Enzymology

T. DEVASENA
Assistant Professor
Centre for Nanoscience and Technology
Anna University, Chennai

OXFORD
UNIVERSITY PRESS

Brief Contents

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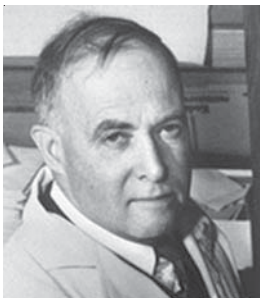
Introduction to Enzymology



James Batcheller Sumner was the first scientist to crystallize enzymes.

Learning Objectives

After reading this chapter, the reader would be able to understand and appreciate the following:

- Definition for enzyme and terminologies involved in enzymology
- Nomenclature and classification of enzymes
- Influence of substrate concentration, temperature and pH on enzyme activity
- Models for monosubstrate and bisubstrate reactions
- Histoenzymology and enzyme assay
- Techniques used in enzyme assays, such as spectrophotometry, fluorimetry, luminometry, manometry, enthalpimetry, and radiometry
- Steps and protocols involved in the purification of enzymes from natural resources
- Methods used for fractionation and characterization of proteins (enzymes)

1.1 Biocatalysts

Enzymes are a colloidal, high molecular weight, non-dialyzable, denaturable, structurally diverse group of proteins. Biochemical reactions taking place within the living cells are catalyzed by enzymes (hence the name, biocatalysts). Enzymes increase the rate of reaction without undergoing any change themselves and without altering the equilibrium of the reaction.

Like any other protein, an enzyme is an amino acid polymer with added cofactors and other post-translational modifications. Often, most of the amino acid polymers are indirectly involved with the enzyme function, perhaps providing ancillary structure or connectivity, indirect activity regulation, or molecular identification of the enzyme. As a result, most enzymes are large molecules weighing many kilo Daltons (kDa).

Enzymes catalyze metabolic reactions, by which

- Nutrients are degraded
- Chemical energy is conserved and transformed

- Biomolecules are synthesized from their precursors
- Cellular activity is regulated in response to various metabolic signals

1.1.1 Evidence to Show that Enzymes are Proteins

After crystallizing urease, James Sumner discovered that enzymes are purely proteins (ribozyme is an exception). Enzymes share the following properties with proteins:

- The proportion of carbon, hydrogen, nitrogen, and sulphur in enzymes are similar to protein.
- Like proteins, enzymes act as ampholytes
- Enzymes are susceptible to denaturation with a consequent loss of activity
- Enzymes can elicit specific antibodies on being injected into an animal body

Enzyme units

The actual molar amount of the enzyme cannot be determined and therefore, it is unknown. However, the amount can be expressed in terms of its activity. There are four ways of expressing enzyme activity.

International Unit (IU) One IU of an enzyme is the amount of enzyme that catalyzes the formation of one micromole of product in one minute under optimal conditions of pH, temperature, and ionic strength.

Katal One Katal is the amount of enzyme catalyzing the conversion of one mole of substrate to product in one second. 1 Katal = 6×10^7 IU.

Turnover number (k_{cat}) In enzymology, the turnover number (also termed k_{cat}) is defined as the maximum number of moles of substrate that an enzyme can convert to product per catalytic site per unit time. It is also called molecular activity. For example, carbonic anhydrase has a turnover number of 400,000 s^{-1}, which means that each carbonic anhydrase molecule can produce up to 400,000 molecules of product (CO_2) per second.

Specific activity (U/mg) Specific activity is the number of units of enzyme activity per milligram of total protein present. Enzyme concentration [E] is units/mg protein × mg protein/ml.

1.1.2 Properties of Enzymes

Enzymes possess the following distinctive properties:

- Catalytic power
- Specificity
- Regulatory power
- Milder reaction conditions

- Reversibility
- Colloidal nature
- Denaturation

Catalytic power

Catalytic power is also called as the *catalytic efficiency* or the *rate enhancement power*. The remarkable property of the cells to carry out reactions at low temperature, pressure and in dilute aqueous medium is obviously due to the catalytic power of the enzymes. An enzyme increases the reaction rate by 10^6 to 10^{12} times over an uncatalyzed reaction. The ratio of *the rate of catalyzed reaction to the rate of uncatalyzed reaction* is called *the catalytic power of an enzyme*. The catalytic power of some enzymes is shown in Table 1.1. Turnover numbers of selected enzymes are shown in Table 1.2. (See Chapter 2 for the energy level diagram.)

Table 1.1 Catalytic power of some enzymes

Enzymes	Uncatalyzed rate ($K_{uncat/s}$)	Catalyzed rate ($K_{cat/s}$)	Catalytic power ($K_{uncat/s} : K_{cat/s}$)
AMP nucleotidase	1.0×10^{-11}	60	6×10^{-11}
Carboxy peptidase A	3.0×10^{-9}	578	1.9×10^{-11}
Triose phosphate isomerase	4.3×10^{-6}	4300	1×10^{-11}
Carbonic anhydrase	1.3×10^{-1}	1×10^{6}	7.7×10^{-11}
Urease	3×10^{-10}	3×10^{4}	10^{14}

Specificity

Specificity refers to the *extraordinary ability* of the enzyme to recognize a specific substrate to catalyze a specific reaction. Enzymes exhibit six types of specificity:

Absolute specificity Specificity of an enzyme towards a single substrate is called *absolute specificity*. For example, urease is specific to urea.

$$\text{Urea} \xrightarrow{\text{Urease}} \text{Carbon dioxide} + \text{Ammonia}$$

Dual specificity The recognition of two different substrates by an enzyme is called *dual specificity.* For example, sucrase acts on sucrose and raffinose.

$$\text{Sucrose} \xrightarrow{\text{Sucrase}} \text{Glucose} + \text{Fructose}$$

$$\text{Raffinose} \xrightarrow{\text{Sucrase}} \text{Fructose} + \text{Mellibiose}$$

Group specificity Specificity of an enzyme towards a group of closely related compounds is called *group specificity*. For example, alcohol dehydrogenase (ADH) acts on ethanol and other higher alcohols.

Table 1.2 Turnover number of selected enzymes

Enzymes	Turnover number
Carbonic anhydrase	6 ,000,000
Catalase	93,000
Acetylcholine esterase	25,000
Lactate dehydrogenase	1,000
RNAse	800
β-galactosidase	200
DNA polymerase	15
Tryptophan synthetase	2
Lysozyme	0.5

$$\text{Ethanol} + NAD^+ \xrightarrow{\text{ADH}} \text{Acetaldehyde} + NADH + H^+$$

$$\text{Propanol} + NAD^+ \xrightarrow{\text{ADH}} \text{Propanaldehyde} + NADH + H^+$$

Geometrical specificity Recognition of cis-trans isomers by enzymes is called *geometrical specificity*. For example, fumarase acts on fumarate (*cis*-form) and malate (*trans*-form).

$$\text{Fumarate (}cis\text{-form)} \xleftrightarrow{\text{Fumarase}} \text{Malate (}trans\text{-form)}$$

Optical specificity Specificity of enzymes towards chiral substances (D&L isomers) is called *optical specificity*. For example, racemase acts on D and L isomers.

$$\text{D-Alanine} \xleftrightarrow{\text{Ala racemase}} \text{L-Alanine}$$

Bond specificity Selectivity of enzymes to a specific type of bond in a compound for subsequent hydrolysis is called *bond specificity*. For example, glycosidase cleaves glycosidic bond, peptidase (pepsin, trypsin) cleaves peptide bond, and esterase cleaves ester bond.

$$\underset{\text{(inactive)}}{\text{Chymotrypsinogen}} \xrightarrow[\text{Pepide bond cleavage}]{\text{Trypsin}} \underset{\text{(active)}}{\text{Chymotrypsin}}$$

Regulatory power

Another important property of an enzyme is its susceptibility to regulation, i.e. the catalytic power is *controllable* depending on the metabolic needs of the cell. Enzymes can be regulated by several mechanisms—covalent modification, post-translational modification, proteolytic cleavage, and genetic control. (All these methods are discussed in Chapter 5).

Milder reaction conditions

Enzyme catalysis occurs under milder conditions of temperature, atmospheric pressure, and nearly neutral pH, except for *extremozymes*, *which are active under extreme conditions* (see Chapter 14.) (A chemically catalyzed reaction, *in vitro*, requires extremes of temperature, pressure, and pH)

Reversibility

Enzymes can catalyze reactions in both directions depending upon the availability of a suitable energy source and suitable solvent conditions.

Colloidal nature

Enzymes, being high molecular weight proteins, exhibit colloidal properties, due to which they present a large surface area for the reaction to take place.

Denaturation

Enzyme denaturation is commonly defined as *any noncovalent change in its structure*. This change may alter the secondary, tertiary, or quaternary structure of the enzyme molecules.

Enzymes are thermolabile and pH sensitive. Hence, they are denatured by high temperature, strong acids, and strong alkali. When proteins are exposed to increasing temperature (except hyperthermozyme—Chapter 14), interactions responsible for tertiary structure are broken. A further rise in temperature results in breaking of the hydrogen bond, increasing the flexibility of the protein, thus leading to denaturation. When enzymes are exposed to very high pH at elevated temperatures, it results in alkaline hydrolysis of peptide bonds.

Proteins containing acid labile groups irreversibly lose their function even with mild acid treatment. This is due to the breaking of specific covalent bonds. Exposure to strong acid at elevated temperatures will first release amide nitrogen from glutamine and asparagine groups, and eventually lead to the hydrolysis of peptide bonds.

1.1.3 Nomenclature and Classification of Enzymes

Enzymes are generally named by adding the suffix—'ase' to the substrate of the reaction. For example urease. Other enzymes acquired names bearing little resemblance to their activity, such as the peroxide-decomposing enzyme *catalase*, or the proteolytic enzymes (*proteases*) of the digestive tract, *trypsin* and *pepsin*, or the hydrolytic enzyme, hydrolase.

Major classification

According to the International Enzyme Commission constituted by the International Union of Biochemistry (1956), enzymes are divided into *six major classes* depending on the type of reaction catalyzed. Major classification of enzymes is shown in Table 1.3.

Subclass and sub-subclass

Each major class is further divided into subclass and each subclass is redivided into subsubclass. Subclasses are shown in Table 1.4.

Table 1.3 Major classification of enzymes

Class	Name of the reaction catalyzed	Example	Model reaction catalyzed by the specific class of enzyme
Oxidoreductase	Redox	Alcohol dehydrogenase Cyt. oxidase	$A_{Red} + B_{oxd} \leftrightarrow A_{Oxd} + B_{red}$ $A^- + B^+ \leftrightarrow A^+ + B^-$
Transferase	Group transfer between two substrates	Hexoseaminase Transaminase	$A - B + C \leftrightarrow A + B - C$
Hydrolase	Hydrolytic bond cleavage	Alkaline phosphotase, Urease	$A - B + H_2O \leftrightarrow A - H + B - OH$
Lyase (Desmolase)	Non-hydrolytic bond cleavage and generation of double bonds	Aldolase, Histidase	$A X - BY \leftrightarrow A = X - Y$
Isomerase	Intramolecular rearrangement of groups	Triose phosphate isomerase Phosphohexose isomerase	$A X - BY \leftrightarrow AY - BX$
Ligase (Synthetase)	Bond formation at the expense of ATP*	Glutamine synthetase, Carbamoyl Phosphate synthetase	$A + B + ATP \leftrightarrow A - B + ADP + Pi$

* An enzyme that catalyzes bond formation without the expense of ATP is called synthase

Table 1.4 Subclasses of enzymes

Class	Subclass	Example	Reaction catalyzed
Oxidoreductase	Dehydrogenase	Lactate dehydrogenase Malate dehydrogenase	Removal of 2 hydrogen atoms with double bond formation
	Oxidase	Cytochrome oxidase	Reduction of oxygen
	Peroxidase	Catalase	Reduction of H_2O_2
	Hydroxylase	Phenylalanine-4-hydroxylase	Introduction of OH groups
	Oxygenase	Tryptophan oxygenase	Incorporation of molecular oxygen
	Oxidative deaminase	Amino acid oxidase	Oxidation of amino acid with the liberation of NH_3
Transferase	One carbon transferase	Methyl transferase Hydroxy methyl transferase Formyl transferase	Transfer of one-carbon group
	Aldehyde and ketone transferase	Acetaldehyde transferase	Transfer of aldehyde/keto group
	Acyl transferase	Aminoacyl transferase	Transfer of acyl/acetyl group to a suitable acceptor
	Glycosyl transferase	Hexosyl transferase	Transfer of glycosyl group
	Alkyl transferase	Ethyl transferase	Transfer of alkyl other than methyl
	N-transferase	Amino transferase	Transfer of nitrogenous group
	Phospho transferase	Kinase	Transfer of phosphoryl group
	Sulpho transferase	Dehydro epiandrosterone Sulphotransferase Choline sulphokinase	Transfer of sulphur containing group
Hydrolase	Esterase	Acetylcholine esterase	Hydrolysis of ester
	Peptidase	Trypsin	Hydrolysis of peptide bond
	Glycosidase	Lysozyme	Hydrolysis of glycosidic bond

(Contd.)

Table 1.4 (Contd.)

Class	Subclass	Example	Reaction catalyzed
	Phosphatase	ALP	Hydrolysis of phosphoric acid ester
	Deaminase	Glucosamine-6-P-deaminase, Glutaminase	Hydrolysis of amines
	Deamidase	Nicotinamide deamidase	Hydrolysis of amides
Lyase	C = C lyase	Aldolase	Cleavage of carbon-carbon bond
	C = O lyase	DNA lyase	Cleavage of carbon-oxygen bond
	C = N lyase	Ammonia lyase	Cleavage of carbon-nitrogen bond
Isomerase	Racemase	Alanine racemase	Interconversion of optical isomers
	Epimerase	Maltose epimerase	Interconversion of epimers
	Cis-trans Isomerase	Fumarase	Interconversion of geometrical isomers
Ligase	C = O ligase	Tyrosine-RNA ligase	Formation of C – O bond
	C = S ligase	Acetate CoA ligase	Formation of C – S bond
	C = N ligase	NAD^+ synthase Tryptophan synthase	Formation of C – N bond
	C = C ligase	Pyruvate carboxylase	Formation of C – C bond

Enzyme commission code (EC)

Each enzyme is assigned a code by the enzyme commission. The code number is called the *enzyme code*. It has *four digits*. The first integer represents the major class, the second integer represents the subclass, the third integer represents the sub-subclass, while the fourth integer represents the individual serial number of that enzyme in the sub-subclass. Thus, a series of four numbers serves to specify a particular enzyme. To illustrate, consider the enzyme that catalyzes this reaction:

$$\text{ATP + D-Glucose} \xrightarrow{\text{D-Glucose-6-phospho transferase}} \text{ADP + D-Glucose-6-phosphate}$$

Class In the above reaction, a phosphate group is transferred from ATP to the – OH group of the sixth carbon of glucose, so the enzyme is a *transferase* (Class 2).

Subclass Enzymes transferring phosphorus-containing groups are called phosphotransferases which comes under seventh category of transferase class, so the subclass is 7.

Sub-subclass If an alcohol group is the acceptor of the phosphate group, it refers to the first category of the phosphotransferases. Therefore, the subsubclass is 1. Entry 2 in this sub-subclass is ATP. Therefore, the EC number for D-glucose-6-phosphotransferase is 2.7.1.2.

1.1.4 Factors Influencing Enzyme Activity

Various factors influence the activity of enzymes. These factors include substrate concentration, reaction temperature, pH of the buffer or reaction medium, oxidation, and radiation exposure. The effect of these factors on the rate of enzyme catalyzed reaction is discussed below.

Effect of substrate concentration

The effect of substrate concentration on enzyme activity can be described by plotting velocity (V) against substrate concentration [s], as shown in Fig. 1.1. The plot is a rectangular hyperbola with two distinct regions: (a) linear region, and (b) plateau region.

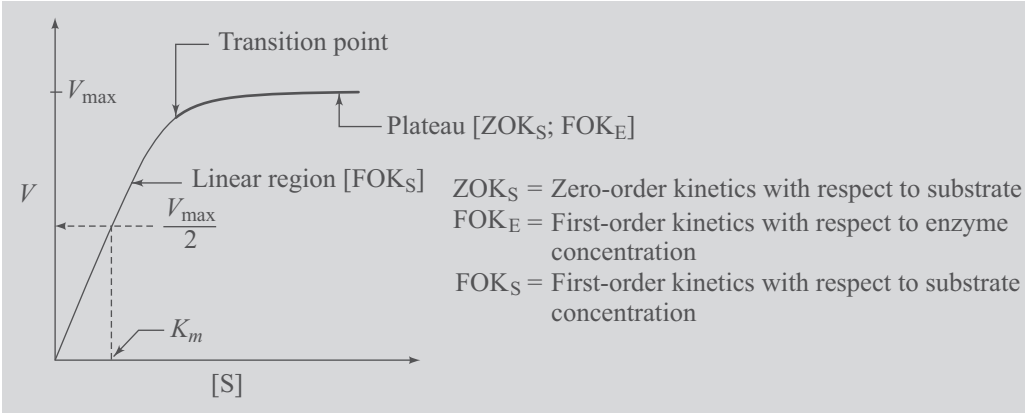


Fig. 1.1 Effect of substrate concentration on enzyme activity

Linear region At the initial stage of the reaction the velocity of the enzyme catalyzed reaction increases *linearly* with an increase in substrate concentration (shown as the linear region). This is also termed initial velocity of the reaction. At this stage, the substrate concentration is low and the reaction follows *first order* kinetics with respect to substrate concentration. Generally, it is assumed that the velocity of the enzyme-catalyzed reaction is directly proportional to the enzyme concentration in a reaction mixture.

Plateau region As the substrate concentration is raised gradually, the velocity reaches a maximum (V_{max}). After V_{max} the velocity is *constant* even with an increase in substrate concentration. This leads to a plateau region. At this region, the reaction rate follows *zero order* kinetics with respect to substrate, but *first order* kinetics with respect to enzyme concentration.

The point in the graph, where the linear plot is *transformed* into a plateau is called the *saturation point*. At this point the active site of the enzyme is completely saturated with the substrate. Substrate concentration at which the rate is $V_{max}/2$, i.e. the rate is half of the maximal velocity is called k_m.

Effect of temperature

The effect of temperature on the rate of the enzyme catalyzed reaction is represented as plot of velocity versus. temperature. This is shown in Fig. 1.2.
The plot is *bell shaped* with three regions:

- ascending segment,
- peak, and
- descending segment.

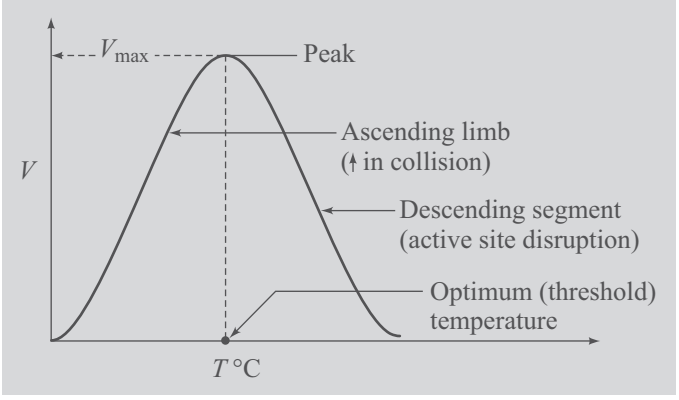


Fig. 1.2 The effect of temperature on the rate of an enzyme-catalyzed reaction

The rate of enzyme-catalyzed reaction increases with the initial rise in temperature in accordance with the *Arhenius equation*:

$$K = A_e^{-\Delta G^*/RT}$$

where, K is the kinetic rate constant for the reaction,
A is the Arrhenius constant, also known as the frequency factor,
ΔG^* is the standard free energy of activation (kJ M^{-1}), which depends on entropic and enthalpic factors,
R is the gas law constant, and
T is the absolute temperature.

The initial rise in temperature increases the probability of effective *collision* between the reactive groups due to increase in kinetic energy, and hence the velocity increases *linearly* (shown as an ascending segment). The velocity 'V' is enhanced for every 10°C is called *temperature coefficient*, denoted as Q_{10}. But after a certain limit of temperature, called the *optimum temperature*, the velocity reaches a *maximum* (peak), then decreases gradually (the descending segment). The descending segment represents that beyond the optimum temperature there is a fall in velocity. It may be due to covalent changes such as the deamination of asparagine residues, or noncovalent changes, such as the rearrangement of the protein chain, or inactivation by heat denaturation.

Effect of pH

The effect of pH on the velocity of an enzyme-catalyzed reaction is represented in Fig. 1.3.

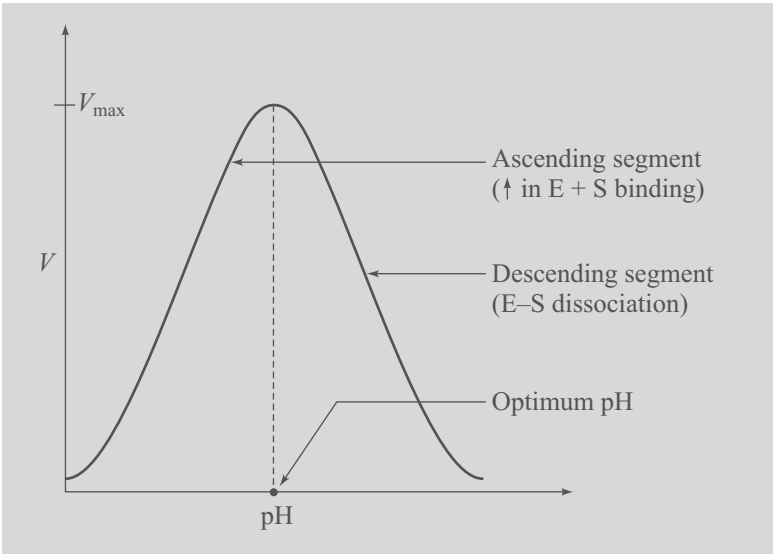


Fig. 1.3 The effect of pH on the velocity of an enzyme-catalyzed reaction

The *bell-shaped* curve of pH versus *V* is similar to that of *T* versus *V*. In the initial stage of the reaction the velocity increases with the increase in pH due to increase in enzyme-substrate *binding*. At a particular pH, called the *optimum pH* (usually 6–8) the velocity reaches a maximum. Beyond the optimum pH the 3-D structure of the enzyme is altered, leading to a *dissociation* of E–S complex and a fall in velocity.

As the optimum pH is between 6–8 most of the enzymes display a bell-shaped curve, but pepsin is an exception. The optimum pH of pepsin is 2 (which exists in acidic gastric juice for digesting proteins), and the curve, is shown in Fig. 1.4.

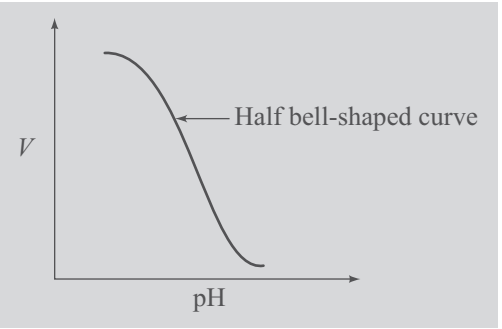


Fig. 1.4 The effect of pH on the velocity of pepsin

Effect of oxidation

Oxidation of the sulphydryl group (–SH) in the active site by the oxidizing agent leads to disulphide bridging (S–S), resulting in loss of enzyme activity.

Effect of radiation

Exposure to high energy (short wavelength) radiations like X-rays, β-rays and γ-rays, leads to *conformational change* and loss of enzyme activity. UV rays also *inactivate* enzymes.

1.2 The Structure of Enzymes

Enzymes have *properly folded* active conformation. Part of the enzyme responsible for catalysis is called *active site*. Active site is functionally divided into two subsites.

Specificity subsite (*substrate binding site*) is the part of the active site where recognition of the substrate takes place.

Reaction subsite is the part of the active site where chemistry occurs. A scheme of enzyme-substrate binding leading to catalysis is shown in Fig. 1.5.

Electrostatic forces and torques can steer the substrate (negatively charged) into its binding site on the protein. Some binding sites are normally shielded from the solvent and can be kept *closed* by salt links between groups on the protein surface. If the correct substrate disrupts these salt links it can gain access to the binding site. This is known as *gated binding*. Electrostatic interactions, particularly salt links and hydrogen bonds between the ligand and the protein can contribute to the affinity and specificity of binding and to the orientation of the ligand in the binding site.

Substrate (S) binds to the active site of the enzyme (E) forming enzyme-substate complex. An enzyme-substate complex is converted into a *high-energy* state called *transition state* complex ($ES^{\ddagger}$). Subsequently, the transition state is converted into product (P) and the free enzyme (E) is released.

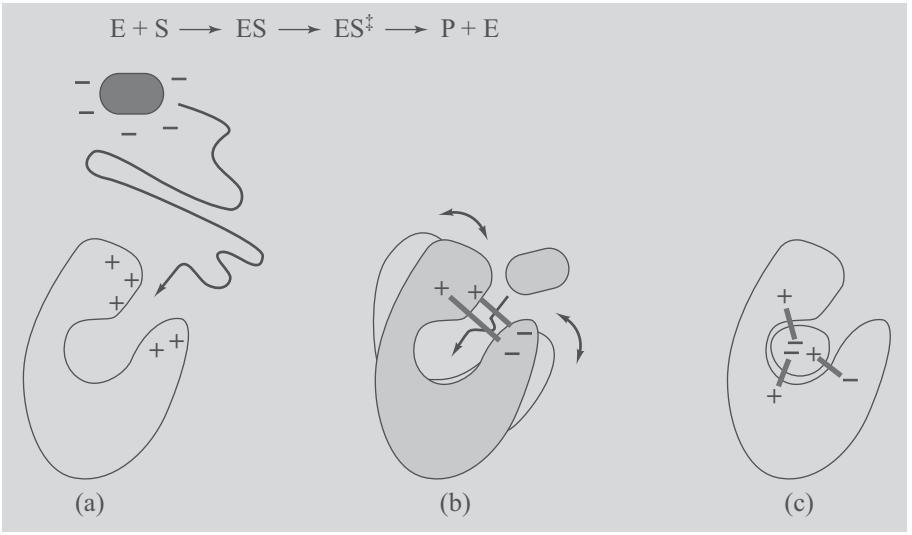


Fig. 1.5 Enzyme-substrate binding steered by electrostatic forces—gated binding

1.3 Models for Monosubstrate Reaction

Monosubstrate reaction is given as:

$$E + S \longrightarrow ES \longrightarrow P + E$$

A monosubstrate reaction is explained by two models:

- Fischer's lock and key model, and
- Koshland's induced fit model.

1.3.1 Fischer's Lock and Key Model

According to this model the active site of the enzyme is rigid and *predetermined* to be complementary to the substrate. Thus, the substrate fits into the active site as the key fits into the lock. This model is shown in Fig. 1.6.

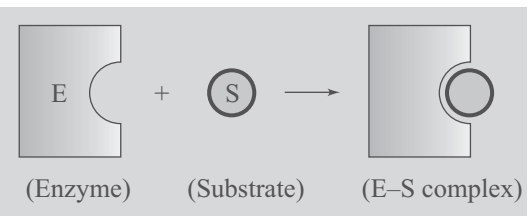


Fig. 1.6 Fischer's lock and key model for a monosubstrate reaction

1.3.2 Koshland's Induced Fit Model

In this model, the active site of the enzyme is flexible and changes shape on substrate binding, i.e. the complementarity is induced after the binding of the substrate to the enzyme. This model is shown in Fig. 1.7.

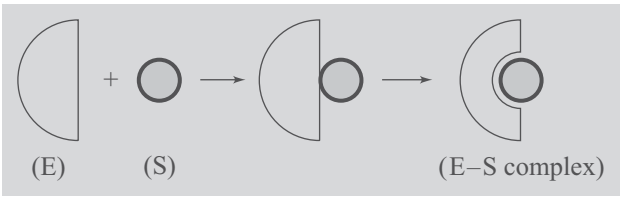


Fig. 1.7 Koshland's induced fit model for monosubstrate reaction

1.4 Models for Bisubstrate Reaction

A bisubstrate reaction is represented as

$$A + B \xrightarrow{E} P + Q$$

A bisubstrate reaction is described by three models. All the models are explained by the *Cleland diagram* or *Cleland notation*.

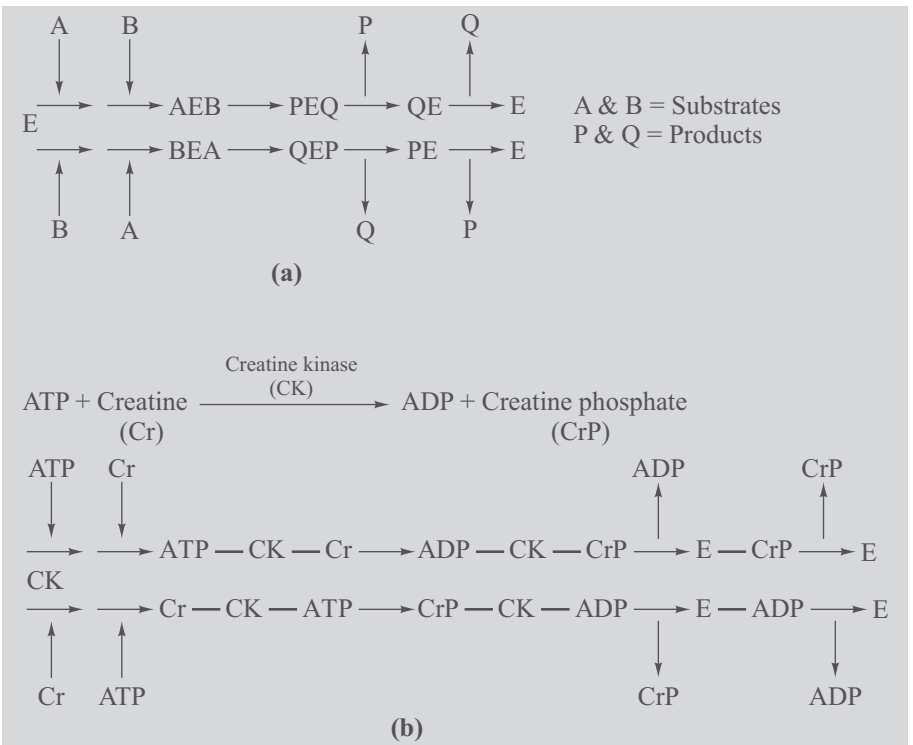


Fig. 1.8 (a) Cleland notation for random sequential model (Random single displacement model) and (b) Cleland notation for creatine kinase

1.4.1 Random Sequential Model

Ternary complexes are formed in this model. Creatine kinase is the best example for this model. It is also known as random single-displacement model.

The Cleland notation for this model along with the example, creatine kinase, is shown in Figs 1.8 (a) and (b).

- Substrate (A/B) binds to enzyme (E) in random order
- *Enzyme-substrate* ternary complex is formed (AEB/BEA)
- Enzyme-substrate ternary complex is converted into *enzyme-product* ternary complex (PEQ/QEP)
- The products are released one-by-one at random (Q+P/P+Q)
- Free enzyme is regenerated (E)

1.4.2 Ordered Sequential Model

Alcohol dehydrogenase is the best example for this model. The Cleland notation for this model is depicted in Figs 1.9 (a) and (b). It is also known as ordered single-displacement model.

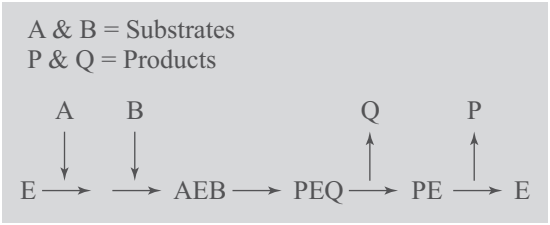


Fig. 1.9 (a) Cleland notation for ordered sequential model

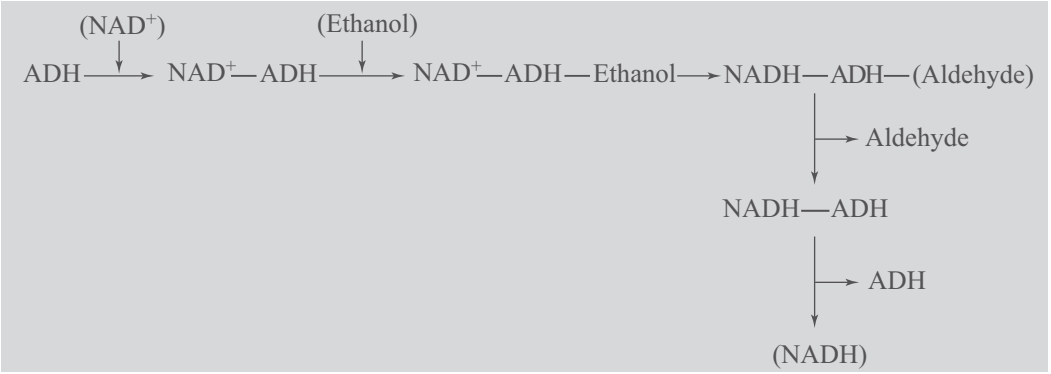


Fig. 1.9 (b) Cleland notation for alcohol dehydrogenase (Here the cofactor NAD is considered as one of the two substrates.)

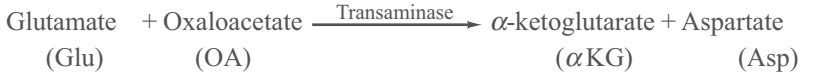
- The substrate binds to the enzyme (E) in a *definite order.*
- The *leading substrate* is A (obligatory substrate or compulsory substrate).
- The *trailing substrate* is B.
- The enzyme-substrate *ternary complex* (AEB) is formed.
- AEB is converted into enzyme-product ternary complex (PEQ).

- The products are released in definite order (Q is released first, followed by P).
- Free enzyme is regenerated (E).

1.4.3 Ping-pong Model

A binary complex is formed in this model. *Transaminase* is the best example for this model. In this model, one of the substrates should be converted into the product before the next substrate binds to the enzyme. Cleland notation for this model is shown in Figs 1.10 (a) and (b).

In this model, one of the substrates should be converted into the product before the next substrate binds to the enzyme.



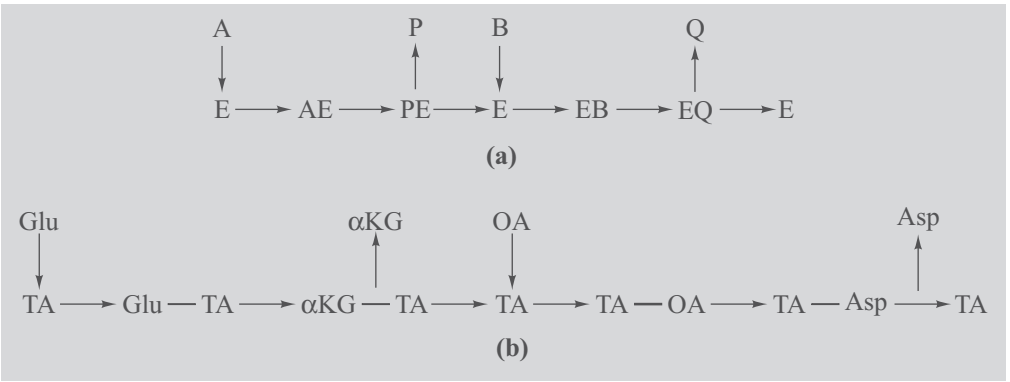


Fig. 1.10 Cleland notations for (a) ping-pong model and (b) for Transaminase

In this model, one of the substrate should be converted into product before the next substrate binds to the enzyme.

- The leading substrate (A) binds to the enzyme (E).
- Enzyme-substrate binary complex is formed (AE).
- Enzyme-product binary complex is formed (PE).
- The product (P) is released with the regeneration of free enzyme.
- The trailing substrate (B) binds to enzyme (E).
- Enzyme-substrate binary complex is formed (BE).
- Enzyme-product binary complex is formed (QE).
- The product (Q) is released.
- Free enzyme is regenerated (E).

1.5 Monomeric Enzyme and Oligomeric Enzyme

Enzymes with single non-dissociable polypeptide chain are called monomeric enzymes. They are usually composed of 100 to 300 amino acids and mostly synthesized as

Table 1.5 Members of monomeric enzymes

Enzyme type	Examples
Protease	Chymotrypsin Trypsin Elastase Thrombin Subtilisin Pepsin Ficin Carboxypeptidase A Carboxypeptidase B
Nuclease	Ribonuclease
Glycosidase	Lysozyme

zymogens and their activities regulated by proteolytic cleavage (see Chapters 3 and 5). Most of the known monomeric enzymes come under the class of *hydrolases*, including peptidase, protease (serine proteases, thiol proteases), nuclease, and glycosidase. Some of the monomeric enzymes are enlisted in Table 1.5.

Enzymes with two or more polypeptide chains are called *oligomeric enzymes*. Their component polypeptide chains are called *subunits*. Examples for oligomeric enzymes and their subunits are shown in Table 1.6. Subunits of an oligomeric enzyme may be identical or nonidentical. Identical subunits are called protomers. Enzymes with two subunits are called dimers and those with three and four subunits are called trimers and tetramers, respectively. Oligomeric enzymes have higher molecular weight than monomeric enzymes. These enzymes are regulated by feed back inhibition or allosteric regulation (see Chapter 5). Examples for oligomeric enzymes include lactose synthase, lactate dehydrogenase, and pyruvate dehydrogenase.

Lactose synthase is a dimer. One subunit is inactive and is called α-lactalbumin and the other one is called N-acetyl lactosamine synthase, which catalyze the following reaction in carbohydrate metabolism:

UDP-galactose + N-acetyl glucosamine ↔ UDP + N-acetyl lactosamine

In the presence of α-lactalbumin, N-acetyl lactosamine synthase catalyze the synthesis of lactose as follows. This reaction is important in a lactating mother:

UDP-galactose + Glucose ↔ UDP + Lactose

Lactate dehydrogenase is a tetramer and each subunit catalyses the same reaction:

$$\text{Lactate} + NAD^+ \leftrightarrow \text{Pyruvate} + NADH + H^+$$

The enzyme subunits exist in two forms based on the difference in amino acid composition. They are M form, which predominates in skeletal muscle, and H form, which predominates in the heart. These forms are called isozymes (see Chapter 7).

Table 1.6 Members of oligomeric enzymes

Oligomeric enzyme	Number of subunits present
Lactate dehydrogenase	Four
Lactose synthase	Two
Tryptophan synthase	Two
Pyruvate dehydrogenase	Multienzyme complex

Pyruvate dehydrogenase is a multienzyme complex, which catalyzes the synthesis of the two-carbon unit acetyl-CoA from pyruvate. Acetyl-CoA formed by this enzyme complex is the precursor for the TCA cycle. Details of this complex are discussed in Chapter 3.

1.6 Multisubstrate Reaction

Multisubstrate reaction is considered as a sequence of individual steps, each of which obeys the single and double displacement model. Pyruvate dehydrogenase is the best example for this reaction (as explained in Chapter 2).

1.7 Enzyme Localization—Histoenzymology

Histoenzymology literally relates *biochemical activity* with histology.

1.7.1 Definition of Histoenzymology

Histoenzymology is defined as *localization* of an enzyme and measurement of enzyme activity in defined cells lying within a complex histology. The subcellular location of enzymes may be revealed by microscopy, after fixation and staining.

1.7.2 Applications

Histoenzymology is used to investigate biochemical cellular changes that may be induced by a drug, a disease, or a toxic agent. Hence, enzyme activity in defined cells is used as *biomarkers*. Uses of histoenzymology are summarized in Table 1.7. For example, alkaline phosphatase can be identified by the formation of a black precipitate. In this reaction β-glycerophosphate is used as a substrate in the presence of lead ions. Acid phosphatase present in the tissue acts on the substrate catalyzing the formation of glycerate and phosphate. Phosphate immediately reacts with lead ions forming insoluble lead phosphate, which is allowed to react with ammonium sulphide. Ammonium sulphide reaction produces a black precipitate of lead sulphide. These reactions are given as follows:

$$\beta\text{-glycerophosphate} \longrightarrow \text{Glycerate} + PO_4^{2-}$$

$$PO_4^{2-} + Pb^{2+} \longrightarrow PbPO_4$$

$$PbPO_4 \xrightarrow{(NH_4)_2S} PbS \longrightarrow \text{black color}$$

Black precipitate is used as cell surface marker. Black particles generated by acid phosphatase are used as a marker of lysosomal activity. Similarly, each enzyme produces a specific color after a specific reaction. Black color produced by succinate dehydrogenase is used as a marker of mitochondrial activity. Blue color produced by glyceraldehyde-3-P dehydrogenase is used as a marker of EMP pathway. Color produced by hydroxylacyl dehydrogenase activity is used as a marker of fatty acid utilization. Na^+ K^+ATPase activity is used as a marker of cell membrane activity. Details of the substrate, coenzyme, buffer, pH, and other components in the reaction mixture used in histoenzymology are summarized in Table 1.8

Table 1.7 Uses of histoenzymology

Enzymes	Uses
Alkaline phosphatase	Cell surface marker
Acid phosphatase	Cell surface marker
Succinate dehydrogenase	Marker of mitochondrial activity
Glyceraldehyde-3-P dehydrogenase	Marker of EMP pathway
Hydroxylacyl dehydrogenase	Marker of fatty acid utilization
Na^+ K^+ATPase	Marker of cell membrane activity

Table 1.8 Reaction mixture used in histoenzymology

Enzymes	Substrate/coenzyme	Buffer-pH	Other components
Alkaline phosphatase	Na-β-glycerophosphate	Barbitone-9.4	Calcium chloride, Magnesium sulphate, Cobalt nitrate, Ammonium polysulphide
Acid phosphatase	Na-β-glycerophosphate	Acetate-5.0	Lead nitrate, Hydrogen sulphide
Succinate dehydrogenase	Sodium succinate	Phosphate-7.8	Phenazine methosulphate and Nitroblue tetrazolium
Glyceraldehyde-3-P dehydrogenase	Fructose –1,6–bisphosphate/aldolase/NAD	Glycyl glycine–8.0	Phenazine methosulphate & Nitroblue tetrazolium
Hydroxylacyl dehydrogenase	Butyryl cysteamine/NAD	Glycyl glycine–8.0	Sodium nitroprusside, menadione, neotetrazolium chloride
Na^+ K^+ATPase	Disodium adenosine-5′ tri-P/lead ammonium citrate/acetate	Tris–7.4	Sodium acetate, Sodium chloride, Magnesium chloride, Potassium chloride
Lactate dehydrogenase	Sodium lactate/NAD	Glycyl glycine–8.0	Phenazine methosulphate & Nitroblue tetrazolium
Glucose-6-P dehydrogenase	Glucose-6-P-disodium salt/NADP	Glycyl glycine–8.0	Phenazine methosulphate & Nitroblue tetrazolium
Guanylate lyase	Guanosine-tri-P lithium salt	Tris–7.4	Sodium acetate, Sodium fluoride, Magnesium chloride

1.8 Enzyme Assay

Enzyme activity can be assayed by the following methods:

- Direct assay
- Coupled assay/linked assay (two-step and three-step coupled assay)

1.8.1 Direct Assay

In the direct method, enzyme activity is assayed by directly measuring the absorbance of light by the product.

Dehydrogenases

Dehydrogenase can be assayed by measuring the *absorbance* of NADH at 340 nm.

$$\text{Substrate (reduced)} + NAD^+ \xrightarrow{\text{Dehydrogenase}} \text{Product (oxidized)} + NADH + H^+$$

$$\downarrow$$

Absorption at 340 nm

Amount of light absorbed by NADH at 340 nm is proportional to dehydrogenase activity.

1.8.2 Two-step Coupled Assay or Linked Assay

In some cases neither the product nor the substrate can be quantitatively measured by determining the light absorption. In such cases, the enzyme reaction is coupled to a *second reaction*, which can very well produce a light-absorbing product. The product of the first reaction becomes the substrate for the second reaction. The amount of light absorbed by the product of the second reaction is proportional to the enzyme activity. This method is called coupled assay or *linked assay*. This method is generally not applicable to measurements in crude preparations, which contain NADH-oxidases and other NADH-utilizing enzymes. One important example is the pyruvate dehydrogenase complex.

Hexokinase and glucose oxidase

Hexokinase can be assayed by coupling to a dehydrogenase reaction as shown in Fig. 1.11. Hexokinase converts glucose into glucose-6-P, which cannot absorb light. However, when glucose-6-P is coupled to a second enzyme-glucose-6-P dehydrogenase, 6-P-gluconolactone and NADPH are formed. Absorption of NADH measured at 340 nm is proportional to hexokinase activity.

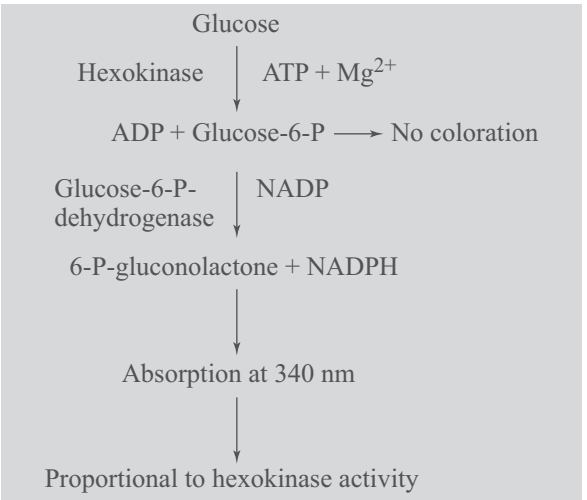


Fig. 1.11 Coupled assay of hexokinase

Coupled assay of glucose oxidase is illustrated in Fig. 1.12. Glucose oxidase converts glucose to gluconic acid (a non-absorbing product). Gluconic acid is coupled to peroxidase via hydrogen peroxide and a color-producing dye, *4-amino antipyrine*. The dye is oxidized to colored form *quinoneimine*. Absorbance of quinoneimine is proportional to glucose-oxidase activity.

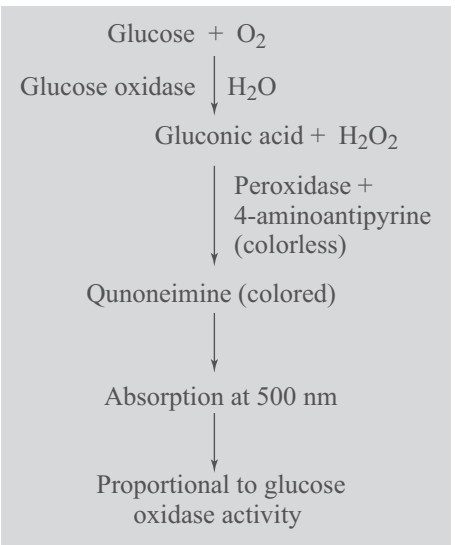


Fig. 1.12 Coupled assay of glucose oxidase

In the three-step coupled assay method the logic is similar to the two-coupled assay, but it involves three inter-linked sequential reactions. Activity of the enzyme catalyzing the first reaction is assayed using the product of the third reaction.

Uricase and cholesterol esterase

As shown in Fig. 1.13, in the first reaction *uricase* converts uric acid into allantoin and H_2O_2. In the second reaction, H_2O_2 interacts with ethanol in the presence of *catalase*, forming acetaldehyde and water. In the third reaction, acetaldehyde combines with $NADP^+$ in the presence of *dehydrogenase*, forming acetate, $NADPH + H^+$. Absorption of NADPH at 340 nm is proportional to uricase activity.

As shown in Fig. 1.14, in the first step of the assay cholesterol *esterase* hydrolyzes cholesterol ester into cholesterol and fatty acid. In the second reaction, cholesterol *oxidase* oxidizes cholesterol into 4-choestenone, simultaneously liberating H_2O_2. In the third reaction, *peroxidase* catalyzes the reaction of H_2O_2 with phenol and 4-aminoantipyrine, forming quinoneimine. Absorption of quinoneimine at 500 nm is proportional to the activity of the first enzyme, cholesterol-oxidase.

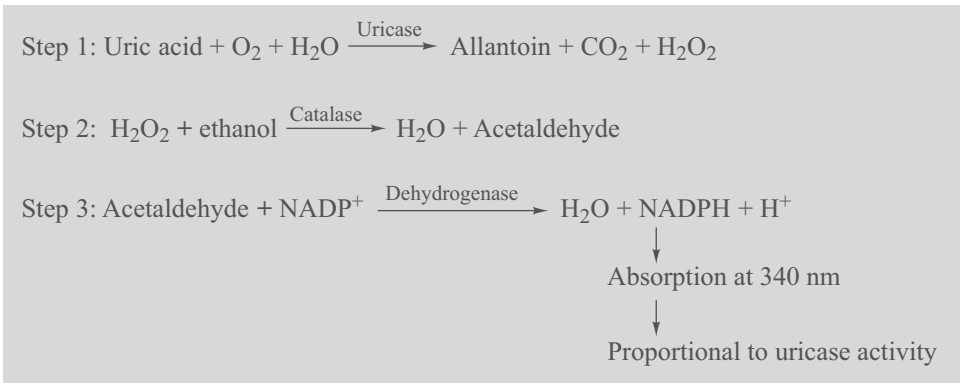


Fig. 1.13 Three-step coupled assay of uricase

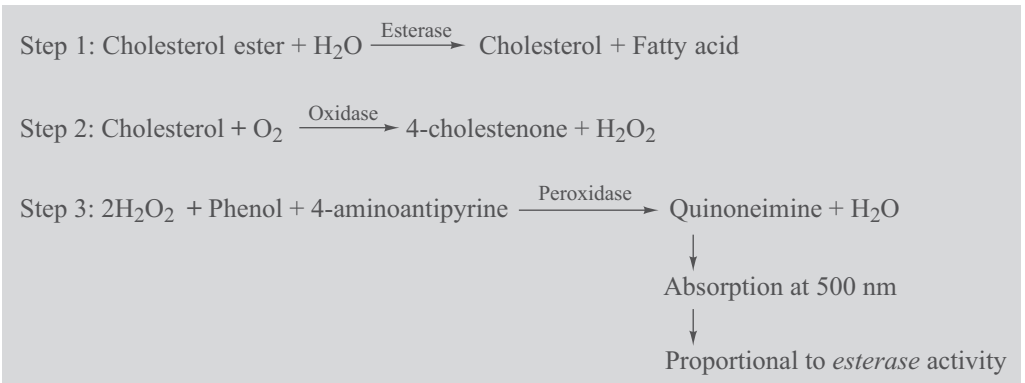


Fig. 1.14 Three-step coupled assay of cholesterol esterase

1.8.2 Instrumental Techniques used in Enzyme Assay

Various techniques used in enzyme assay are as follows:

- Spectrophotometry
- Fluorimetry

- Luminometry
- Radiometry
- Manometry
- Enthalpimetry/Micro-calorimetry

Spectrophotometry

Spectrophotometry is a technique in which the amount of a substance is quantified using the light absorbed by the substance (absorbance, A) based on Beer-Lambert's Law. Thus, if the substrate or the product of an enzyme-catalyzed reaction absorbs light then the amount of the light absorbed is proportional to the enzyme activity. The absorbance, A is given by a simple formula,

$$A = \log_{10}(I/I_0)$$

where I_0 is the intensity of the incident light and I is the intensity of the light transmitted through the sample.

According to Beer-Lambert's law

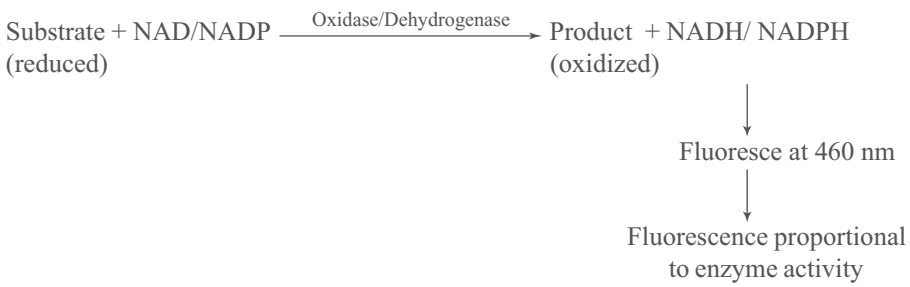
$$A = \varepsilon cl$$

where ε is the molar extinction coefficient, c is the molar concentration and l is the length of the light path through the sample in cm.

Spectrophotometry is used in the assay of enzymes using NAD^+ or NADH *as coenzyme*.

Fluorimetry

Fluorescence is a process by which molecules absorb light of a particular wavelength and re-emit light of a longer wavelength. Fluorimetry involves measurement of the fluorescence of the product of an enzyme catalyzed reaction using a fluorimeter. Fluorescence is related to the enzyme activity. Reduced forms of nicotinamide coenzymes-NADH and NADPH absorb light at 340 nm and fluoresce at 460 nm. Therefore, enzymes utilizing NAD and NADH as coenzymes can be assayed by measuring the fluorescence of the products—NADH and NADPH. Oxidases and dehydrogenases can be assayed by this technique.



For example, β-galactosidase can be assayed using a synthetic substrate 4-methyl umbelliferyl-β-D-galactoside. Derivatives of 4-methyl umbelliferone are also used in the assay of esterases, phosphatases, and sulphatases. Lipase can be assayed by using a non fluorescent substrate dibutyryl fluorescein, which is hydrolyzed to a fluorescent product, fluorescein.

Dibutyryl fluorescein $\xrightarrow{\text{Lipase}}$ Fluorescein $\longrightarrow$ Fluorescence $\longrightarrow$ Intensity proportional to lipase activity

Luminometry

Some of the products emit light during the course of the reaction. This process is called luminescence. This phenomenon is called bioluminescence for a biological reaction and chemiluminescence for a chemical reaction. The intensity of luminescence is measured by a luminometer, which in turn is proportional to the enzyme activity. For example, luciferase acts on its substrate to produce a luminescent product—oxyluciferin. The intensity of the luminescence is proportional to the luciferase activity.

$$\text{Luciferin} + \text{ATP} \longrightarrow \text{Luciferyl adenylate} + PP_i$$

$$\text{Luciferyl adenylate} + O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{LIGHT}$$

$\downarrow$

Intensity proportional to luciferase activity

Radiometry

Radiometry involves assay of enzymes whose substrate is radioactively labeled. In such cases the product will be radiolabeled and the radioactivity is proportional to the enzyme activity. β-emitters like tritium (^3H), carbon (^{14}C), phosphorous (^{32}P), sulphur (^{35}S), and iodine (^{135}I) are used as radiolabels and the radioactivity is measured by using Geiger Muller counter, or more preferably scintillation counter. For example, acetyl choline esterase is assayed by using the ^{14}C labeled substrate—acetylcholine, and measuring the radioactivity of the ^{14}C-labeled product—acetate.

$$^{14}\text{C–Acetylcholine} \xrightarrow{\text{Acetylcholine esterase}} \text{Choline} + {}^{14}\text{C–Acetate}$$

$\downarrow$

Radioactivity

$\downarrow$

Measured by scintillation counter

$\downarrow$

Proportional to acetylcholine esterase activity

Manometry

Manometry is used to assay enzymes whose substrate or product is a gas. This technique makes use of a pressure-measuring apparatus, called a manometer, for measuring the pressure changes produced during the reaction. This method is used to assay the reactions where gas is consumed or liberated. For example, glucose oxidase, which uses molecular oxygen as a substrate or decarboxylases, which produce carbondioxide as a product, may be assayed by manometry. Sometimes, reactions that produce acid can be assayed by adding bicarbonate to the reaction mixture and measuring the evolution of carbon dioxide.

Enthalpimetry

Enthalpimetry, also called *microcalorimetry*, is an assay technique that measures the amount of heat liberated during an enzyme-catalyzed reaction, with the help of a microcalorimeter provided with a highly sensitive temperature-sensor-like thermistor. Usually, the product of the primary reaction may be linked to a secondary reaction with a buffer to liberate more amounts of heat than the primary reaction. This results in higher sensitivity. Hexokinase activity can be measured by coupling the primary reaction (phosphorylation reaction) to a Tris-buffer reaction via protons (H^+).

Primary reaction

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose-6-phosphate} + \text{ADP} + H^+ \ (\Delta H = -28 \text{ kJmol}^{-1})$$

Secondary reaction

$$H^+ + \text{Tris} \longrightarrow \text{TrisH}^+ \ (\Delta H = -47 \text{ kJmol}^{-1})$$

Here, the enthalpy of the secondary reaction is higher than the primary reaction, which can be measured with high sensitivity.

1.9 Purification of Enzymes from Natural Resources

Purification of enzymes from their sources is a crucial part of enzymology, which is needed for investigation on the structure and function of enzymes. This section describes different steps involved in enzyme purification like: choosing the sources used for extracting enzymes, methods used for separating enzymes and other impurities.

1.9.1 Need for Enzyme Purification

Enzyme purification is done in order to isolate an enzyme with maximum catalytic activity or specific activity.

1.9.2 Steps in Enzyme Purification

Enzyme purification is a *multistep* process as shown below:

Step 1 Choosing of a suitable source and extraction of enzyme from the source
Step 2 Removal of whole cells (undisturbed cells) and cell debris from the enzyme extract
Step 3 Removal of nucleic acids and lipids
Step 4 Concentration and primary purification of enzyme
Step 5 Final purification by column chromatography and quality check

These five steps in enzyme purification are shown as a flow-sheet in Fig. 1.15.

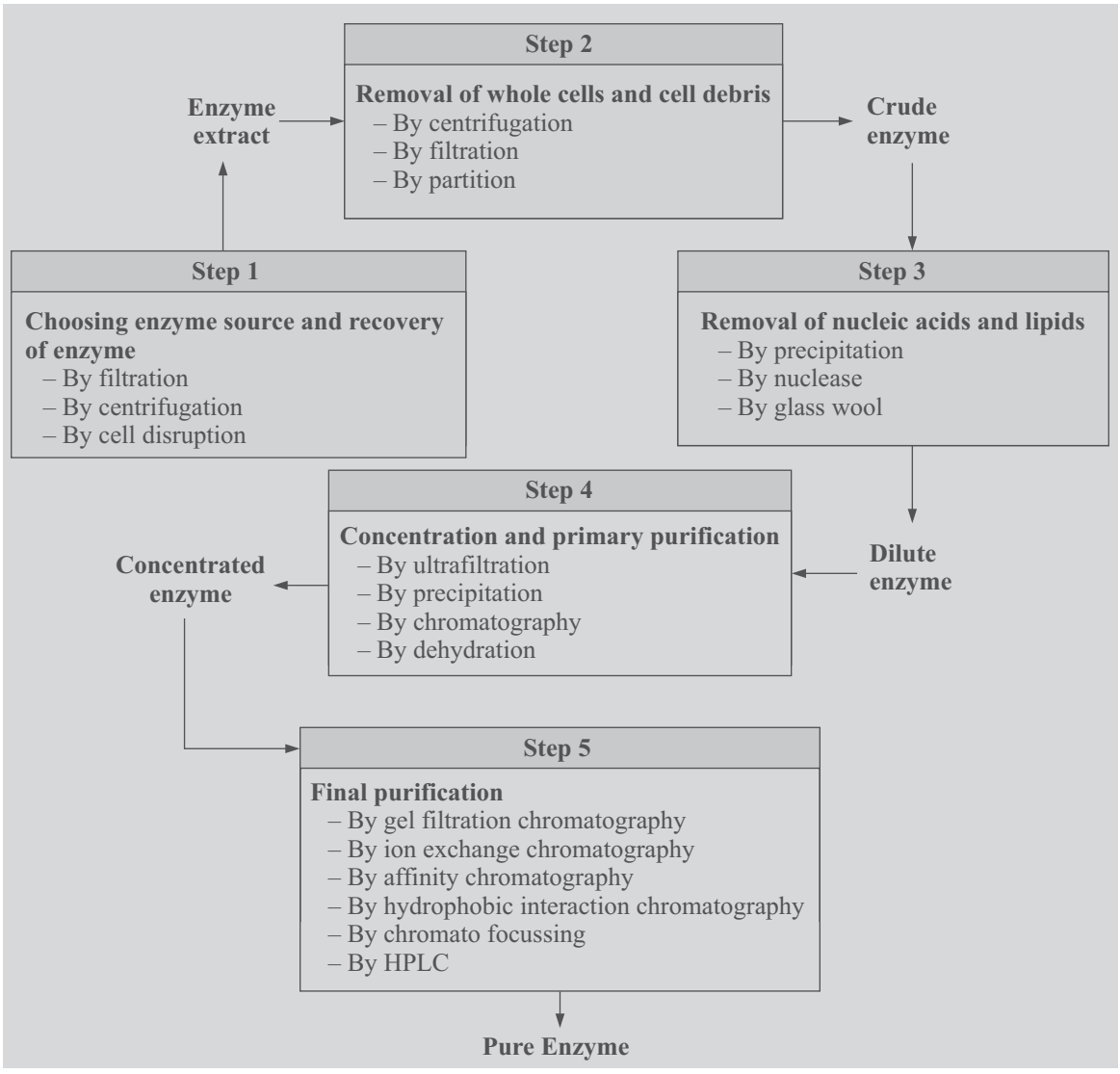


Fig. 1.15 Flow-sheet for enzyme purification

Step 1 Extraction (initial recovery) of enzyme from natural source

A prerequisite for the initial recovery of any enzyme is the identification of a suitable source that is rich in the target enzyme. Enzymes are usually recovered from a microbial source, a plant source, or an animal source.

Microbial source

Many enzymes of industrial interest, especially extracellular enzymes, are obtained from microbial sources as they can be cultured in large quantities over a short time period by fermentation. Suitable microbial sources are identified and isolated by screening strategies. Microbial enzymes are often more stable than analogous enzymes obtained from plant or animal tissue. Generally Recognized As Safe–(GRAS) certified microbes are chosen for enzyme recovery. GRAS-listed microbes are non-pathogenic, nontoxic, and generally they do not produce antibiotics. GRAS-listed bacteria includes *Bacillus subtilis, B. amyloliquifaciens* and *Lactobacillus* species. GRAS-listed fungi for enzyme sourcing include *Aspergillus* species, *Penicillium* species, *Mucor, Rhizopium* species, and *Saccharomyces cerevesiae*. This is shown in Table 1.9. Enzymes purified from a range of microbes are shown in Table 1.10.

Table 1.9 GRAS-certified microbes used for enzyme sourcing and purification

Bacteria	*Bacillus subtilis* *Bacillus amyloliquifaciens* *Lactobacillus* species
Fungi	*Aspergillus* species *Penicillium* species *Mucor* species *Rhizopium* species *Saccharomyces cerevesiae*

Table 1.10 Enzymes recovered and purified from a range of microbial sources

Enzyme	Microbial source	Enzyme	Microbial source
Lactate dehydrogenase Cholesterol esterase Glucose-6-P-dehydrogenase	*Yeast* *Pseudomonas* species *Yeast* *Leuconostac mesentroides*	Asparaginase	*Escherechia coli*
Glucose oxidase Glycerol kinase	*Aspergillus niger* *Candida* *Mycoderma* *Arthobacter* species	Collagenase	*Clostridium* species
		α-amylase	*Bacillus subtilis* *Asereillus* species
Phytase	*Aspergillus flavus* *Aspergillus candidus*	Aminoacylase	*Aspergillus oryzae* *Bacillus stearothermophilus*
Hexokinase	*Yeast*		
Cellulase	*Trichoderma viridae* *Trichoderma reesei* *Trichoderma koningii* *Penicillium fumiculosum*	Lactase	*Kluveromyces lactis* *Kluveromyces fragilis* *Aspergillus niger*

Table 1.10 (Contd.)

Enzyme	Microbial source	Enzyme	Microbial source
Pullulanase	*Aerobacter aerogenes* *Klebsciella aerogenes*	Protease	*Bacillus licheniforms* *Bacillus lentus* *Bacillus alcalophilas* *Bacillus amyloliquifaciens*
Invertase	*Saccharomyces* species		
Pectinase	*Aspergillus niger* *Aspergillus fusarium*		
Phytase	*Aspergillus niger*	Xylanase	*Clostridium thermocellum*
Lipase	*Humicola languinosa*	Thermolysin	*Bacillus thermoproteoliticus*
Cellulase	*Humicola insolens*	Cyclodextrin glycosyl transferase	*Bacillus megatarium* *Bacillus circilans*
Glucoamylase	*Aspergillus niger*	Penicillin acylase	*Kluvera* species *Aerobacter* species *Brevibacterium* species *Proteus rettgeri* *Proteus morganii*
Glucose isomerase	*Aerobacter* species *Bacillus* species *Lactobacillus brewis* *Actinoplanes missouriensis*		

Microbial enzymes are useful in industrial process as well as for therapeutic *purposes.* Microbial source of some important enzymes and their applications are given in Table 1.11.

Table 1.11 Applications of enzymes sourced from microbes

Enzyme	Source	Application
Streptokinase	*Hemolytic Streptococci*	Thrombolytic agent
Staphylokinase	*Staphylococcus aureus*	Thrombolytic agent
Asparaginase	*Erwinia chrysanthemi*	Treatment of leukemia
Glucose oxidase	*Aspergillus niger*	Quantification of blood glucose
Alcohol dehydrogenase	*Aspergillus niger*	Determination of blood alcohol level
Amylase	*Bacillus* species *Aspergillus oryzae*	Degradation of starch
Protease	*Bacilli*, *Aspergilli*	Degradation of proteins for food and detergent
Cellulase	*Trichoderma* species *Aspergillus niger* *Actinomyces*	Degradation of cellulose

Heterologous enzymes in microbes

Expression of enzymes in cells that are not a natural source of that enzyme is called recombinant enzyme production. Such enzymes are produced by recombinant technology and are called heterologous enzymes. *Escherechira coli* is the commonly employed organism of heterologous enzyme production. The enzyme expressed accumulates in the cytoplasm from which it can be recovered or the enzyme is secreted into the medium using signal peptide (recombinant DNA technology). Other microbial sources for heterologous production include *Lactobacillus lactis*, *Cornybacterium glutamaticum*, *Bacillus* species, and *Streptomyces* species. Yeast such as *Saccharomyces cerevasiae* and *P. pastoris* are suitable candidates for heterologous production of α–1 antitrypsin and streptokinase, respectively. Filamentous fungi are used for producing extracellular enzymes. For example, triglyceride lipase and aspartic protease.

Plant source

Plants represent a traditional source of a wide range of enzymes. Plant tissues are chosen as a source for subsequent purification of various enzymes like ascorbate oxidase, phytase, peroxidase, urease, bromealin, amylase, lipase, sucrase, nitrate reductase, aldehyde oxidase, protesse, xanthine dehydrogenase, sulphate oxidase, fructose bisphosphate aldolase, sedoheptulose bisphosphatase, fructose bisphosphatase and papain. The plant source of these enzymes are given in Table 1.12. Among these, papain and ficin (protease) assume much significance because they are the most widely studied enzymes. Like microbes, plants can also serve as a source for heterologous enzymes. For example, α-amylase, chymosin (rennin), cyclodextrin transferase, glucoamylase, lysozyme, xylanase, and phytase are heterologous enzymes expressed in plants. The original source and the plant expression system of some enzymes are shown in Table 1.13.

Table 1.12 Enzymes purified from plant source

Enzyme	Plant Source
Ascorbate oxidase	*Curcubita* species
Peroxidase	Horseradish
Phosphoglycerol pyruvate carboxylase	Maize leaves
Urease	Jack bean
Bromealin	Pineapple stem/fruit
β-amylase	Barley
Pectin esterase	Citrus fruits
Phytase	Wheat, rye, triticale
Papain	Latex and leaves and unripe fruits of papaya
Ficin	Latex of certain tropical trees

Table 1.13 Heterologous enzymes expressed in plants

Enzyme	Original source	Plant expression system
α-amylase	*Bacillus licheniforms*	Tobacco
Chymosin	Calf	Tobacco
Cyclodextrin	*Klebsciella pneumoniae*	Potato
Glucoamylase	*Aspergillus niger*	Potato
Lysozyme	Chicken	Tobacco
Phytase	*Aspergillus niger*	Tobacco
Xylanase	*Clostridium thermocellum*	Tobacco

Animal source

Animal tissues are a source of several enzymes of industrial use and therapeutic use. The choice of tissue depends on the extent of expression of a particular enzyme in that tissue. For example, the pancreas is a good source for the purification of digestive enzymes; liver is the best source for recovery and purification of detoxification enzymes (like SOD). Other organs like stomach, placenta, heart, kidney, or cells like erythrocytes can be sources for specific enzymes. Table 1.14 summarizes the list of enzymes purified from animal sources.

Methods for initial recovery

Any of the above-mentioned sources that are essentially rich in the target enzyme is chosen and the enzymes are recovered by several methods.

Table 1.14 Enzymes purified from animal sources

Enzyme	Animal Source
Alkaline phosphatase	Calf intestine and kidney
Acetyl choline esterase	Bovine erythrocytes
Arginase	Beef liver
Cholesterol esterase	Pig beef pancreas
Creatine kinase	Rabbit muscle, beef heart, pig heart
Glutamate dehydrogenase	Beef liver
Glycerol-3-P-dehydrogenase	Rabbit muscle
Uricase	Porcine liver
Xanthine oxidase	Buttermilk
Pepsin	Mammalian stomach
Reinin (chymosin)	Mammalian stomach
DNAse	Mammalian pancreas
Chymotrypsin	Mammalian pancreas
Trypsin	Mammalian pancreas
Glucocerebrosidase	Placenta

These methods are mentioned in Table 1.15 and discussed below.

Table 1.15 Methods of cell disruption for enzyme recovery

Enzyme	Method
Intracellular enzyme	Filtration
Cytosolic enzyme	Differential centrifugation
Subcellular organelle enzyme	Differential centrifugation
Extracellular enzyme Secretory enzyme	Cell disruption using 1. Blender 2. Mortar and pestle 3. Dynomill or bead mill 4. High-pressure homogenizer 5. Osmotic shock 6. Enzymes 7. Detergents or chemicals 8. Sonicator 9. Freeze-thaw cycle

Recovery of extracellular enzymes

Most of the microbial enzymes are produced by fermentation and released into the fermentation medium. Such secretory enzymes can be recovered by *filtration* or by *centrifugation* of the fermentation medium.

Recovery of intracellular enzymes

Intracellular enzymes present within plant cells, animal cells or microbial cells are recovered by *cell disruption* in which cell architecture is disorganized in the presence of a buffer in order to release the cellular content and form crude extract. Cell disruption can be done by using blenders, homogenizers or other methods, based on the nature of the enzymes. Mechanical methods should be strong enough to break the cell wall and the membrane, but should not physically damage the enzymes. Also, cold conditions are preferable to avoid heat-induced damage during the mechanical operation. Various methods of cell disruption for enzyme recovery are listed in Table 1.15.

Blender A blender has a small chamber equipped with a set of blades of different shapes. The chamber is filled with the specimen and the blender is operated electronically. The blades rotate and exert a *shearing force* to disturb the cell architecture and release the homogenate. Plant cells and mammalian cells may be homogenized using a blender.

Mortar and pestle A small amount of the tissue sample is taken in a mortar and hand-ground with the pestle in the presence of *abrasives* like fine glass pieces and a buffer to form a homogenate. Plant leaves, liver, kidney, and other organs can be disrupted on a

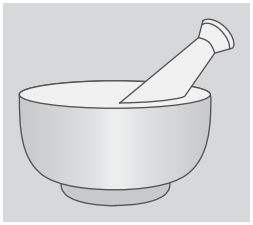


Fig. 1.16 Mortar and Pestle for small scale cell disruption

small scale by this method. Figure 1.16 shows a picture of mortar and pestle.

Dynomill or bead mill Components of a dynomill are shown in Fig. 1.17. It is made up of a chamber provided with a rotating impeller disc.

The source tissue and glass beads of 0.5µm diameter are loaded in the chamber. The impeller disc is rotated at high speed at which the velocity is approximately around 10m/sec. The beads collide with the disc and the cells are *trapped* between the beads and the disc, leading to disruption of cellular architecture.

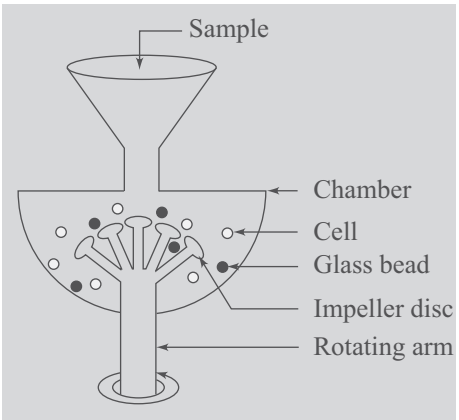


Fig. 1.17 A dynomill

High-pressure homogenizer A high-pressure homogenizer is shown in Fig. 1.18. It has an upper chamber and a lower chamber, both connected by a small orifice.

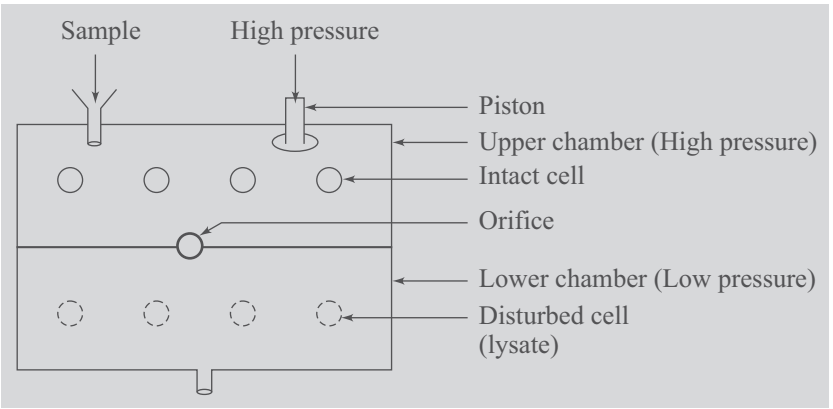


Fig. 1.18 High-pressure homogenizer

The cell suspension is introduced through an inlet into the upper chamber and subjected to a high pressure created by a mechanically driven piston. The cells are thus mechanically pushed through the orifice into the lower chamber, where the pressure drops suddenly. As a result, the cells *burst abruptly* due to *turbulence* and *shearing force*. The crude extract is collected *through* the outlet. Plant cells and microbial cells can be disturbed by this method. The French Press and the Hughes Press are commercially available high-pressure homogenizers that can be used for a large volume of cells.

Osmotic shock Animal cells can be lysed by osmotic shock. Cells are placed in a buffer of high osmotic pressure, for example, a buffer with 20% sucrose for half an hour to one hour. Consequently, water molecules *diffuse* from the cells into the buffer causing the cells to shrink. The shrunken cells are then transferred to a buffer of low osmotic pressure or into distilled water. As a result, water molecules diffuse into the cell causing the cells to swell and rupture.

Table 1.16 Enzymes used in cell disruption

Enzyme	Cells disrupted
Lysozyme	Gram-negative bacteria
Zymolase or lyticase	Yeast cell
Chitinase	Fungal cell

Enzymes Specific enzymes interact with a wide range of microbial cells and induce cell disruption. Lysozyme cleaves the cell wall of Gram-positive bacteria. Zymolase or lyticase disturbs yeast cell. Chitinase is used to disturb the cell wall architecture of fungal cells. This is shown in Table 1.16.

Table 1.17 Chemicals used in cell disruption

Detergents	Sodium lauryl sulphate Polysorbate Triton
Solvents	Toluene Acetone
Chaotropic agents	Urea Guanidine

Detergents and chemicals Detergents and chemicals used to disturb microbial cells for recovering intracellular enzymes are shown in Table 1.17. Detergents like sodium lauryl sulphate, polysorbate, triton, and solvents like toluene acetone, etc. and chaotropic agents like urea or guanidine are used to disturb microbial cells on a large scale.

Sonicator Sonicator is a device that generates sound waves. Cells are exposed to high-frequency sound waves of greater than 20 Hz for 30–60 seconds in the presence of ice. Sound waves create a *shearing force* and disturb the cell architecture.

Freeze-thaw cycle The freeze-thaw cycle involves repeated freezing and thawing of cell suspension. This results in intracellular ice crystal growth leading to membrane damage and cell rupture.

As a result of cell disruption the enzyme will be present in the form of a crude extract. It should therefore be separated from cell debris and the whole cells.

Step 2 Removal of cell debris and whole cells

After cell disruption the intracellular enzymes are released into the medium (homogenate) and thereafter the cell debris and whole cells can be removed by centrifugation, filtration, or aqueous two-phase partitioning. Details of the methods used for removing cell debris during enzyme purification are given in Table 1.18.

Table 1.18 Methods used for removing cell debris during enzyme purification

Method	Type	Apparatus/materials involved
Centrifugation	Continuous flow centrifugation	Continuous-flow centrifuge, Disc-type centrifuge, Hollow-bowl centrifuge, Basket centrifuge
Filtration	Fiber depth filtration Polymer membrane filtration Modern cartridge filtration	Cellulose fiber, glass fiber Cellulose acetate membrane system Polytetrafluoro ethylene membrane system Nylon membrane system
Partitioning	Double aqueous phase partitioning system	Two incompatible water soluble polymers

Centrifugation Centrifugation is usually done in a continuous flow centrifuge. The homogenate is continuously pumped and the clarified solution is continually collected. The deposited solids (whole cells and cell debris) can be removed from the centrifuge bowl by periodically stopping centrifuge and manually removing the pelleted material. Modern centrifuge allows intermittent discharge of solids through a discharge valve or a peripheral nozzle.

Filtration Filtration is done through a depth filter, a membrane filter, or a modern cartridge filter. A depth filter is made up of randomly cross-linked cellulose fibers or glass fibers forming a mesh work of channels. It retains whole cells, cell debris, denatured proteins, aggregates, and precipitates not only on the surface but also within the depth of the filter. The enzyme is then filtered out.

Membrane-like sheets of polymers like cellulose acetate, nylon, or polytetrafluoro ethylene with pore size 0.02–10 μm are used to filter cell debris under pressure. This method is also called microfiltration.

Modern cartridge filtration system involves filter sheets of different pore size folded into a single cylindrical cartridge system by joining both ends. This is useful in efficient filtration. This method is shown in Fig. 1.19.

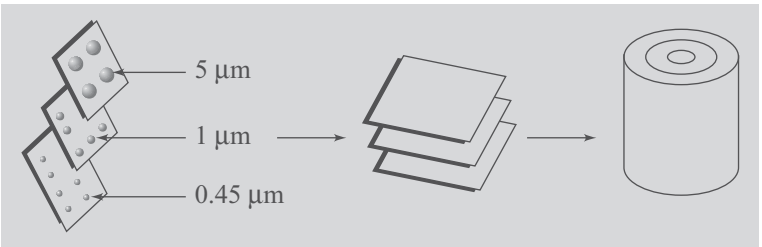


Fig. 1.19 Modern cartridge filtration system

Aqueous two phase partitioning Aqueous polymer solutions are similar to solutions of some polar organic solvents like alcohol, acetone, and acetonitrile. These organic solvents are completely miscible in water, and so are the aqueous polymers like polyethyleneglycol and polyvinylpyrrolidone. In both cases (aqueous organic solvent and aqueous polymer solutions) the phase partitioning can be induced by salt addition of salt. Aqueous two-phase partitioning is shown in Fig. 1.20.

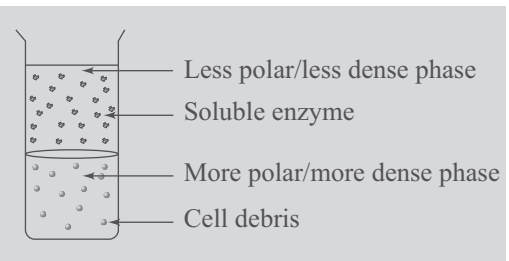


Fig. 1.20 Aqueous two-phase partitioning of cell debris and enzyme

When the homogenate is passed through such a partitioning system, debris settles to the lower phase, while soluble *enzymes* are retained on the less dense *upper phase*. Subsequently, the two phases are separated.

Step 3 Removal of nucleic acids and lipids

Nucleic acids present in the homogenate are removed by precipitation with cationic polymer-like polyethylenimine, and subsequent separation by centrifugation or filtration. Compounds like streptomycin, protamine, magnesium chloride, or manganese chloride can also be used for precipitation. Alternatively, treatment with nucleases is also used.

Removal of lipid is aided by passing the homogenate through glass wool. The solution obtained after step 3 is called *crude enzyme*.

Step 4 Concentration of enzymes

The crude enzyme obtained in the third step is more dilute and hence it is concentrated by various methods like ultrafiltration, precipitation, and dehydration. The principles of these methods are summarized in Table 1.19.

Ultrafiltration This method is used to concentrate and purify the enzyme preparation by removing impurities of smaller size. Membranes of cellulose acetate, cellulose nitrate, polyvinyl chloride, and polycarbonate with pore size of 1 to 20 nm are used for ultrafiltration. The pore size should be less than the molecular size of the target enzyme. The crude preparation is passed through the membrane filter and pressure is applied using nitrogen gas and peristaltic pump. Molecules with low molecular size than the filter cut-off pore size pass through the ultra filter, while high molecular size enzymes are concentrated and retained within the filter.

Table 1.19 Methods used for concentration of enzymes

Method	Principle leading to concentration	Apparatus
Ultrafiltration	Filtration under pressure based on molecular weight	Polymer membrane like polyvinyl acetate
Precipitation	Increased protein-protein interaction by salting in and salting out. Precipitation below 0°C by lowering dielectric constant of aqueous solution	Ammonium sulphate solution Organic solvents
Dehydration	Removal of water from enzyme by adding sephadex	Sephadex G-25 beads

Precipitation Proteins in crude solution are precipitated using neutral salt, usually ammonium sulphate. Addition of a specific quantity of ammonium sulphate to the crude solution increases the protein solubility. This is called *Salting in*. However, when salt concentration in the crude solution is further increased above an optimal level the salt molecules effectively compete with protein molecules for *water of hydration*. This promotes increased protein-protein interaction, eventually leading to precipitation. This process is called *Salting out*. A Diagrammatic representation of salting out is shown in Fig. 1.21.

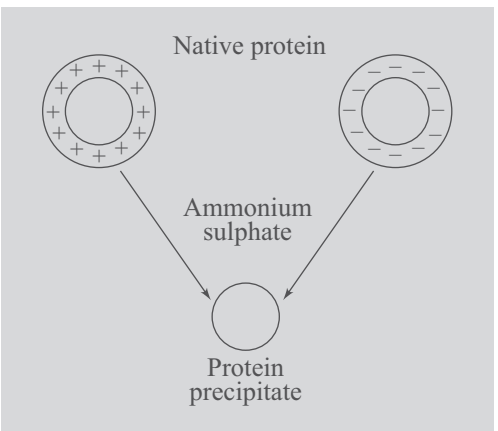


Fig. 1.21 Precipitation of protein by salting out

Addition of various organic solvents like ethanol, acetone, isopropanol, or diethyl ether at or below 0°C precipitates protein from the solution by lowering the dielectric constant of the aqueous solution. Thermal denaturation can be carried out to precipitate contaminant proteins and nontarget proteins. This is used if the target proteins are extremozymes.

Adding dry sephadex Addition of dry sephadex G-25 beads to crude preparation results in the entry of water into the beads, while the enzymes that are too large to enter into the beads remain concentrated.

Step 5 Final purification by column chromatography

Column chromatography makes use of a column packed with a stationary phase into which the sample to be purified and the mobile phases are passed through, and the sample separated. In this technique, the separation is based on various principles like gel filtration or exclusion based on molecular size, ion exchange mechanism based on surface charge, biological affinity based on specific biological interaction, high pressure or hydrophobic interaction. This section explains different types of column chromatography principles used in enzyme purification.

Gel filtration chromatography

Gel filtration chromatography is also called gel permeation chromatography, or gel exclusion chromatography, or molecular sieve chromatography. It is used to separate and purify enzymes based on molecular size exclusion through a gel bead column. A column of porous gel beads is used as a stationary phase, while a liquid distributed inside and outside the gel pores (sieves) act as a mobile phase. The principle of gel filtration chromatography is shown in Fig. 1.22.

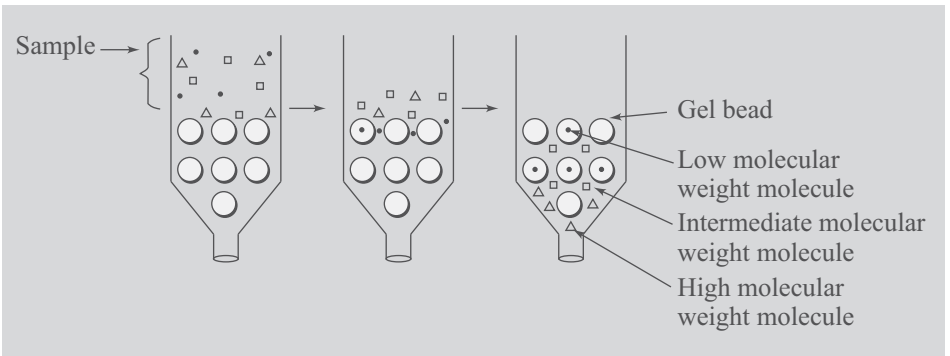


Fig. 1.22 Principle of gel filtration chromatography (the pore size of the gel bead can be chosen to retain the target enzyme to be purified)

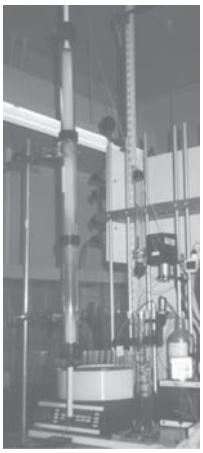


Fig. 1.23 Gel filtration chromatography equipment

When a sample mixture is passed through such a gel filtration column, molecules are distributed between the inner and the outer mobile phase depending on their molecular size. Compounds of high molecular size are completely excluded from the sieve and come out at a faster rate, while compounds of low molecular size are completely retained within the inner mobile phase of the sieve and come out at a slower rate after elution. Compounds of intermediate molecular size pass through the interstitial space between the gel beads and come out at a moderate speed. If the pore size of the gel beads is chosen to retain the enzyme molecule, the latter can easily be purified and recovered by elution. Porous glass granules or porous gel beds of polymeric organic compounds are used for enzyme purification by gel filtration chromatography. This is shown in Table 1.20. In addition, this method is also used to determine the molecular weight of enzymes and other proteins. Some amount of water and low molecular weight impurities can be removed using sephadex G-25 column. A picture of the gel filtration chromatography equipment is shown in Fig. 1.23.

Table 1.20 Gel materials used for enzyme purification by gel filtration chromatography

Gel	Trade Name
Agarose	Sepharose Biogel-A
Polyacrylamide	Biogel-P Sephacryl
Dextran	Sephadex Sephacryl
Polystyrene	Biobead

Ion exchange chromatography

Enzymes with net positive or negative charge are separated and purified due to the attraction between the charged group of the target molecule and the oppositely charged group present in the solid matrix stationary phase called the ion exchangers. For example, sulphonated polystyrene, carboxymethyl cellulose, and DEAE cellusose (diethylaminoethyl cellulose). Enzyme purification by ion exchange chromatography is shown in Fig. 1.24.

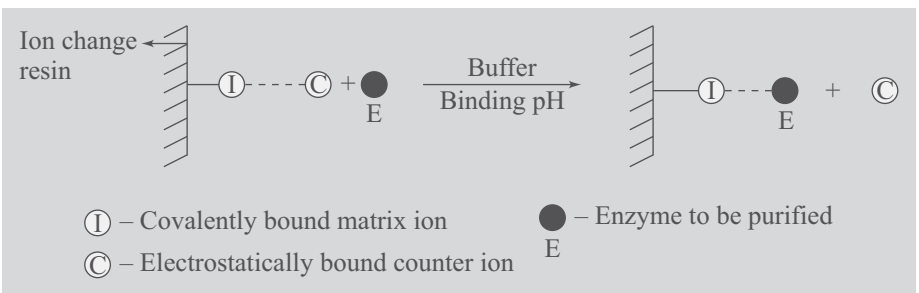


Fig. 1.24 Enzyme purification by ion exchange chromatography

Ion exchangers are cross-linked inert matrix containing covalently bound ions, which in turn is *electrostatically* bound to exchangeable counter ions. Matrix that has covalently bound positive ion, which in turn is electrostatically bound to a negative counter ion is called an *anionic exchanger*. For example, DEAE cellusose: $OC_2H_5–N^{(+)}H(C_2H_5)_2$. Matrix that has covalently bound negative ion, which in turn is electrostatically bound to a positive counter ion is called as *cationic exchanger*. For example, carboxymethyl (CM) cellulose: $–OCH_2CO_2^{(–)}$.

The DEAE exchanger is highly positively charged at pH 6–8, so DEAE cellulose is most useful for enzymes that are negatively charged in this range. Carboxy/methyl cellulose is highly negatively charged, at around pH 4.5, hence CM cellulose is applicable in purifying enzymes that are positively charged at around pH 4.5. This is shown in Fig. 1.25.

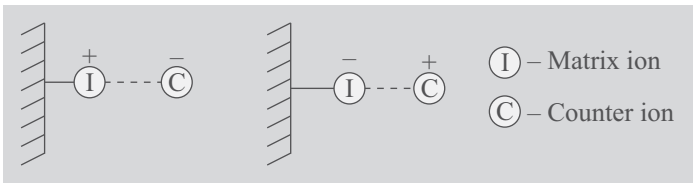


Fig. 1.25 Ion exchangers used for enzyme purification

Ion exchangers are treated with 0.5 N NaOH and 0.5 N HCl and allowed to swell. The swollen exchanger is suspended in water to expose the functional group and equilibrated with suitable counter ions. Excess counter ions can be removed by water-wash and the exchanger packed into the column. The column is equilibrated with a low concentration buffer of suitable binding pH at a rate of 60 ml/hr in order to allow uniform diffusion of the sample into the column and to get a good resolution. A buffer with a pH that is one unit above or below the isoelectric point of the enzyme to be separated is used as a mobile phase.

A crude sample is dissolved in the mobile phase and applied on the column and diffused in it. At this stage, the charged group of the sample compound undergoes an exchange with the counter ion and adsorbs reversibly to the column. Bound enzyme is eluted by changes in either salt concentration or pH. NaCl salt can be used for elution. In this procedure, as the concentration of the salt (NaCl) increases, the enzyme protein is displaced from DEAE cellulose by anion ($Cl^–$) and from CM cellulose by cation (Na^+). In pH elution, the pH is altered over the relatively narrow working range and the proteins are eluted as their isoelectric point is reached, since they then have no net charge to bind the resin.

Affinity chromatography

Affinity chromatography is the best method for enzyme purification. This method involves separation of enzyme molecules based on their specific noncovalent reversible biological interaction towards ligands, which are immobilized in a solid stationary phase.

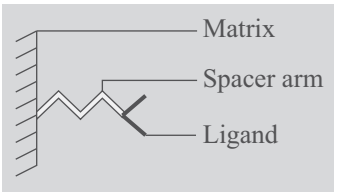


Fig. 1.26 Affinity column used for enzyme purification by affinity chromatography

Matrix such as agarose, dextran, polystyrene, and polyacrylamide are used as the stationary phase. The stationary phase chosen should have a suitable chemical group to bind with the ligand. The stationary phase is first activated by treatment with cyanogen bromide. Ligands possessing chemical groups like amino, carboxyl, thiol, or hydroxyl are covalently immobilized at the stationary phase by means of spacer arms (hydrophobic or hydrophilic compounds and packed into a column. A column with a stationary phase, a ligand, and a spacer arm can be collectively called an affinity column. This is shown in Fig. 1.26. Tryptophan is used as a ligand for purifying chymotrypsin. (Tryptophan is the substrate for chymotrypsin and capable of binding, but not hydrolyzing, significantly, the enantiomeric substrate analog.) Avidin can be used for separating and purifying biotin containing enzymes because it has a high affinity towards biotin. Metal ions are used as a ligands for superoxide dismutase, (so-called metal affinity chromatography) because the histidine residue of the enzyme forms coordinate covalent bonding with the metal. Cibacron blue dye is used as a ligand for kinases, dehydrogenases, and DNA polymerase (so-called dye ligand chromatography). Dye ligands may act as substrate analogs, offering affinity interactions with their corresponding enzymes. After binding the enzyme with its ligand the affinity column is percolated with a suitable buffer. As a result, enzymes bound to a ligand are retained within the column, while other nonspecific impurities are washed away. Subsequently, enzyme molecules are eluted by percolating the column with a deforming buffer at a pH, that a changes the characteristics of the enzyme and no longer allows it to bind to the immobilized ligand, or by the use of a competitive counter-ligand like reversible inhibitor, a for which the enzyme has more affinity than the column ligand. An overview of enzyme purification by affinity chromatography is shown in Fig. 1.27.

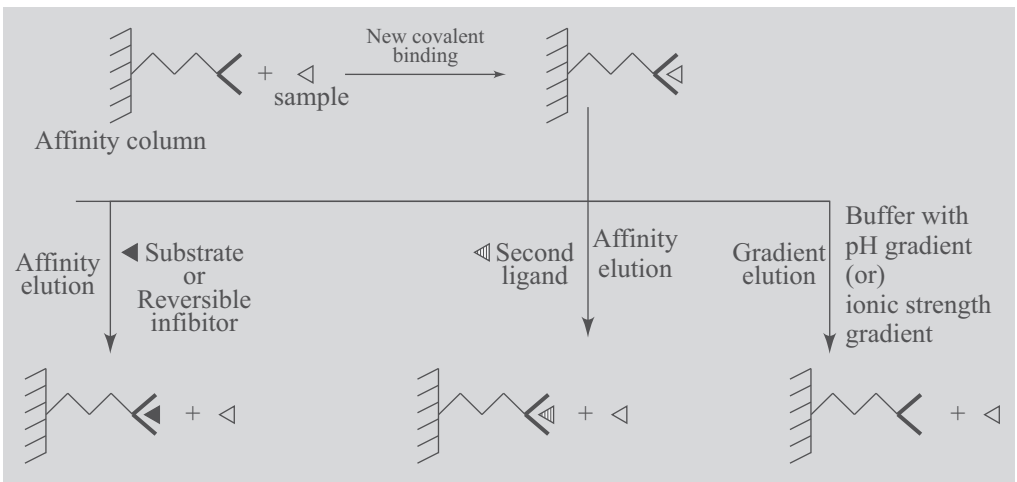


Fig. 1.27 An overview of enzyme purification by affinity chromatography

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography is a type of adsorption chromatography. It involves separation of compounds based on the difference in interaction between the *surface specific hydrophobic amino acids* of the protein with a solid support called adsorbents. Hexyl, octyl, or phenyl groups supported on an agarose column can be used as adsorbents. Enzymes initially precipitated using ammonium sulphate (step 4) have their hydrophobic amino acid residues on the surface of the protein *exposed*. The precipitate is then dissolved in a buffer. The pH of the buffer should be equal to the isoelectric point of the protein. The sample is then loaded to the column at high ionic strength. At high ionic strength, nonpolar amino acids form hydrophobic interactions with the column package. Thus, protein can be separated based on the difference in hydrophobic interaction with the adsorbent. The column is eluted with the buffer by continuously decreasing the ionic strength or increasing the pH, as this will decrease the hydrophobic interaction and detach the protein from the column. Alternatively, the column may be percolated with a nonionic detergent such as Tween 20 or Triton × 100. As the adsorbent has greater affinity for the detergent than for the protein, the latter can be eluted out.

HPLC

Conventionally, chromatography is not carried out under high pressure. In a unique technique called HPLC (high pressure liquid chromatography), purification and separation can be done under high pressure using microparticulate stationary phase of diameter, based on any one of the principles described for other chromatography. Here the high pressure is used in order to increase the flow rate and to decrease back pressure and get a better resolution than that of low pressure chromatography (conventional chromatography).

After the final step of purification by chromatography, the degree of purification of the enzyme can be calculated from the specific activities of the crude and purified extracts using the following formula:

$$\text{Degree of purification} = \frac{\text{Specific activity of purified extract}}{\text{Specific activity of crude extract}}$$

Percentage recovery of any enzyme can be calculated from the total units in the crude and purified extracts by using the following formula.

$$\%\ \text{recovery} = \frac{\text{Total units in purified extract}}{\text{Total units in crude extract}} \times 100$$

1.10 Fractionation and Characterization of Proteins (Enzymes)

Proteins can be fractionated by various methods and various properties like molecular weight, electrophoretic mobility, isoelectric point, and charge to mass ratio can be determined. Fractionation and characterization can be done by the following methods:

1.10.1 Analytical Ultracentrifugation

Analytical ultracentrifugation is done to find purity, sedimentation rate, molecular weight, and conformational change. An analytical ultracentrifuge operates at a very high speed of 70,000 rpm. This is shown in Fig. 1.28. The protein sample to be characterized is suspended in a suitable medium (solvent) and taken in the analytical cell, which is counter-balanced by the counterpoise cell. The sample is centrifuged for sedimentation. The molecules are fractionated into density zones. A parallel beam of light illuminates the sedimenting particle and gets refracted in the zone, and the refractive index is plotted against the distance of the analytical cell and monitored as the peak. The area of the peak gives the concentration of the sample. The appearance of a single sharp peak indicates purity of the protein, while appearance of an additional peak, shouldering of main peak, and asymmetric peak, represents impurity. Shift in the peak or movement of the peak gives the sedimentation velocity (v) of the particles at time (t). The sedimentation coefficient (S) is calculated using the equation:

$$S = v/\omega^2 \text{ r}$$

where v = sedimentation velocity
ω = angular velocity of the rotor
r = radial distance moved by the molecules from the axis of rotation

S is substituted in the following equation in order to determine the molecular weight

$$M = [RTS/D(1-\upsilon)\rho]$$

where M = Molecular weight of the molecule
R = Molar gas constant
T = Absolute temperature in Kelvin
S = Sedimentation coefficient
D = Diffusion coefficient of the molecule
υ = Partial specific volume of the molecule
ρ = Density of the medium at 20 °C

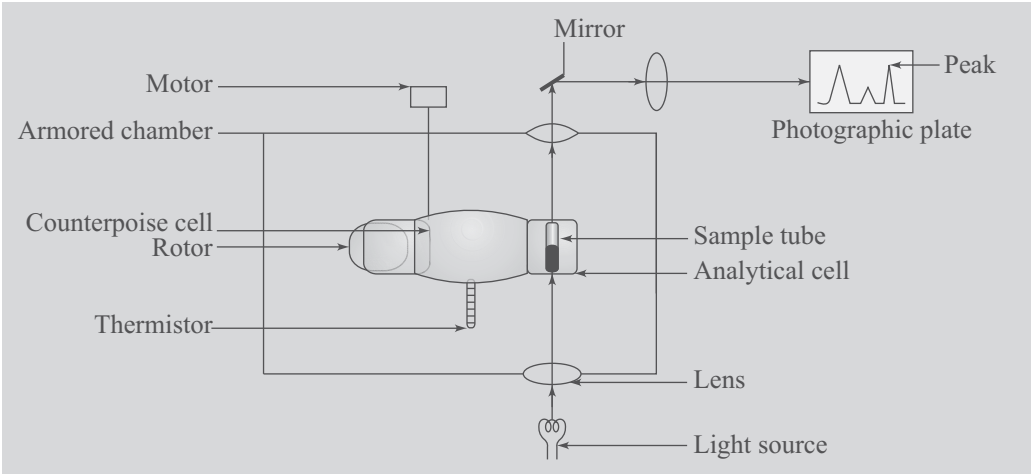


Fig. 1.28 Analytical ultracentrifuge

The native protein molecule is compact and the sedimentation rate is faster. However, when there is any conformational change in the native molecule, it becomes disorganized, leading to a decrease in the sedimentation rate. Therefore, the difference in the sedimentation rate, before and after the conformational change gives us an idea of the extent of conformational changes. For example, denaturation of aspartate transcarbamoylase can be detected.

1.10.2 Gel Filtration Chromatography

Proteins of high, low, and intermediate molecular weights are fractionated using distribution coefficient (K_D) value and elution volume. The K_D value for high-molecular weight molecules is equal to zero, and hence are completely excluded from the inner mobile phase. For low-molecular weight molecules, the K_D value is equal to one, and gain complete access to the mobile phase within the gel pore. Intermediate-molecular weight molecules have K_D values between zero to one and gain partial access to the inner mobile phase with the distribution coefficient value.

Elution volume is the volume of liquid (buffer or mobile phase) required to elute a particular compound with a K_D value between 0 and 1. It is given by the equation

$$V_e = V_o + K_D \times V_i$$

where V_o is the volume external to the gel bead (called void volume).

For two compounds having different molecular weight and hence different K_D values, say K_D' and K_D'' Ve is given by the equation

$$V_e = K_D'' - K_D'' \times V_i$$

Molecular weight of the fractionated protein can be determined based on the linear relation between log molecular weight (M_w) and elution volume (V_e). The equilibrated gel column is loaded with standard protein of known molecular weight, e.g. β-galactosidase (116 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and lysozyme (143 kDa). Ve is determined for standard protein markers and for unknown protein. A plot of log molecular weight versus V_e of the standard protein is constructed to get a linear relation, as shown in Fig. 1.29. The plot is extrapolated and the molecular weight (M_w) of the test protein is identified.

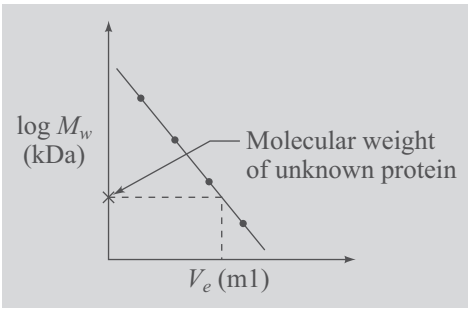


Fig. 1.29 Determination of molecular weight of protein by gel filtration chromatography using elution volume

1.10.3 Isoelectric Focusing

Isoelectric focusing (IEF) is a type of gel electrophoresis in which compounds are separated in a gel with pH gradient, based on differences in isoelectric point. Isoelectric focusing gel has preselected pH range. *Immobilized pH gradient strips* (IPG strips) can also be used for ctrophoresis. IPG strips are available in different lengths to accommodate various gel sizes and different pH ranges to allow optimal separation. IPG strips reduce the preparation time and reagent waste. Proteins are electrophoresed on IEF gel or IPG strip.

Protein molecules with a pH lower than the isoelectric point acquire a net positive charge and move towards the cathode, while those with pH higher than the isoelectric point acquire net negative charge and move towards the anode. Migration continues until the molecules reach a pH region in the gel, which is equal to their isoelectric point. At the region, where the pH of the gel is equal to the isoelectric point of the molecule, the molecules become *stationary* and exist as *zwitter ions*, as shown in Fig. 1.30. Thus, proteins could be fractionated based on their isoelectric point.

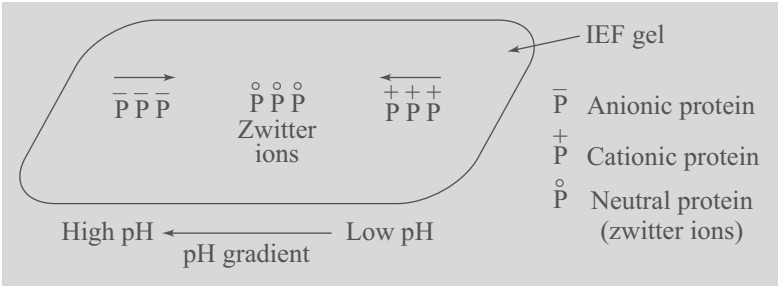


Fig. 1.30 Protein fractionation based on isoelectric point using isoelectric focusing

1.10.4 One-Dimensional PAGE

One-dimensional PAGE is usually done using two different gels of different pore size; upper *stacking gel* with larger pore size and lower *separating gel* with smaller pore size. The secondary structure of protein is stabilized by hydrophobic interaction, hydrogen bond, and disulphide bond. So the secondary structure of protein is disturbed by the addition of denaturing agents like SDS, β-mercaptoethanol, and urea in order to aid electroophotetic mobility. Disturbance of secondary structure leads to increase in intrinsic viscosity and increased entropy. Function of denaturing agents are shown in Table 1.21.

Table 1.21 Role of denaturing agent in protein fractionation

Denaturing of agent	Function
SDS	Disturbs hydrophobic interaction Confers negative charge to the protein that is proportional to the molecular size
β-mercaptoethanol	Break disulphide bridges
Urea	Break hydrogen bonds

The *denatured sample* is loaded to the upper stacking gel where protein molecules migrate down towards the anode and are tightly stacked into a sharp distinct disc, which in turn enters into the separating gel and gets resolved based on the molecular-sieving property of the gel. Low molecular weight proteins pass through the gel pores easily and migrate faster, while high molecular weight proteins are retarded due to *frictional resistance* and migrates, slowly. To determine the molecular weight of proteins a plot of molecular weight of standard protein markers against distance traveled (electrophoretic mobility) in the gel is constructed to get a linear relation as shown in Fig. 1.31. This plot gives the *inverse relationship* between the molecular weight and the distance traveled (electrophoretic mobility). The molecular weight range of the marker proteins should cover the molecular weight of the unknown proteins.

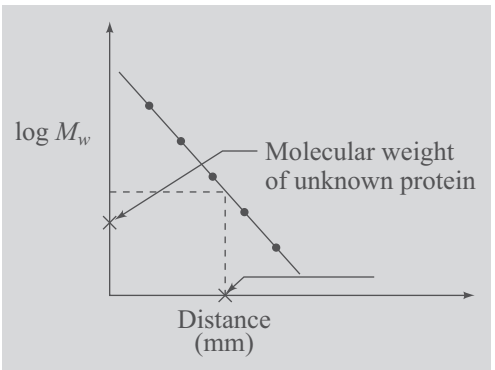


Fig. 1.31 Determining the molecular weight of protein by SDS PAGE using standard markers

1.10.5 Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis separates proteins, first based on their isoelectric point in a horizontal direction and in the second dimension based on their molecular mass in a vertical direction. In this technique, the protein molecules are first separated in an IEFgel with pH gradient based on differences in isoelectric point. The gel is then incubated in SDS to denature the protein. The separation in the second dimension by molecular mass is performed in slab SDS-PAGE. Under applied voltage the denatured protein subunits are separated on the second gel, based on mass. Thus, in 2-D PAGE whole proteins are fractionated. This method is useful for characterization of *isoelectric point and molecular mass of proteins*.

If the identity of the protein is desired, usually the method of *in-gel digestion* is applied, where the protein spot of interest is excised, and digested proteolytically. The peptide masses resulting from the digestion can be determined by mass spectrometry using peptide mass fingerprinting. If this information does not allow unequivocal identification of the protein, its peptides can be subject to tandem mass spectrometry for *de novo* sequencing.

1.10.6 HPLC/MS

Characterization of protein mixtures using HPLC/MS is also called *shotgun proteomics* and *mudpit* (multidimensional protein identification technology). In this method peptides (obtained after enzymatic digestion) are fractionated by one or two steps of liquid chromatography. The eluant from the chromatography stage can be either directly introduced to the mass spectrometer through electrospray ionization, or analyzed using MALDI.

1.10.7 Mass Spectrometry

Mass spectrometry is an analytical technique that measures the mass-to-charge ratio of charged particles. It is generally used to find the composition of a physical sample by generating a mass spectrum representing the masses of sample components. The mass spectrum is obtained by a mass spectrometer. Components of a mass spectrometer are schematically shown in Fig. 1.32.

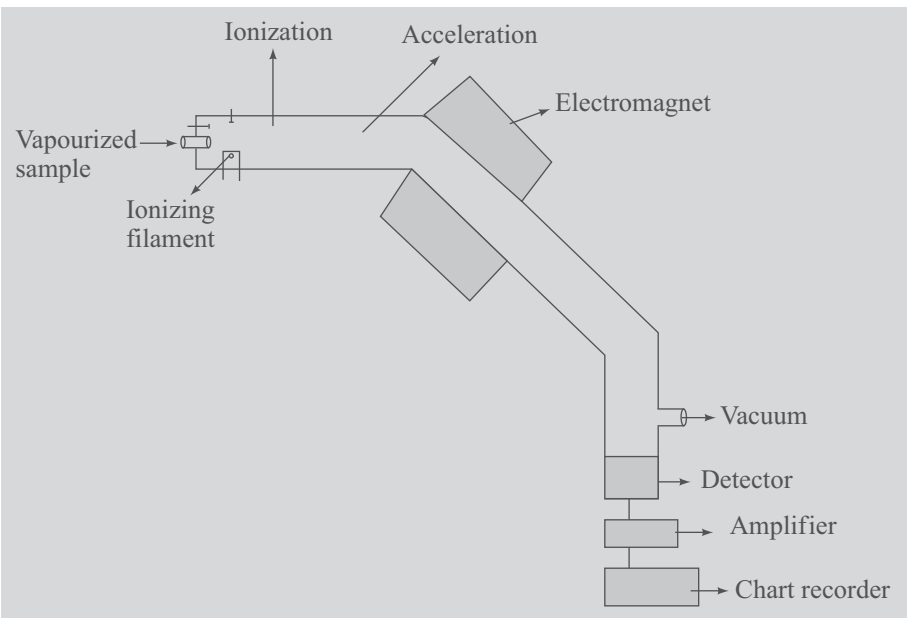


Fig. 1.32 Components of mass spectrometer

Mass spectrometers consist of three basic parts: an *ion source*, a mass analyzer, and a detector system. The stages within the mass spectrometer are:

- Production of ions from the sample
- Separation of ions with different masses
- Detection of the number of ions of each mass produced
- Data analysis

Mass spectrometry is used for the characterization of proteins. Methods for ionization of whole proteins are *electrospray ionization* (ESI), *matrix-assisted laser desorption/ionization* (MALDI), and *surface-enhanced laser desorption/ionization* (SELDI).

Two approaches are used for characterizing proteins:

- Top-down approach
- Bottom-down approach

In the top-down approach, intact proteins are ionized by either of the two techniques described above, and then introduced to a mass analyzer.

In the bottom-down approach, proteins are enzymatically digested into smaller peptides using *proteases* such as trypsin or pepsin, either in solution or in gel, after electrophoretic separation. Other proteolytic agents are also used. The collected peptide products are then introduced to the mass analyzer. When the characteristic pattern of peptides is used for the identification of the protein the method is called *peptide mass fingerprinting* (PMF). If the identification is performed using the sequence data determined in *tandem MS* analysis, i.e. analysis without the help of data base, it is called *de novo sequencing*.

Whole protein mass analysis is primarily conducted using either *time-of-flight* (TOF) MS, or *Fourier transform ion cyclotron resonance* (FT-ICR). These two types of instruments are preferable here because of their wide mass range, and in the case of FT-ICR, its high mass accuracy. Mass analysis of proteolytic peptides is a much more popular method of protein characterization, as cheaper instrument designs can be used for characterization. Additionally, sample preparation is easier once whole proteins have been digested into smaller peptide fragments. The most widely used instrument for peptide mass analysis are the MALDI time-of-flight instruments as they permit the acquisition of PMFs at high pace (1 PMF can be analyzed in approximately 10 sec). Multiplestage quadrupole-time-of-flight and the quadruple ion trap also find use in this application. Given below is a list of various mass analyzers used for protein characterization.

- Scanning mass analyzer
- MALDI time-of-flight mass analyzer
- Trapped ion mass analyzer
- Fourier transform ion cyclotron resonance
- Quadrupole mass analyzer

Proteins of interest to biological researchers are usually part of a very complex mixture of other proteins and molecules that coexist in the biological medium. This presents two significant problems. First, the two ionization techniques used for large molecules only work well when the mixture contains roughly equal amounts of constituents, while in

biological samples, different proteins tend to be present in widely differing amounts. If such a mixture is ionized using electrospray or MALDI, the more abundant species have a tendency to '*drown*' or suppress signals from less abundant ones. The second problem is the difficulty in interpretation of mass spectrum. Enzymatic digestion of a protein gives rise to a large number of peptide products in the form of complex mixture whose mass spectrum is very difficult to interpret. To contend with this problem, *Two-dimensional gel electrophoresis* (2-DGE) and HPLC/MS are used to fractionate proteins, or their peptide products from an enzymatic digestion.

1.10.8 Protein Identification by Peptide Mass Fingerprinting

Peptide mass fingerprinting is used to identify proteins. This method uses the masses of proteolytic peptides as input to a search of a database of predicted masses that would arise from digestion of a list of known proteins. If a protein sequence in the reference list gives rise to a significant number of predicted masses that match the experimental values, there is some evidence that this protein was present in the original sample.

Recent Developments

Intestinal enzymes are used in controlling obesity. An international team has pinpointed an enzyme that determines whether what fat people eat is burnt off as energy or stored in the body, a breakthrough that raises the prospect of a pill to target the enzyme, allowing people to eat without the risk of putting on weight. In their study on laboratory rodents, the team focused on MGAT2, an enzyme found in the intestines of mice as well as humans. Mice without the MGAT2 enzyme protein were able to eat a high-fat diet, while remaining slim and healthy. The fat they absorbed was burnt off as energy, rather than stored. (Source: *Deccan Chronicle*, March 17th, 2009).

Summary

Enzymes are biocatalytic proteins. They possess a recognition site for recognizing the substrate and an active site for catalyzing the reaction of the substrate. Activity of enzymes can be expressed in terms of international units, or Katal, or turnover number, or specific activity.

Enzymes possess remarkable properties like catalytic power, specificity, regulatability, milder reaction condition, reversibility, denaturability, and colloidal nature. Enzyme activity is influenced by various factors like substrate concentration, temperature, pH, and radiation. Thus, each enzyme has its own optimum parameters (like temperature, pH, optima, and k_m value). Based on the type of reaction, catalyzed enzymes are classified into six major classes—oxidoreductase, transferase, hydrolase, isomerase, lyase, and ligase. Each major class, in turn, is further classified into subclasses and sub-subclasses.

Enzyme-substrate interaction takes place either by lock and key model (predetermined active site) or by induced fit model (substrate-induced complementarity). Binding of substrate and release of product during enzyme-catalyzed reaction is explained by the Cleland notation.

Histoenzymology refers to the localization of enzymes and the measurement of enzyme activity in defined cells lying within a complex histology.

Histoenzymology is used to investigate biochemical cellular changes that may be induced by a drug, a disease, or a toxic agent.

Enzyme activity in any source can be assayed by three methods: direct assay, coupled assay/linked assay, and multistep coupled assay. After the assay, the enzyme can be purified from the relevant source by initial recovery of the enzyme, separation of cell debris and whole cells, and concentration and final purification by column chromatography.

Enzymes can be fractionated and characterized by several standard methods like SDS PAGE, 2D-PAGE, isoelectric focusing, analytical ultracentrifugation, gel filtration chromatography, and mass spectrometry.

Key Terms

Substrate A substance upon which the enzyme acts.

Simple enzyme An enzyme made up of protein alone.

Conjugated enzyme enzyme conjugated to a non-protein part is called conjugated enzyme or holoenzyme.

Apoenzyme The protein part of a conjugated enzyme is called apoenzyme.

Cofactors Inorganic prosthetic groups of enzymes are called cofactors.

Coenzymes Organic prosthetic groups are called coenzymes.

Endoenzymes Endoenzymes are those whose site of synthesis and site of action are similar. For example, metabolic enzymes.

Exoenzymes Exoenzymes are those whose site of synthesis and site of action are different. For example, digestive enzymes.

Adaptive enzymes An adaptive enzyme is one that is not constantly present in the cell, and instead, is produced only when its substrate is present.

Constitutive enzymes Enzymes that are always present in relatively constant amounts in the cell, regardless of the presence of the substrate, are called constitutive enzymes.

Chromogenic enzymes Enzymes that convert a colorless substrate into a colored product are called chromogenic enzymes.

Light-emitting enzymes Enzymes that convert normal substrate into a glowing light-emitting product are called light emitting enzymes.

Mechanochemical enzymes Enzymes that convert chemical energy into mechanical energy are called mechanochemical enzymes. For example, ATPase couples hydrolysis of ATP to induce a conformational change in muscle protein.

Modulators Modulators are small molecules that modulate the enzyme activity.

Activators Activators are modulators that enhance enzyme activity.

Inhibitors Inhibitors are modulators that decrease the enzyme activity.

Active site An active site is the area of the enzyme to which the substrate binds for the subsequent reaction.

Homotropic enzyme A homotropic enzyme is one that has a structural similarity with the substrate and the modulator.

Heterotropic enzyme A heterotropic enzyme is one that has no structural similarity with its substrate and its modulator.

Regulatory enzymes Enzymes that respond to various cellular metabolic signals and adjust their catalytic activity are called regulatory enzymes.

Plasma-specific enzyme Enzymes that are synthesized in the tissues but present in the blood in equivalent or higher concentration than in the tissues are called plasma-specific enzymes. For example, Lipoprotein lipase

Non-plasma-specific enzyme Enzymes that are present in a lower amount in blood than in tissues are called nonplasma-specific enzymes. For example, Pancreatic lipase.

Homogenous catalysis In homogenous catalysis, the catalyst and the substrate are in the same phase without any phase boundary.

Heterogenous catalysis In heterogenous catalysis, the catalyst is in a phase with the surface boundary. For example, hydrogenation of a ethylene in the presence of a metal catalyst like palladium.

International Unit (IU) One IU of an enzyme is the amount of enzyme that catalyzes the formation of one micromole of product, in one minute, under optimal conditions of pH, temperature, and ionic strength.

Katal One Katal is the amount of enzyme catalyzing the conversion of one mole of substrate to product in one second. One Katal = 6×10^7 IU.

Turnover number (K_{cat}) It is defined as the number of substrate molecules converted into product per enzyme molecule, per unit time, when the enzyme is fully saturated with the substrate.

Reactive groups These are the functional groups of the enzyme and the substrate that collides for the reaction to occur.

Heterologous enzymes Enzymes that are expressed by recombinant technology in cells that are not natural sources of that enzyme are called heterologus enzymes.

Isoelectric point It is the pH at which the protein bears no net charge.

Homogenization Disturbance of cell architecture to release the cell content/cell lysate is called homogenization.

Analytical ultracentrifugation It is the technique by which biomolecules are characterized by sedimentation under a very high centrifugal field in a refrigerated condition.

Sedimentation rate The velocity with which the particles sediment during centrifugation is called as sedimentation rate

Sedimentation coefficient It is the sedimentation rate per unit centrifugal field.

Elution volume It is the volume of the liquid required to elute a compound with kD value between 0 to 1.

Isoelectric focusing It is an electrophoretic technique in which molecules are separated, based on their isoelectric point.

2D PAGE It is a technique in which molecules are subjected to two different types of electrophoresis-isoelectric focusing followed by SDS-PAGE.

pKa It is the negative logarithm of the acid dissociation constant, Ka. Just like the pH, the pKa tells the acidic or basic nature of solutions. (pKa < 2 means strong acid; pKa > 2 but < 7 means weak acid; pKa > 7 but < 10 means weak base; and pKa > 10 means strong base.

Solved Problems

1. An enzyme of molecular weight 46000, at a concentration of 1 mg catalyzed a reaction at a velocity of 0.25 μ moles/min under optimum conditions. Calculate the following:

(a) Specific activity of the enzyme in terms of units/ mg protein.
(b) Specific activity of the enzyme in terms of units/ mole.
(c) Turnover number

Solution

Calculation of specific activity (in terms of unit/mg protein):

$$\text{Specific activity} = \frac{V_{max}}{\text{mg enzyme}}$$

$$= \frac{0.25\,\mu\text{moles/min}}{10^{-3}\text{mg}}$$

$$= \frac{0.25\,\mu\text{moles/min} \times 10^3}{\text{mg}}$$

$$= \frac{250\text{ units}}{\text{mg protein}}$$

Calculation of specific activity (in terms of units/mole):

$$\text{Specific activity} = \frac{250\text{ units}}{\text{mg protein}} \times \frac{\text{Mw}}{\text{mole}}$$

$$= 2.5 \times 10^5 \times \frac{\text{units}}{\text{g protein}} \times \frac{\text{Mw}}{\text{mole}}$$

$= (2.5 \times 10^5 \text{ units/g protein})(4.6 \times 10^4 \text{g/mole})$

$= 2.5 \times 4.6 \times 10^5 \times 10^4 \text{ units/g protein} \times \text{g/mole}$

$= 11.5 \times 10^9 \text{ units/g protein} \times \text{g/mole}$

$= 1.15 \times 10^{10} \text{ units/mole enzyme}$

Calculation of turnover number:

$$1\ \mu\text{g enzyme} = \frac{10^{-6}\text{g}}{\text{Mw mol/g}} = \frac{10^{-6}\text{g}}{4.6 \times 10^4 \text{ mol/g}}$$

$$= \frac{1}{4.6} \times 10^{-6} \times 10^{-4}$$

$$= 0.21 \times 10^{-10}$$

$$= 2.17 \times 10^{-11} \text{ moles enzyme}$$

Substitute this value for $[E]t$ in the turnover number formula.

$$\text{Turnover number} = \frac{V_{max}}{[E]_t}$$

$$= \frac{0.25 \times 10^{-6} \text{ moles/min}}{2.17 \times 10^{-11} \text{ moles enzyme}}$$

$$= \frac{0.25}{2.17} \times \frac{10^{-6} \times 10^{-11} \text{ moles/min}}{\text{moles enzyme}}$$

$$= \frac{0.115 \times 10^{-6} \times 10^{11} \text{ moles/min}}{\text{moles enzyme}}$$

$$= 0.115 \times 10^5 \text{ min}^{-1}$$

$$= \mathbf{1.15 \times 10^4 \text{ min}^{-1}}$$

2. An enzyme preparation contains 6 mg of protein/ml. The specific activity of the enzyme = 21 units/mg protein. Calculate the initial velocity of the reaction in a standard 1 ml reaction mixture containing (i) 20 µl of preparation and (ii) 5 µl of preparation.

Solution

Enzyme concentration in a preparation is given as:

[Enzyme] = units/mg protein × mg protein/ml

$$= \frac{21 \text{ units}}{\text{mg protein}} \times \frac{6 \text{ mg protein}}{\text{ml}}$$

[Enzyme] = 126 units/ml

Consider that the velocity of the enzyme-catalyzed reaction is directly proportional to the enzyme concentration in a reaction mixture.

i.e. [Enzyme] = Velocity

Therefore, 20 µl of preparation contains

$$\frac{126 \text{ units}}{\text{ml}} \times 20\mu\text{l}$$

$$= \frac{126 \text{ units}}{\text{ml}} \times 0.02 \text{ ml}$$

$$= 2.52 \text{ units enzyme}$$

or

$$V = 2.52\ \mu \text{ moles} \times \text{min}^{-1} \times \text{ml}^{-1}$$

Similarly, 5 µl of preparation contains

$$\frac{126 \text{ units}}{\text{ml}} \times 5\ \mu\text{l}$$

$$= \frac{126 \text{ units}}{\text{ml}} \times 0.005 \text{ ml}$$

$$= 0.63 \text{ units enzyme}$$

$$V = 0.63\ \mu \text{ moles} \times \text{min}^{-1} \times \text{ml}^{-1}$$

3. 10 µl of a crude enzyme extract from a tissue catalyzed a reaction at a rate of 0.10 µ moles /min under standard optimum assay condition. 50 ml of the crude extract is fractionated by ammonium sulphate precipitation and the precipitate when redissolved in 10 ml of the buffer forms a purified extract. 10 µl of the purified extract catalyzed the same reaction at a rate of 0.18 µ moles/min. Calculate the percentage recovery of the enzyme.

Solution

$$\% \text{ recovery} = \frac{\text{Total units in purified extract}}{\text{Total units in crude extract}} \times 100$$

Crude enzyme extract contains:

$$\frac{0.10\ \mu \text{ mole/min}}{10\ \mu\text{l}}$$

$$= \frac{0.10\ \mu \text{ mole/min}}{0.01 \text{ ml}} = 10\ \mu \text{moles ml}^{-1}\text{min}^{-1}$$

$$= \mathbf{10 \text{ units/ml}}$$

Total units = units in 50 m l of the extract

Therefore,

$$\frac{10\,\text{units}}{\text{ml}} \times 50\,\text{ml} = \textbf{500 Total units}$$

Total units in crude enzyme extract = 500 Total units

Purified enzyme extract contains

$$\frac{0.18\,\mu\text{moles/min}}{10\,\mu\text{l}}$$

$$= \frac{0.18\,\mu\text{moles}/\text{min}}{0.01\,\text{ml}} = 18\ \mu\text{moles ml}^{-1}\,\text{min}^{-1}$$

= 18 units/ml

Total units = units in 10 ml of the extract

Therefore,

$$\frac{18\,\text{units}}{\text{ml}} \times 10\text{ml} = 180\ \text{Total units}$$

Total units in purified enzyme extract

= 180 Total units

$$\%\ \text{recovery} = \frac{\text{Total units in purified extract}}{\text{Total units in crude extract}} \times 100$$

$$= \frac{180}{500} \times 100 = 36\%$$

Enzyme recovery = **36%**

4. A crude cell-free liver extract contains 30 mg protein/ml. 10 µl of the crude extract catalyzed a reaction at a rate of 0.15 µ mole/min under standard optimum assay condition. 50 ml of the crude extract is fractionated by ammonium sulphate precipitation. The fraction precipitating between 20% and 40% saturation was redissoved in 10 ml buffer. This solution, which forms the purified extract contains 40 mg protein/ml. 10 µl of the purified extract catalyzed the reaction at a rate of 0.60 µ mole/min. Calculate the degree of purification obtained by the fraction.

Solution

Crude enzyme extract contains:

$$\frac{0.15\ \mu\ \text{moles/min}}{10\ \mu\text{l}}$$

$$= \frac{0.15\ \mu\ \text{moles/min}}{0.01\ \text{ml}} = 15\ \mu\ \text{moles ml}^{-1}\,\text{min}^{-1}$$

= 15 units/ml

1 ml of crude extract contains 30 mg protein.

Specific activity of crude extract

$$= \frac{\text{units/ml}}{\text{Mg protein/ml}} = \frac{15\text{units/ml}}{30\text{mg protein/ml}}$$

= 0.5 units/mg protein

Purified enzyme contains

$$\frac{0.60\,\mu\text{moles/min}}{10\,\mu\text{l}}$$

$$= \frac{0.60\,\mu\text{moles/min}}{0.01\,\text{ml}} = 60\,\mu\text{moles ml}^{-1}\,\text{min}^{-1}$$

= 60 units/ml

1 ml of purified extract contains 40 mg protein.

Specific activity of purified extract =

$$\frac{\text{Units/ml}}{\text{Mg protein/ml}} = \frac{60\ \text{units/ml}}{40\ \text{mg protein/ml}}$$

= 1.5 units /mg protein

Degree of purtification =

$$\frac{\text{Specific activity of purified extract}}{\text{Specific activity of crude extract}} = \frac{15}{0.5} = 3$$

Degree of purification = **3 fold**

5. Effect of temperature on V_{max} of an enzyme catalyzed reaction is given below:

Temperature (T °C)	V_{max} (µ moles min^{-1})
25	5.50
30	10.40
35	14.15
40	19.15
45	26.00

Calculate the Q_{10} value.

Solution

Q_{10} values are calculated for different 10°C

$$Q_{10} = \frac{V_{max}\ \text{at}\ T_2}{V_{max}\ \text{at}\ T_1}$$

where the difference between T_2 and T_1 is 10, i.e $T_2 - T_1 = 10°C$.

Therefore, if 35°C and 45°C are considered, then

$$Q_{10} = \frac{V_{max} \text{ at } 45°C}{V_{max} \text{ at } 35°C}$$

$$Q_{10} = \frac{26.00}{14.15} = 1.83$$

Q_{10} between 35°C and 45°C is **1.83**.

6. One gram of fresh weigh of liver contains 20 units of an enzyme with turnover number of 5×10^4 min^{-1}. Estimate the intracellular concen-tration of the enzyme.

Solution

Generally it is assumed that fresh tissue has 80% water, all of which is intracellular. That is, fresh tissue has

$$\frac{80 \text{ ml water}}{100 \text{ g wet wt.}} = 0.80 \text{ ml/g}$$

1 g of tissue has 20 units of enzyme and 1 g of tissue corresponds to 0.8 ml of water.

Therefore,

$$\frac{20 \text{ units}}{0.8 \text{ ml}} = \frac{25 \text{ units}}{\text{ml}}$$

$$\frac{25 \times 10^{-3} \text{units}}{\text{litre}} = 25 \times 10^3 \text{moles} \times \text{litre}^{-1} \times \text{min}^{-1}$$

Intracellular concentration of enzyme is

$$\frac{V_{max}}{\text{Turnover number}}$$

$$= \frac{25 \times 10^{-3} \text{ moles} \times \text{litre}^{-1} \times \text{min}^{-1}}{5 \times 10^4 \text{ min}^{-1}}$$

$$= 5 \times 10^{-3} \times 10^{-4} \text{ moles} \times \text{litre}^{-1}$$

$$= 5 \times 10^{-7} \text{ M}$$

Intracellular concentration of enzyme

$$= \mathbf{5 \times 10^{-7} \text{ M}}$$

Review Questions

1. Give evidences to show that enzymes are proteins.
2. How does an active site differ from a regulatory site?
3. Explain various terms used to measure enzyme activity.
4. Define holoenzymes, apoenzymes, plasma- specific, non-plasma specific-enzymes, homotropic enzymes, and heterotropic enzymes.
5. What is meant by enzyme specificity? Explain its types with suitable examples.
6. How are enzymes classified based on the type of reaction they catalyze
7. Give the systemic name and the E-C number of the enzyme catalyzing the following reactions:

   UDP-galactose ↔ UDP-glucose

   Pyruvate + ATP + CO_2 + H_2O ⟶ Oxaloacetate + ADP + Pi

   D-glyceraldehyde-3-P ↔ dihydroxyacetone phosphate
8. How is the rate of an enzyme-catalyzed reaction influenced by substrate concentration, temperature, and pH?
9. Explain the lock and key model and the induced fit model for enzyme substrate interaction.
10. What do you mean by histoenzymology? Give its applications.
11. How is an enzyme activity assayed?
12. Describe the steps involved in enzyme purification, with a flow sheet.
13. Explain the methods involved in fractionation and characterization of proteins.
14. Velocity of a reaction catalyzed by 1 μg of enzyme of molecular weight 30,666 is equal to 0.166 μ mole/min.
    (i) Find the specific activity of the enzyme in terms of: (a) units/mg and (b) units/mole.
    (ii) Calculate the turnover number.

    [**Ans:** *(i) : (a) 166 units/mg protein; (b) 0.507×10^{10} units/mole enzyme (ii) 0.5×10^4 min^{-1}*]

15. An enzyme preparation has a specific activity of 6.3 units/mg protein and contains 18 mg proteins/ml. Calculate the initial velocity of the reaction in a standard 1ml reaction mixture containing (i) 20 µl and (ii) 5 µl of the preparation.

[**Ans:** *(i) 22.68 µ moles × min^{-1} × ml^{-1} (ii) 5.67 µ moles × min^{-1} × ml^{-1}*]

16. Velocity of a reaction catalyzed by 10 µl of a crude enzyme extract from a tissue is 0.21 µ moles /min under standard optimum assay condition. 40 ml of the crude extract is fractionated by ammonium sulphate precipitation and the precipitate when redissolved in 10 ml of the buffer, forms a purified extract. 10 µl of the purified extract catalyzed the same reaction at a rate of 0.32 µ moles/min. Calculate the percentage recovery of the enzyme.

[**Ans:** *76.1% recovery*]

17. A crude enzyme extract from a tissue contains 25 mg protein/ml. 10 µl of the crude extract catalyzed a reaction at a rate of 0.24 µ mole/min under standard optimum assay condition. 50 ml of the crude extract is fractionated by ammonium sulphate precipitation. The fraction precipitating between 20% and 40% saturation was redissolved in 10 ml buffer. This solution, which forms the purified extract, contains 45 mg protein/ml. 10 ml of the purified extract catalyzed the reaction at a rate of 0.75 µ mole/min. Calculate the degree of purification obtained by the fraction. [**Ans:** *1.72 fold*]

18. The following table gives the values of V_{max} of an enzyme-catalyzed reaction at different temperatures.

Temperature (T °C)	V_{max} (µ moles min^{-1})
25	6.50
30	12.00
35	16.50
40	22.30
45	30.00

Calculate the Q_{10} values between (i) 25 °C and 35 °C (ii) 30 °C and 40 °C

[**Ans:** *(i) 2.46 (ii) 1.85*]

19. A tissue sample has 60 units of an enzyme with turnover number of 5×10^4 min^{-1}. Calculate the intracellular concentration of the enzyme.

[**Ans:** 9.37×10^{-7} *M*]

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