds between enzymes and their matrices include cyanogen bromide, glutaraldehyde, and other compounds lised in Table 1.

Table 02: Covalent grafting method of enzymes

Functional groups		**Activation Agents**
Enzyme	**Support**	
-NH2	-OH	Cyanogen bromide
-NH2	-NH2	Glutaraldehyde
– COOH	-NH2	Carbodiimide
-NH2	– COOH	Acyl azide carbodiimide
-SH	-SH	4,4′-Dithiodipyridine

The matrices commonly used in this immobilization method are either:

- **Natural** such as sephadex, agarose, Sspharose and cellulose.
- **Synthetics** such as acrylamide, methacrylic acid and styrene.
- **Inorganic substances** including glass and aluminosilicates.

Covalent immobilization by cyanogen bromide

The covalent immobilization method for enzymes often relies on the activation of polysaccharide matrices (such as cellulose, dextran, starch, or agarose) are activated using cyanogen bromide (CNBr). These natural polymers possess numerous OH groups, but their

reactivity is insufficient for direct binding to enzymes. CNBr activates these groups by forming a reactive derivative (cyclic imidocarbonate), which then enables covalent binding of the enzyme via its free amino groups (Figure 37).

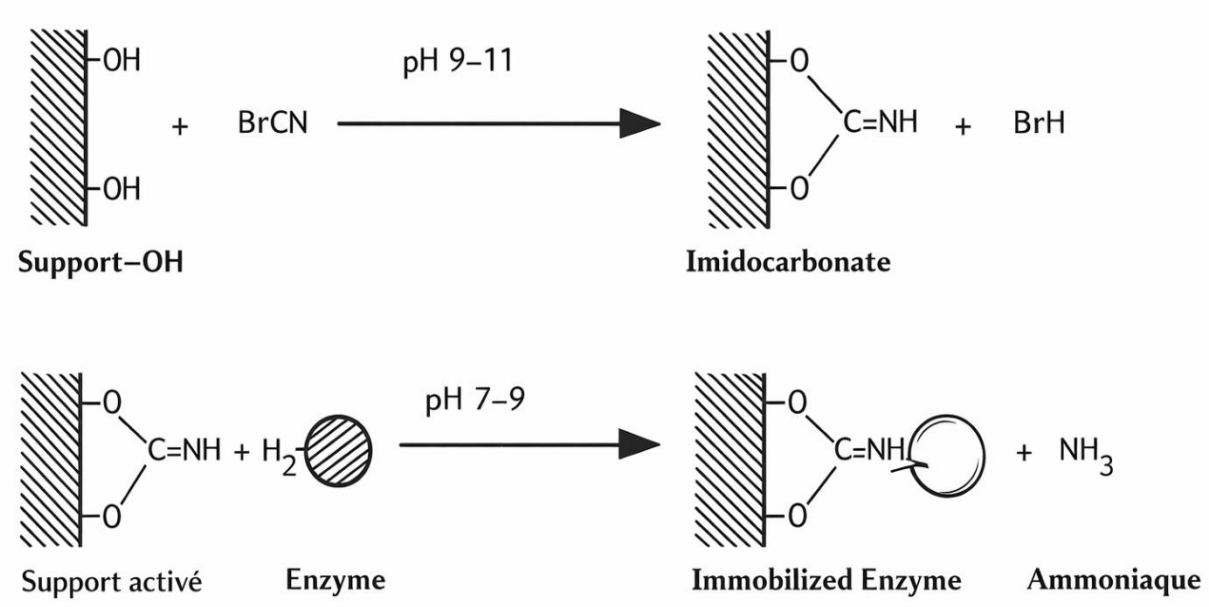


Figure 37: Covalent immobilization of enzyme by cyanogens bromide(CNBr) activation (*Adapted from* Sabotič et al., 2012)

2.3.2. Immobilization by cross-linking

Crosslinking is an immobilization method that uses cross-linking agents to link enzymes together through covalent bonds, without the need for a matrix or support. This process leads to the formation of an insoluble, three-dimensional enzymatic network, unlike free enzymes.

Two main forms can be distinguished:

• Direct cross-linking, where enzymes are linked together by bi- or multifunctional agents.
• Co-cross-linking, which involves adding an inert protein (often bovine serum albumin, BSA) in addition to the cross-linking agent, usually glutaraldehyde, to improve the efficiency of the process. The resulting complex has a high molecular weight and is insoluble.

Advantages

- Increased stability due to the covalent bonds.
- Wide range of immobilization strategies can be employed.

- Wide variety of mineral (glass, silica, ceramic…) and organic (cellulose, synthetic polymer…) supports.
- It is possible to perform immobilization in the presence of a substrate to avoid inactivation (protection of the active site).
- Strength of the enzyme-substrate bond.
- Gives the enzyme a longer lifespan,

Disadvantages

- Implementation of often complex chemical reactions
- Experiment length (support activation stage)
- Risk of chemical changes to the enzyme (loss of activity)
- The enzyme needs to be purified beforehand.
- Immobilization is more complex to implement (choice of groups to activate…)
- The quantities of immobilized enzymes are lower compared to the two previous methods with fixation yields generally below 100%.

2.4. Areas of application of immobilized enzymes

Owing to their high specificity of action (biospecificity), enzymes are indispensable tools in manufacturing and analysis, playing a role in many fields such as research, quality control and industrial production of metabolites.

2.4.1. Analytical

In medicine, enzyme-impregnated supports (papers or strips) are widely used in various clinical tests, particularly for the quantification of analytes such as cholesterol, uric acid, or certain hormones (enzyme-based test strips used for the determination of insulin).

Furthermore, ELISA-type techniques rely on the use of fixed enzymes, generally linked to antibodies themselves adsorbed onto the walls of plastic microplates, enabling for sensitive and specific clinical assays (detection of human chorionic gonadotropin (hCG) in pregnancy tests.

2.4.2. Therapeutic

Administering enzymes to treat certain pathologies related to enzyme deficiencies faces several obstacles, such as their degradation by proteases or their capture and hydrolysis by macrophages.

To overcome these limitations, enzymes can be associated with protective molecules (albumin, dextran, polyethylene glycol), encapsulated in microcapsules, or even incorporated into red blood cells. These strategies improve their stability and their *in vivo* efficiency.

2.4.3. Chemical synthesis

Immobilized enzymes are widely used in production units for compounds of pharmaceutical interest, such as amino acids, organic acids, antibiotics, and steroid hormones.

For example, L-aminoacylase extracted from Aspergillus oryzae is immobilized by ionic bonds on DEAE-Sephadex and used for the continuous production of L-amino acids (L-methionine, L-alanine, L-phenylalanine, L-tryptophan, L-valine, etc).

2.4.4. Agri-food

In the food industry, immobilized enzymes play a major role:

- In glucose production, fungal α-amylase hydrolyzes starch into sweet syrups.
- In the dairy industry, lactase (of fungal or yeast origin) hydrolyzes lactose into glucose and galactose.
- Finally, yeast invertase enables the production of invert sugar, characterized by a higher sweetening power and less crystallization.

2.5. Enzyme immobilization and use in bioreactors

2.5.1. Bioreactor

A bioreactor is a closed system that enables biological reactions to be carried out under controlled conditions in order to produce substances of interest. It is widely used in biotechnology, food processing, pharmaceuticals, and environmental applications.

There are two main types:

- the fermenter, which uses living microorganisms under sterile conditions;
- the enzymatic reactor, where enzymes (often immobilized) catalyze the transformation of a substrate into a product, generally continuously.

The choice of a reactor depends on technical and economic factors, including cost and target productivity, the nature of the enzyme and reaction kinetics, the properties of the support, and the operating conditions (pH, temperature, flow and stability).

Performance is evaluated through several essential criteria: substrate conversion rate, final product concentration, productivity, biocatalyst utilization efficiency and energy efficiency.

2.5.2. Role of bioreactors

Bioreactors play a central role in:

-Optimizing enzymatic reactions to maximize yield and productivity.

-Precise control of operating conditions (pH, temperature, agitation, etc.) to ensure optimal enzymatic activity.

-Improving enzyme stability, particularly by preventing their denaturation.

-The possibility of using immobilized enzymes, promoting their reuse and increasing the efficiency of the process.

2.5.3. The components of a bioreactor

A bioreactor consists of several essential elements that ensure the proper functioning of biological reactions:

- **The tank**: main chamber where biochemical reactions and cell growth take place; it is usually made of steel at pilot scale and of glass at laboratory scale.
- **The lid**: airtight device ensuring protection of the environment against any external contamination.
- **The injection and sampling system**: often equipped with a syringe and a catheter, it enables the introduction and removal of solutions during culture.
- **The agitation system**: guarantees effective homogenization of the environment, ensuring good distribution of heat and species present.
- **The sensors (or probes)**: devices for measuring key parameters such as temperature, pH and dissolved oxygen concentration, in order to maintain optimal conditions.
- **The control system**: automated system, usually connected to a computer, enabling the monitoring, recording and regulation of the operating parameters of the bioreactor.

2.5.4. Types of bioreactors

In enzyme engineering, bioreactors are classified according to their mode of operation, the nature of the enzymes used, and the contact methods between the enzyme and the substrate.

a. According to the operating method

-Batch bioreactor: the enzyme and the substrate are introduced at the beginning of the process, and the reaction continues until the substrate is exhausted.

-Semi-continuous bioreactor (fed-batch): the substrate is added gradually in order to limit inhibition phenomena and optimize enzymatic activity.

-Continuous bioreactor: the substrate is continuously fed while the products are simultaneously evacuated, ensuring stable production over long periods.

b. Depending on the nature of the enzyme

-Free enzyme bioreactors: the enzyme is dissolved in the reaction medium; this system is suitable for short-duration processes or those using inexpensive enzymes.

-Immobilized enzyme bioreactors: the enzyme is fixed to a solid support or encapsulated which enables for its reuse and improves its stability.

c. According to the kind of mixing and enzyme-substrate contact

-Agitated bioreactor (STR: Stirred-Tank Reactor): equipped with a mechanical stirring system ensuring homogenization of the medium; widely used on an industrial scale.

-Fixed bed bioreactor (FBR: Fixed Bed Reactor): the immobilized enzyme is held on a fixed support, while the substrate flows through it.

-Fluid bed bioreactor (FBR: Fluidized Bed Reactor): the particles supporting the enzyme are put into suspension, promoting exchanges and the efficiency of the reaction.

-Membrane bioreactor: a membrane separates the enzyme from the substrate or products, enabling better process control and facilitating separation operations.

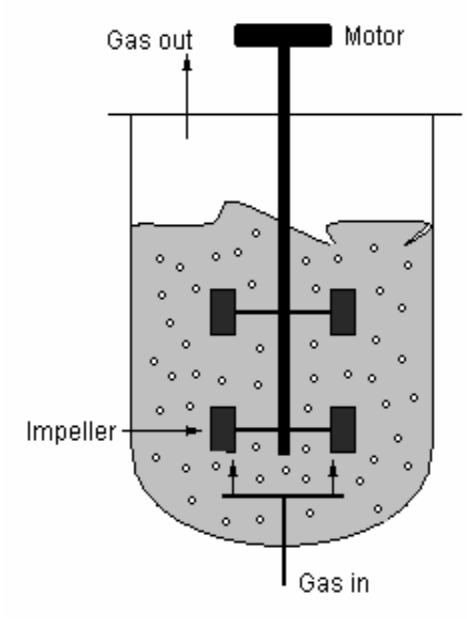


Figure 38: A typical stirred-tank bioreactor (Ratledge & Kristiansen 2006).

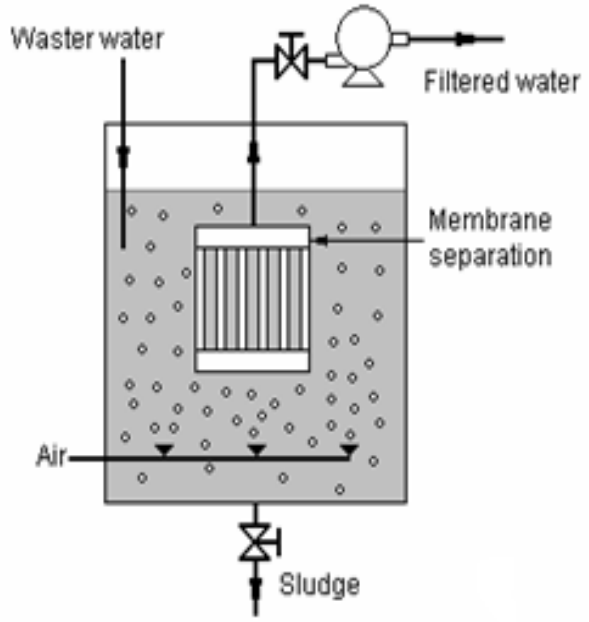


Figure 39: Typical membrane bioreactors (Ratledge & Kristiansen 2006).

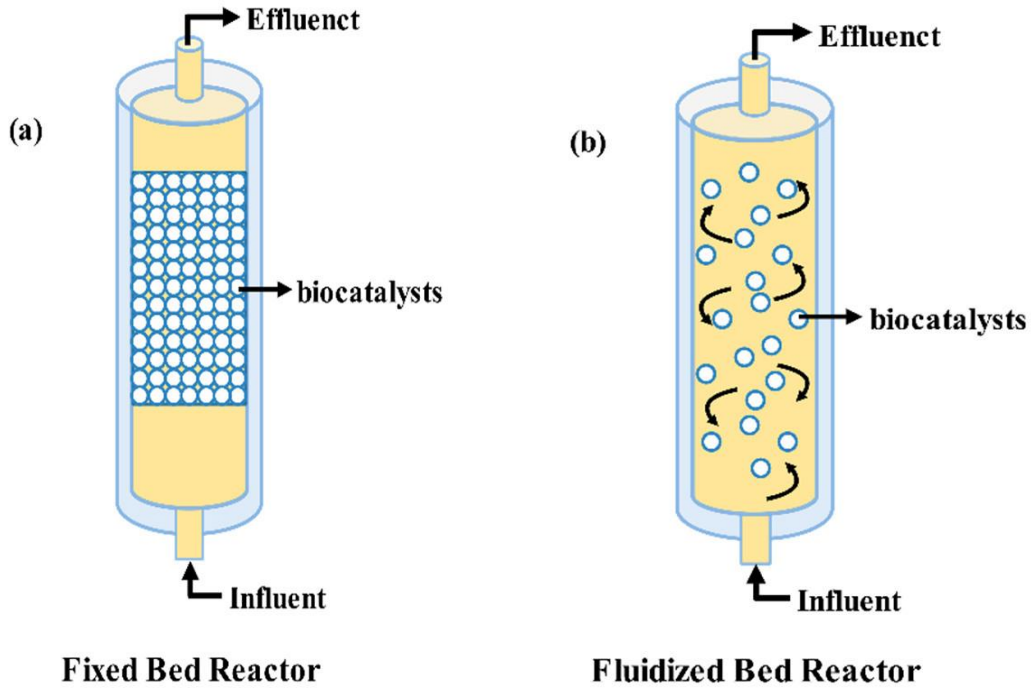


Figure 40: Schematic diagram of fixed bed reactor and fluidized bed reactor (Liu et al., 2022).

3. Applications of enzymes in biotechnology

3.1.Industrial preparation and production of enzymes

The concept of "industrial enzyme" emerged in the late 19th and early 20th centuries, with the isolation and large-scale production of the first enzymes such as invertase and amylase.

3.1.1. Origin and importance of industrial enzymes

The enzymes used in industry can come from plant, animal, or microbial sources. However, microorganisms are widely preferred because they have a high capacity for enzyme secretion, thus facilitating recovery processes. Microbial enzyme production offers several significant advantages, including independence from seasonal and geographical constraints, the ability to use inexpensive substrates, and great flexibility in optimizing culture conditions. Furthermore, these processes enable for high yields while ensuring rigorous control of production parameters.

3.1.2. Stages of industrial enzyme production

a. Definition of the characteristics of the enzyme

The first step is to precisely define the properties of the enzyme of interest. Enzyme specificity is a fundamental criterion, particularly in applications involving the hydrolysis of complex macromolecules. Furthermore, it is essential to determine the optimal conditions for activity, especially pH and temperature, which directly influence stability and catalytic efficiency. In many industrial applications, good resistance to high temperatures is required to ensure the sterility of the reaction medium.

b. Selection of the microbial strain

The selection of the microbial strain relies on appropriate isolation techniques, such as enrichment cultures followed by cultures on selective media. The chosen strain must be able to grow on simple and cost-effective media, while producing a minimal amount of undesirable secondary metabolites. It must also efficiently secrete the enzyme to facilitate its recovery and offer safety guarantees by being non-pathogenic and devoid of any production of toxic substances.

c. Production by fermentation

The industrial production of enzymes relies primarily on fermentation processes. Surface cultures, used particularly for filamentous fungi, have limitations related to the

control of physicochemical parameters. Consequently, submerged cultures in agitated liquid media are now preferred. They enable for precise control of temperature, pH, and aeration, reduce the risk of contamination, and facilitate the transition from laboratory to industrial scale.

d. Extraction and recovery of enzymes

After fermentation, the enzymes must be separated from the biomass and the culture medium. This separation is achieved through processes such as filtration, centrifugation, or precipitation. When enzymes are extracellular, their recovery is relatively simple since they are present in the culture medium. However, for intracellular enzymes, a preliminary cell lysis step is necessary, using mechanical, physical, or chemical methods. The choice of method depends on the enzyme's location and the characteristics of the biological system used.

e. Enzyme concentration

After extraction, the resulting enzyme solutions are often diluted and require a concentration step. This step must be carried out in a way that preserves the enzyme activity. Several techniques can be used, such as precipitation with salts, particularly ammonium sulfate, precipitation with organic solvents, isoelectric point precipitation, or ultrafiltration using semi-permeable membranes. These methods reduce the volume while increasing the concentration of the active enzyme.

f. Purification of enzymes

The degree of purification required depends on the enzyme's final application. Industrial applications generally tolerate partially purified preparations, while the medical and analytical fields require highly purified enzymes. Among the techniques used, chromatography plays a central role due to its high efficiency. Other methods, such as crystallization or electrophoresis, can also be employed, although their use is often limited to the laboratory scale.

g. Formulation of enzyme preparations

Enzymes intended for industrial use are formulated in various forms to facilitate their use, transport, and storage. They can be presented in solid form, particularly as powder or granules, in liquid form with a high concentration of active ingredient, or immobilized on

solid supports. This last form has the advantage of improving the enzyme's stability and enabling its reuse in industrial processes.

3.1.3. Regulation of industrial enzymes

The use of enzymes, particularly in the food industry, is subject to strict regulations designed to ensure consumer safety. Raw materials of animal origin must come from healthy animals that meet sanitary standards, while plant-based materials must be derived from healthy plants and edible parts. The microorganisms used must be non-pathogenic and non-toxin-producing. Finally, enzyme preparations must meet rigorous chemical and microbiological quality criteria, and their use must not compromise food safety or quality.

3.2. Applications in industrial sectors (pharmaceutical, cosmetic, agricultural)

3.2.1. Agri-food field

Enzymes are widely used in the food industry due to advances in extraction and production techniques, which has enabled their use as biocatalysts in many industrial processes improving the quality, yield and technological properties of food.

a. Bread making

In bread making, amylases (α and β) are added to flour to increase the fermentable sugar content, thereby improving fermentation, gas production, and bread volume. Simultaneously, proteases partially hydrolyze gluten, reducing its elasticity and improving dough extensibility. Furthermore, xopeptidases promote the release of amino acids, influencing the Maillard reaction and, consequently, the sensory characteristics of the bread. Hemicellulases, particularly xylanases, convert insoluble pentosans into soluble forms, thus improving dough texture and the final product quality. Furthermore, oxidoreductases, such as glucose oxidase, contribute to dough firming and color modification. Finally, invertase hydrolyzes sucrose into glucose and fructose, thereby increasing sweetness and improving fermentation properties.

b. Drinks

In fruit juice clarification, pectinases, such as polygalacturonase and pectin esterase, break down pectins, reducing viscosity and facilitating juice clarification. Their use also improves filterability, yield, and the stability of the finished products.

c. Dairy products

In cheese production, chymosin catalyzes the specific hydrolysis of κ-casein, leading to milk coagulation. The different sources of rennet (animal, microbial, recombinant, or vegetable) are chosen according to technological, economic, or religious constraints.

3.2.2. Pharmaceutical field

In the pharmaceutical field, enzymes play a central role in therapeutics, diagnostics, and the industrial production of drugs. Enzyme replacement therapies, such as glucocerebrosidase or α-galactosidase, are used to treat certain hereditary metabolic diseases by compensating for an enzyme deficiency, thereby limiting the accumulation of toxic substrates in cells.

Furthermore, thrombolytic enzymes, such as streptokinase and urokinase, are used in the treatment of cardiovascular diseases by catalyzing the conversion of plasminogen to plasmin, the enzyme responsible for breaking down fibrin, a component of blood clots. Similarly, digestive enzymes, such as pancreatic amylases, lipases, and proteases, are administered to patients with pancreatic insufficiency to ensure efficient hydrolysis of nutrients and improve their intestinal absorption.

Therapeutic proteases, particularly trypsin and collagenases, are used for the enzymatic cleansing of wounds, removing necrotic tissue and promoting healing. In the field of diagnostics, enzymes such as glucose oxidase, peroxidase, and transaminases are widely used for the specific measurement of biomolecules and as biomarkers, enabling the diagnosis and monitoring of numerous pathologies.

Furthermore, enzymes play a major role in the pharmaceutical industry as biocatalytic agents. Enzymes such as penicillin acylase, lipases, and transaminases are exploited for their stereospecificity, enabling the synthesis of chiral molecules with high purity and yield.

Moreover, microbial fermentation processes involving enzymes enable for the industrial production of antibiotics, vitamins, and other therapeutic compounds.
Enzymes also play a role in the activation of prodrugs, where biocatalysts such as esterases or cytochrome P450 catalyze biotransformation reactions that convert inactive compounds into active forms. Finally, some enzymes possess antimicrobial properties, such as lysozyme, contributing to the fight against infections. Enzymes coupled to antibodies, such as peroxidase

used in ELISA tests, enable for highly sensitive and specific detection of target molecules through colorimetric or luminescent reactions.

3.2.3. Cosmetics

In the cosmetics industry, enzymes are widely used for their specific properties that enhance the efficacy and safety of formulations. In cleansing and exfoliating products, proteases, such as papain and bromelain, catalyze the hydrolysis of proteins in the stratum corneum, ensuring gentle removal of dead cells and promoting cell renewal. Simultaneously, lipases contribute to the degradation of skin lipids, thereby improving the effectiveness of cleansing products and maintaining the balance of the hydrolipidic film.

Enzymes also play a role in skin protection and anti-aging care. Antioxidant enzymes, such as superoxide dismutase and catalase, neutralize reactive oxygen species, limiting oxidative stress, which is responsible for premature skin aging. Furthermore, in depigmenting treatments, tyrosinases and especially their inhibitors are used to modulate melanin synthesis, thus reducing pigmentation spots and improving skin tone uniformity.

Furthermore, certain enzymes play an important role in the preservation and microbiological stability of cosmetic products. Oxidative enzymes, such as glucose oxidase and peroxidase, exhibit antimicrobial properties and help limit the growth of microorganisms in formulations.
Finally, in hair care, enzymes are used to improve coloring processes. Oxidases and peroxidases, such as glucose oxidase, enable for the controlled production of hydrogen peroxide directly at the hair shaft, resulting in milder and less damaging coloration. Furthermore, tyrosinases and laccases catalyze the oxidation of chromogenic precursors, facilitating the formation of colored pigments while ensuring greater effectiveness and better adaptation to the characteristics of the hair fiber.

3.2.4. Agronomic field

In agronomy, enzymes play a fundamental role in improving soil fertility. Phosphatases, particularly acid phosphatase and alkaline phosphatase, catalyze the hydrolysis of organic phosphate esters present in the soil, releasing inorganic phosphate that is directly assimilable by plants and contributes to optimizing their mineral nutrition. Furthermore, cellulases (endoglucanases) and hemicellulases (xylanases) are involved in the decomposition

of plant residues, promoting humus formation and improving the physical structure and biological activity of the soil.

Enzymes also contribute to crop protection by playing a role in defense mechanisms against phytopathogenic agents. Chitinases hydrolyze the chitin that makes up the cell walls of fungi, thus limiting their development, while β-1,3-glucanases degrade the glucans in these cell walls, strengthening plant resistance to fungal infections.

Finally, enzymes play a key role in the processing and valorization of agricultural residues. Ligninases, such as lignin peroxidase and manganese peroxidase, enable the degradation of lignin, a complex and particularly resistant polymer, thus facilitating the transformation of lignocellulosic residues. Furthermore, cellulases and xylanases are widely used in composting processes to accelerate the decomposition of plant waste, enabling its conversion into rich organic amendments and promoting the sustainable management of agricultural resources.

3.3. Enzymatic biosensors

3.3.1. Definition

A biochemical biosensor, also called a biological sensor, is an analytical device situated at the interface of several scientific disciplines, including physics, chemistry, and biology. It relies on the integration of various technologies, such as molecular biology, microelectronics, optics, and computer science.

A biosensor consists of two fundamental elements:

- **The bioreceptor**, which ensures the specific recognition of the analyte (target molecule).
- **The transducer** which converts the biological signal resulting from this interaction into a measurable physical signal. The latter can be optical, electrochemical, electromagnetic, piezoelectric, calorimetric or acoustic in nature.

Thus, the general principleof biosensors is based on the transformation of a biochemical event (specific interaction between the bioreceptor and the analyte) into a quantifiable signal. This conversion enables the detection and/or quantification of biomolecules in various matrices such as food, the environment, or biological fluids.

According to the International Union of Pure and Applied Chemistry (IUPAC), a biosensor must meet several criteria: it must be small, compact, provide a reversible signal, ensure accurate measurements, and ensure effective interaction between the biological system and the transducer.

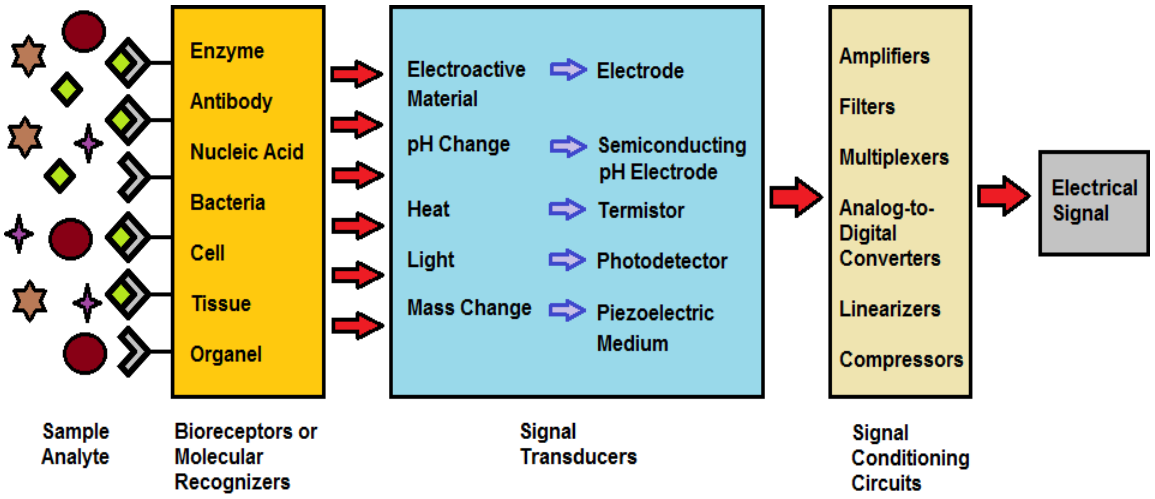


Figure 41: Schematic representation of the operating principle of a biosensor (Vargas-Bernal et al., 2012).

The figure 41 illustrates the general operating principle of a biosensor. The device extracts relevant information from the species to be detected, enabling its evaluation. This information is then processed, recorded, and potentially stored.

The choice of bioreceptor depends directly on the nature of the targeted analyte. Various types of bioreceptors have been developed over time, including:

-Enzymes, which catalyze specific reactions;

-Nucleic acids, involved in recognition by hybridization;

-Biological receptors (hormones, toxins, growth factors, neurotransmitters);

-Whole cells or cellular organelles;

-Antibodies, characterized by their high antigen-antibody specificity.

These biological elements interact with the target analyte, inducing physicochemical changes (variations in pH, charge, mass, potential, etc.). These variations are detected by the transducer, converted into electrical signals, then amplified and processed to provide a usable analytical response.

3.3.2. Biosensor performance parameters

Like any analytical method, biosensors are evaluated based on several essential parameters:

- **Selectivity**: the ability of the biosensor to specifically recognize the target analyte in the presence of other species. It depends mainly on the bioreceptor, but can be influenced by the type of transducer used.
- **Sensitivity**: the ability of the sensor to detect small variations in the concentration of the analyte. It corresponds to the ratio between the variation of the measured signal and that of the analyzed quantity.
- **Reproducibility**: ability to obtain similar results in repeated measurements under the same experimental conditions.
- **The detection limit**: lowest concentration of the analyte that can be reliably and significantly detected.
- **Accuracy**: degree of agreement between the measured value and the actual value. The observed difference corresponds to the absolute error.
- **Lifespan**: time interval during which the biosensor maintains satisfactory analytical performance.
- **The drift**: gradual variation of the sensor's baseline signal in the absence of analyte, which can affect the reliability of measurements over time.

3.3.3. Classification

Bioreceptors can be classified according to their mode of interaction with the analyte. We thus distinguish affinity bioreceptors, which recognize and bind the analyte without transforming it; metabolic bioreceptors, which possess catalytic activity and transform the analyte; and biomimetic bioreceptors, more recently developed, which mimic the behavior of natural biological systems.

3.3.3.1. Affinity Bioreceptors

Affinity bioreceptors are characterized by their ability to specifically bind the analyte without inducing chemical transformation. Their function relies on non-covalent interactions (hydrogen bonds, ionic interactions, Van der Waals forces, and hydrophobic interactions), which, although weak individually, confer high affinity and specificity to the whole.

a. Antibodies

Antibodies, or immunoglobulins, are glycoproteins produced by the immune system in response to the introduction of an antigen. They possess specific recognition sites capable of binding to this antigen with high affinity, thus enabling its neutralization.

In biosensors, antibodies are widely used for direct immunological detection, without the need for markers. The formation of the antigen-antibody complex must induce a measurable change in a physical property (mass, charge, refractive index, etc.), which is detected by the transducer.

b. Membrane receptors

Membrane receptors are non-catalytic proteins of non-immune origin, located in the plasma membrane, cytoplasm, or cell nucleus. They are capable of interacting with a wide variety of ligands, such as hormones, neurotransmitters, growth factors, or odorant molecules.

Among them, G protein-coupled receptors (GPCRs) constitute the largest family of receptors in mammals, representing approximately 3.4% of the genome. They ensure the transmission of extracellular signals to intracellular effectors, thus triggering biological responses involved in various functions (vision, taste, smell, neuronal activity, metabolism, immune response).

There are three main classes of receptors, classified according to their mechanism of action:

-Ion channel-activating receptors: their activation leads to the opening or closing of ion channels;

-Enzyme-linked receptors: ligand binding induces catalytic activity;

-G protein-coupled receptors: the active binding of intracellular signaling pathways via G proteins.

Nucleic acids (DNA and RNA) constitute carriers of genetic information whose sequence enables specific recognition by base complementarity.

In biosensors, they can be immobilized:

- in double-strand form, for applications screening toxins or active molecules;

- in single-strand form, in order to detect hybridization phenomena, used in particular in molecular diagnostics and genomics.

These DNA biosensors exhibit high affinity and specificity. They are widely used in pathogen detection, cancer analysis, toxic substance detection, and in the study of DNA alterations induced by pollutants.

3.3.3.2. Metabolic bioreceptors

Unlike affinity bioreceptors, metabolic bioreceptors catalyze a chemical reaction involving the analyte, which is then consumed. The biosensor response therefore is directly dependent on the catalytic activity of the bioreceptor.

These systems are caracterized by their high sensitivity, particularly at low analyte concentrations, due to the signal amplification linked to the enzymatic reaction.

a. Enzymes

Enzymes are biological catalysts, generally protein-based, that accelerate biochemical reactions without being consumed. They possess a specific active site that enables them to recognize a given substrate and transform it into a product.

Some enzymes are made up of proteins only, while others require the presence of a cofactor (metal ion or coenzyme) for catalytic activity.

In enzymatic biosensors, detection can be based on the direct measurement of the substrate or product formed; or on the inhibition of enzymatic activity by the analyte.

Enzymes provide excellent specificity and enable low detection limits. However, their use is limited by their sensitivity to physicochemical conditions (pH, temperature, inhibitors) and their high cost. In addition, denaturation results in an irreversible loss of activity.

b. Microorganisms

Microbial biosensors use living cells (bacteria, yeasts, fungi, or even algae) as recognition elements. These microorganisms contain a set of enzymes capable of catalyzing various metabolic reactions.

The main advantage of these systems is that they do not require enzyme extraction or purification. Furthermore, they can adapt to certain environmental variations and enable for in-situ regeneration of the biocatalyst.
However, they have several limitations including lower selectivity and sensitivity, longer response time, and experimental constraints related to maintaining suitable physiological conditions.

c. Plant or animal tissues

Some biosensors directly utilize biological tissues (plant or animal) rich in natural enzymes. For example: plant tissues (banana for dopamine, cucumber leaf for cysteine); and animal tissues (rabbit muscle or liver for various metabolites).

These systems do not require enzymatic purification, but their implementation remains complex and their selectivity and sensitivity are generally limited.

3.3.3.3. Biomimetic bioreceptors

Biomimetic bioreceptors are synthetic systems designed to replicate the recognition properties of natural biological systems, in terms of affinity and specificity.

a. Aptamers

Aptamers are oligonucleotides (single-stranded DNA or RNA) capable of binding specifically to a target (proteins, small molecules) through their three-dimensional structure.
They offer several advantages over antibodies:
-easy chemical synthesis;
-high stability;
-low immunogenicity and toxicity;
-reduced cost;
-possibility of structural modifications.

b. Molecularly imprinted polymers (MIPs)

Molecularly imprinted polymers are synthetic materials that contain recognition sites specific to a given analyte, mimiking antigen-antibody interaction.

Their principle is based on the polymerization of monomers in the presence of the target molecule (imprint). After removal of this molecule, complementary cavities remain, differing within the polymer matrix, characterized by complementary size, shape and chemical properties, enabling selective recognition of the analyte.

These polymers exhibit high stability, good reproducibility, and resistance to harsh experimental conditions. However, their development requires precise optimization of synthesis parameters to ensure effective imprinting and selectivity.

3.3.4. Applications of enzymatic biosensors

a. Medical and biomedical field

Enzymatic biosensors are widely used for the diagnosis and monitoring of numerous diseases. They enable for the measurement of biomarkers in the blood, such as glucose (diabetes), cholesterol, lactate, or urea. Due to their speed and accuracy, they are integrated into portable devices like glucometers, offering real-time monitoring. They are also used in rapid point-of-care tests, facilitating immediate diagnosis without the need for lengthy laboratory analyses.

b. Agri-food industry

In this field, enzymatic biosensors ensure food quality and safety control. They enable for the measurement of constituents such as sugars (glucose, lactose) or alcohol during fermentation. They also serve to detect contaminants such as pesticides, toxins, or antibiotic residues. Furthermore, they contribute to assessing product freshness by identifying compounds resulting from product degradation.

c. Environmental monitoring

Enzymatic biosensors are used to monitor pollution and analyze the quality of natural environments. In particular, they enable for the measurement of biological oxygen demand (BOD) in water, an indicator of organic pollution. They can also detect pesticides and other toxic substances at very low concentrations. Their ability to provide rapid results directly in the field is a major advantage.

d. Pharmaceutical and biotechnology industry

In this sector, enzymatic biosensors play a crucial role in controlling manufacturing processes, particularly for monitoring enzymatic reactions and fermentations. They are used for the precise quantification of drugs and for screening new molecules, especially for identifying enzyme inhibitors. These applications are essential for the development of new treatments and the optimization of industrial processes.

e. Security and defense

Enzymatic biosensors enable the rapid detection of hazardous substances, including neurotoxic agents such as organophosphate compounds. Their operation often relies on enzyme inhibition, enabling for the rapid identification of toxins. They are used for monitoring sensitive areas and preventing chemical risks.

3.4. Artificial enzymes

3.4.1. Definition

Artificial enzymes are synthetic organic molecules, generally small in size, designed to mimic the active site of natural enzymes. Their principle is based on reproducing enzymatic catalytic mechanisms, particularly proximity and orientation effects, which facilitate the conversion of the substrate into a product.

These systems are built from a host molecule capable of selectively recognizing and binding a substrate. Catalytic functional groups are added to this structure to reproduce enzymatic activity. Among the first host molecules used were cyclodextrins, crown ethers, and calixarenes, which provide cavities suitable for molecular recognition.

Despite significant progress, the performance of artificial enzymes remains generally inferior to that of natural enzymes.

3.4.2. Types of artificial enzymes

3.4.2.1.Enzymatic mimicry

Enzyme mimics constitute the first category of artificial enzymes and fall within the field of biomimetic chemistry. They are designed to reproduce, at least partially, the catalytic properties of natural enzymes. Their development relies on the construction of catalytic sites and molecular recognition sites within a suitable host structure.

This field has evolved towards different approaches, among which nanozymes and chemozymes are particularly prominent.

a. Nanozymes

Nanozymes represent a recent class of artificial enzymes based on nanomaterials. Their emergence dates back to 2007, with the discovery unexpected of Fe_3O_4 nanoparticles exhibiting peroxidase-like activity. They are defined as nanostructures capable of mimicking enzymatic functions.

Compared to natural enzymes, nanozymes offer several advantages, including high stability, reduced production cost, and ease of large-scale synthesis. Their physicochemical properties also provide great design flexibility and a wide range of potential applications. The growing interest in these systems is reflected in the involvement of numerous laboratories worldwide.

b. Chemzymes

Chemzymes are biomimetic systems based on supramolecular architectures and non-protein host molecules. Among these systems, artificial enzymes based on cyclodextrins constitute a particularly representative model.

b.1. Cyclodextrins

b.1.1. Definition

Cyclodextrins are cyclic oligosaccharides made up of D-glucose units linked by α-1,4 glycosidic bonds. Historically described by Schardinger at the beginning of the 20th century, they are produced by enzymatic transformation of starch via cyclodextrin glucanotransferase.

b.1.2. Properties

Cyclodextrins are characterized by their low toxicity, good solubility, and stability, particularly in alkaline media. However, they are susceptible to hydrolysis under acidic conditions, leading to ring opening and the formation of linear oligosaccharides.

b.1.3. Benefits and applications

Due to their ability to form entrapment complexes, cyclodextrins enable the encapsulation of guest molecules, leading to favorable modifications of their properties. In particular, they contribute to:

1. Stabilization of light- or oxygen-sensitive substances.
2. Modification of the chemical reactivity of the guest molecules.
3. Fixation of highly volatile substances.
4. Improvement of the solubility of substances.
5. Transformation of liquid substances into powders.
6. Protection against the degradation of substances by microorganisms.
7. Masking of bad smells and bad tastes.
8. Masking of pigments or the color of substances.
9. Catalytic activity of cyclodextrins with guest molecules.

These properties explain their widespread use in diverse fields such as analytical chemistry, the pharmaceutical industry, the food industry, agriculture, and cosmetics.

3.4.2.2.Designed enzymes

Design enzymes are enzymes developed using a *de novo* design approach, which do not directly replicate (mimic) the activity of natural enzymes. They are developed from integrated structural and functional data, combining the characteristics of several enzymes with a thorough understanding of their catalytic mechanisms.

Their creation is based on an approach iterative structured in several stages:

a. Computational design

This initial phase consists of defining an optimal catalytic mechanism for the targeted reaction, by identifying the key functional groups to be introduced or modified. It leads to the establishment of the conceptual and genetic scheme of the enzyme.

b. Genetic engineering

The gene encoding the designed enzyme is synthesized and then inserted into a heterologous expression system (host cells). The enzyme produced is then isolated and purified for functional analyses.

c. In vitro evaluation

The catalytic activity of the purified enzyme is tested experimentally in the presence of its specific substrate in order to validate its functionality.

d. Directed evolution

Also called *in vivo* evolution, this optimization step relies on iterative cycles of mutagenesis and selection. Directed evolution exploits the catalytic promiscuity of enzymes, that is, their ability to catalyze side reactions. Libraries of variants are generated by random and/or targeted mutagenesis, and then subjected to intensive screening to identify mutants with improved performance.

Approaches combining random and site-directed mutagenesis, often guided by structural data, enable the identification of functional "hot spots." Screening is a crucial step and relies on high-throughput methods or display techniques that ensure genotype-phenotype correlation.

Thus, the integration of a detailed knowledge of structure-function relationships and efficient selection systems makes it possible to effectively optimize the catalytic properties of engineered enzymes.

Key Points

- Enzyme immobilization consists of attaching the enzyme to a support or entrapping it within a matrix to enhance its stability, reusability, and process control. The main methods include adsorption, entrapment, covalent binding, and cross-linking.
- Immobilized biocatalysts are widely used in analytical, therapeutic, chemical synthesis, and food-processing applications, particularly in batch, fed-batch, and continuous bioreactors.
- Industrial enzyme production involves enzyme and production-strain selection, fermentation, extraction, concentration, purification, and final formulation, all under strict regulatory oversight.

- Biosensors combine a bioreceptor with a transducer to convert a specific biological interaction into a measurable signal. Their performance is assessed in terms of selectivity, sensitivity, reproducibility, detection limit, and stability.

- Artificial enzymes encompass biomimetic systems (nanozymes and chemozymes) as well as de novo designed enzymes, which are optimized through genetic engineering and directed evolution to achieve novel or enhanced catalytic activities.

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