COURSE INTRODUCTION

The objective of this course deals basic introduction to enzymes in concerned to enzymatic reactions. The aim is to provide brief introduction of enzyme, nomenclature and classification of enzymes. The course is organized into following blocks:

Block 1 It covers the enzymes nomenclature and multi-enzymes complexes

Block 2 It deals the enzyme kinetics and regulation

Block 3 It describes the industrial enzymes and purification.

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Block- I

Enzymes nomenclature and multi-enzymes complexes

Unit-1
Introduction to enzymes

Unit-2
Multi-enzymes complexes

Introduction

This is the first block on Enzymes nomenclature and multi-enzymes complexes. It consists of following two units as follows:

Unit 1: The cell is the structural and functional unit of life—the basic building block of living systems. Cells have the capability to effectively utilize biocatalysts, known as enzymes, which have outstanding catalytic efficiency and both substrate and reaction specificity. Enzymes have amazing catalytic power and their high level of specificity for

their substrate makes them suitable for biological reactions. They are crucial for cellular metabolism. Each and every chemical reaction that takes place in plants, micro-organisms and animals proceeds at a quantifiable rate as a direct result of enzymatic catalysis. Most of the history of biochemistry is directly or indirectly related to the history of enzyme research. Catalysis in biological systems was initially reported in the early 1800s based on research into the digestion of meat. In this report the catalytic activity of secretions from the stomach, the conversion of starch into sugar by saliva, and various plant extracts were reported.

Unit 2: Multienzyme complexes are discrete and stable structures composed of enzymes associated noncovalently that catalyze two or more sequential steps of a metabolic pathway. They can be considered a step forward in the **evolution of catalytic efficiency** as they provide advantages that individual enzymes, even those that have achieved catalytic perfection, would not have alone. Then, the **rate** at which an enzymatic reaction proceeds is partly determined by the **frequency** with which enzymes and their substrates **collide**. Hence, a simple way to increase it is to increase the concentrations of enzymes and substrates. However, their concentrations cannot be high because of the enormous number of different reactions that occur within the cell. And in fact in the cells most reactants are present in micromolar concentrations (10^{-6} M), whereas most enzymes are present in much lower concentrations.

Unit- 1: Introduction to enzymes

Structure

Objectives

1.1 Introduction

1.2 Brief History of Enzymes

1.3 General Properties of Enzymes

1.3.1 Proteinaceous in nature

1.3.2 Colloidal nature

1.3.3 Substrate specificity

1.3.4 Catalytic properties

1.3.5 Turn over Number

1.1 Introduction

Enzymes are the biological macromolecules which speed up the rate of biochemical reactions in living organisms without undergoing any change. They are also called as

biological catalysts. Enzymes are also called as biocatalyst because they act as a catalyst in living organisms and regulate the rate of chemical reactions without itself being altered in the process. Enzymes are not only used to catalyse biochemical reactions in living cells but also used to catalyse a wide range of commercially important processes such as in the production of sweetening agents and the modification of antibiotics, in washing powders in analytical assays for clinical, forensic and environmental applications.

Enzymes are the complex protein molecules, often called biocatalysts, which are produced by living cells. They are highly specific both in the reactions that they catalyze and in their choice of reactants, which are known as substrates. An enzyme typically catalyzes a single chemical reaction or a set of closely related reactions. Side reactions resulting in the wasteful formation of by-products are rare in enzyme-catalyzed reactions, in comparison to uncatalyzed ones. Enzymes can also be defined as soluble, colloidal and organic catalysts that are produced by living cells, but are capable of acting independently of the cells.

Objectives

This is the first block on enzymes nomenclature and multi-enzymes complexes. It consists of following two units. Under first unit (Introduction to enzymes) we have following objectives. These are as under:

- To know definition of enzymes.
- To know about nomenclature and classification of enzymes
- To know about cofactors and its specificity.
- What are the important criteria for enzyme homogeneity?
- To know multi-enzymes complexes.
- What are enzyme inhibitions and protein mediated transport?

1.2 Brief History of Enzymes

The existence of biological catalysis was first recognized and described during the late 18th century while studying the digestion of meat by secretion of the stomach. Subsequently, Louis Pasteur concluded in 1850s that fermentation of sugar into alcohol by yeast is catalyzed by “ferments”. He postulated that these ferments were inseparable from the structure of living yeast cells, a view called ‘vitalism’ that prevailed for many years. He wrote that "alcoholic fermentation is an act correlated with the life and the organization of

the yeast cells, not with the death or putrefaction. The first enzyme discovered was Diastase from malt by Anselme Payen in 1833.

The German physiologist F.W. Kuhne coined the term enzyme (Greek meaning "in heaven") in 1878 to represent the “ferments”when he was studying the ability of yeast to produce alcohol from sugars. In 1898, Duclaux proposed that all enzymes should have suffix ―ase. The word enzyme is derived from the Greek words *en* (meaning ‘within’) and *zume* (meaning ‘yeast’). In 1897, Eduard Buchner studied the ability of dead yeast extracts to ferment sugar and he found that the sugar was fermented even when there was no living yeast cells in the mixture.

Eduard Buchner isolated the enzyme for the first time in 1903 for which he was awarded Nobel Prize in Chemistry for the discovery of "cell free fermentation". First enzyme recognized as protein was jack bean urease, crystallized in 1926 by James B. Sumner of Cornell University. He later, in 1946, received the Nobel Prize for his work with the enzyme urease. Urease is an enzyme that catalyzes the conversion of urea to ammonia and carbon dioxide. Certain bacteria that convert urea to ammonia as part of the nitrogen cycle contain this enzyme.

1.3 General Properties of Enzymes

The general properties of enzymes can be summarized as follows.

1.3.1 Proteinaceous in nature

All enzymes are proteins, except ribozymes (RNA molecules having catalytic activity).

1.3.2 Colloidal nature

Enzymes exist as hydrophilic colloids in protoplasm.

1.3.3 Substrate specificity

Enzymes possess remarkable specificity in that they generally catalyse the conversion of only one type of substrate molecule into product molecules. For example, maltase acts only on maltose while pancreatic lipase acts in a variety of fats. The substrate specificity of enzyme is based on amino acids sequence in the catalytic site as well as the optical isomeric form of the substrate.

Fig. 1

1.3.4 Catalytic properties

- Enzymes are only required in very low concentrations.
- They don't initiate the catalysis but accelerate the rate of catalysis by lowering the activation energy.
- They remain unchanged at the end of reaction.
- They do not alter the properties of end products.
- Enzymes accelerate the forward or reverse reactions to attain the equilibrium but don't shift the equilibrium.
- Most of the enzymes catalyzed reactions are reversible.

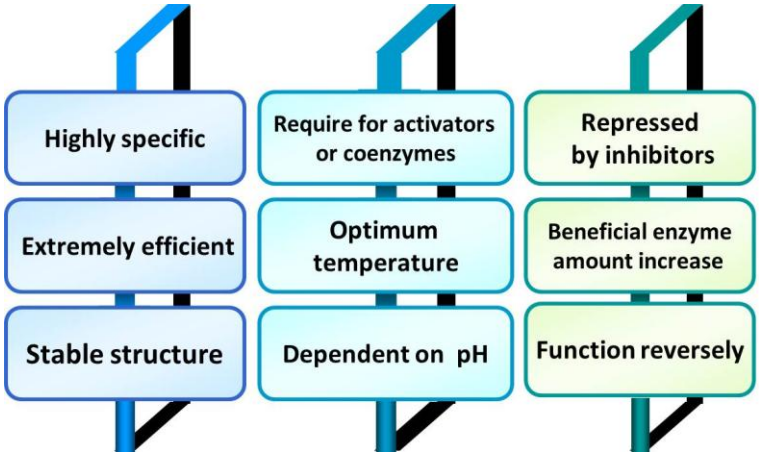

Fig. 2

1.3.5 Turn over Number

It is the number of substrate molecules converted per unit of time when its active site is saturated with substrate. Turn over number varies from 10^2 to 10^3 $sec^{-1.}$ For example the turn over number for sucrase is 10^4, that means, one sucrase molecule converts 10,000 sucrose molecules into products. Similarly, it is 36 million for carbonic anhydrase (fastest enzyme) and 5 million for catalase (2^{nd} fastest enzymes).

Enzyme Turnover Number: Examples		
	Enzyme	**Turnover Number** *(per second)*
1	Carbonic anhydrase	600000
2	Acetylcholinesterase	280000
3	Penicillinase	2000
4	Chymotrypsin	100
5	DNA Polymerase I	15
6	Lysozyme	0.5

Fig. 3

1.3.6 Sensitivity

Enzymes are highly sensitive to change in pH, temperature and inhibitors. Enzymes work best at a narrow range of condition called optimum.

1.3.6.1 Temperature

Enzymes are very sensitive to heat and temperature i.e. they are heat labile. The optimum temp of enzymes is 20-35°C. They become inactivated at very low temperature and denatured (destroyed) at very high temp i.e. greater than 45°C. In archebacterium, Pyrococcus furious, the optimum temperature of hydrogenise is greater than 95°C. This heat-stable enzyme enables *Pyrococcus* to grow at 100°C.

1.3.6.2 pH Value

The optimum pH of most endoenzyme is pH 7.0 (neutral pH). However, digestive enzymes can function at different pH. For example, salivary amylase act best at pH 6.8, pepsin act best at pH 2.0 etc. Sometime a change in pH causes the reverse reaction, e.g. at pH 7.0 phosphorylase break down starch into glucose 1- phosphate while at pH5 the reverse reaction occurs.

1.3.6.3 Inhibitors

Enzymes are also sensitive to certain inhibitors. Inhibitors are any molecules like cellular metabolites, drugs or toxins which reduce or check enzyme activity.

1.4 Chemical Nature

Almost all enzymes are proteins except ribozymes (catalytic RNAs).Ribozymes are RNA molecule having catalytic activity and are discovered during 1980s. Protein enzyme molecule is composed of one or more amino acid chains called polypeptide chains. The amino acid sequence determines the characteristic folding patterns of the protein's structure, which is essential to enzyme specificity. Amino acid-based enzymes are globular proteins that range in size from less than 100 to more than 2 000 amino acid residues. These amino acids can be arranged as one or more polypeptide chains that are folded and bent to form a specific three-dimensional structure, incorporating a small area known as the active site, where the substrate actually binds.

1.5 Cofactors and Coenzyme

A coenzyme is a substance that works with an enzyme to initiate or aid the function of the enzyme. It can be considered a helper molecule for a biochemical reaction. Coenzymes are small, nonproteinaceous molecules that provide a transfer site for a functioning enzyme. They are intermediate carriers of an atom or group of atoms, allowing a reaction to occur. Coenzymes are not considered part of an enzyme's structure. They are sometimes referred to as cosubstrates. Coenzymes cannot function on their own and require the presence of an enzyme. Some enzymes require several coenzymes and cofactors. Some examples of coenzymes are as given below.

Examples: The B vitamins serve as coenzymes essential for enzymes to form fats, carbohydrates, and proteins. An example of a non-vitamin coenzyme is S-adenosyl methionine, which transfers a methyl group in bacteria as well as in eukaryotes and archaea.

1.5.1 Coenzymes, Cofactors, and Prosthetic groups

Some texts consider all helper molecules that bind to an enzyme to be types of cofactors, while others divide the classes of chemicals into three groups:

- Coenzymes are nonprotein organic molecules that bind loosely to an enzyme. Many (not all) are vitamins or are derived from vitamins. Many coenzymes contain

adenosine monophosphate (AMP). Coenzymes may be described as either cosubstrates or prosthetic groups.

- Cofactors are inorganic species or at least non-protein compounds that aid enzyme function by increasing the rate of catalysis. Typically, cofactors are metal ions. Some metallic elements have no nutritional value, but several trace elements function as cofactors in biochemical reactions, including iron, copper, zinc, magnesium, cobalt, and molybdenum. Some trace elements that appear to be important for nutrition do not appear to act as cofactors, including chromium, iodine, and calcium.
- Cosubstrates are coenzymes that bind tightly to a protein, yet will be released and bind again at some point.
- Prosthetic groups are enzyme partner molecules that bind tightly or covalently to the enzyme (remember, coenzymes bind loosely). While cosubstrates bind temporarily, prosthetic groups permanently bond with a protein. Prosthetic groups help proteins bind other molecules, act as structural elements, and act as charge carriers. An example of a prosthetic group is heme in hemoglobin, myoglobin, and cytochrome. The iron (Fe) found at the center of the heme prosthetic group allows it to bind and release oxygen in the lung and tissues, respectively. Vitamins are also examples of prosthetic groups.

An argument for using the term cofactors to encompass all types of helper molecules is that many times both organic and inorganic components are necessary for an enzyme to function. There are a few related terms also related to coenzymes:

- Apoenzyme is the name given to an inactive enzyme that lacks its coenzymes or cofactors.
- The protein part of the enzyme on its own without its cofactor is termed as apoenzyme.
- Holoenzyme is the term used to describe an enzyme that is complete with its coenzymes and cofactors.
- Holoprotein is the word used for a protein with a prosthetic group or cofactor.

A coenzyme binds to a protein molecule (the apoenzyme) to form an active enzyme (the holoenzyme).

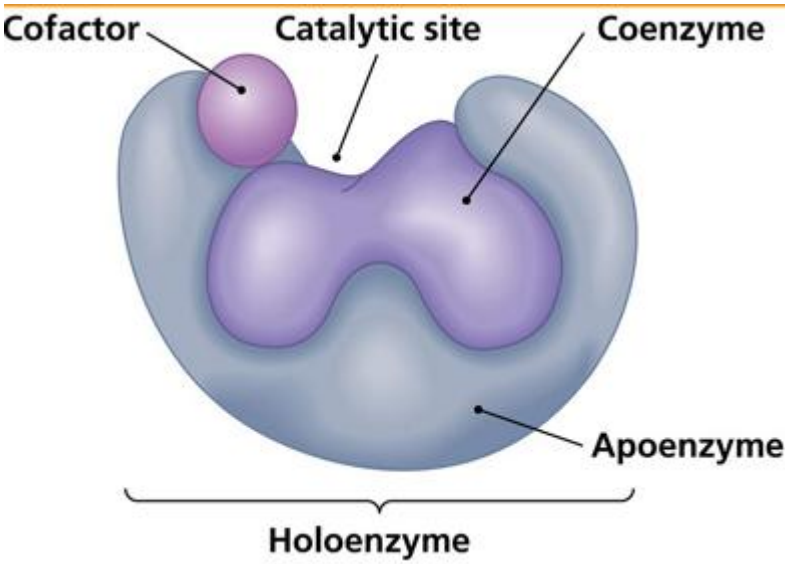

Fig. 4 Holoenzyme

Some inorganic ions that serve as cofactors for enzymes are given in the following table.

Ions	Enzymes
Cu^{2+}	Cytochrome oxidase
Fe^{2+} or Fe^{3+}	Cytochrome oxidase, catalase, peroxidase
K^{+}	Pyruvate kinase
Mg^{2+}	Hexokinase, glucose 6-phosphatase, pyruvate kinase
Mn^{2+}	Arginase, ribonucleotide reductase
Mo	Dinitrogenase
Ni^{2+}	Urease
Zn^{2+}	Carbonic anhydrase, alcohol dehydrogenase, carboxypeptidases A and B

Some coenzymes that serve as transient carrier of specific atoms or functional groups are given in the following table.

Coenzyme	Examples of chemical groups transferred	Dietary precursor in mammals
Biocytin	CO_2	Biotin
Coenzyme A	Acyl groups	Pantothenic acid and other compounds
5′-Deoxyadenosylcobalamin (coenzyme B_{12})	H atoms and alkyl groups	Vitamin B_{12}
Flavin adenine dinucleotide	Electrons	Riboflavin (vitamin B_2)
Lipoate	Electrons and acyl groups	Not required in diet
Nicotinamide adenine dinucleotide	Hydride ion ($:H^-$)	Nicotinic acid (niacin)
Pyridoxal phosphate	Amino groups	Pyridoxine (vitamin B_6)
Tetrahydrofolate	One-carbon groups	Folate
Thiamine pyrophosphate	Aldehydes	Thiamine (vitamin B_1)

1.6 Enzyme Active Sites

Enzyme-catalyzed reactions take place within the confines of a pocket on the enzyme called the active site. Uncatalyzed reactions tend to be slow because most biological molecules are quite stable in the neutral-pH, mild-temperature, aqueous environment inside cells. Enzymes greatly increase the rates of biological reactions by providing a specific environment within which a reaction can occur more rapidly. The active site may well involve only a small number (less than 10) of the constituent amino acids.

The reactant molecule is referred to as the substrate. The surface of the active site is lined with amino acid residues with substituent groups that bind to the substrate and catalyze its chemical transformation. Often, the active site encloses the substrate, sequestering it from solution. The active site of the enzyme chymotrypsin is shown in given fig 5.

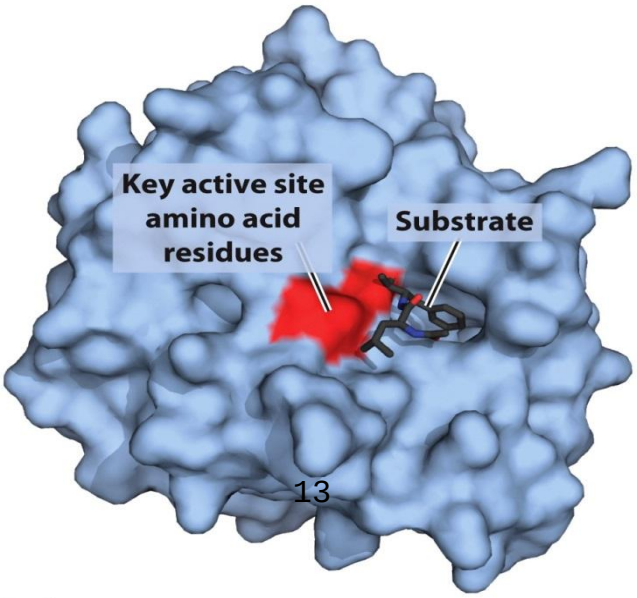

Fig. 5 Active Site of the Enzyme Chymotrypsin

1.7 Nomenclature

Many enzymes have been named by adding the suffix -ase to the name of their substrate which refer to the reaction that they catalyse (e.g. oxidase, dehydrogenase, carboxylase) while some individual proteolytic enzymes generally have the suffix -*in* (e.g. trypsin, chymotrypsin, papain) on their names. Biochemists by international agreement have adopted a system for naming and classifying enzymes based on the type of reaction catalyzed. International Union of Biochemistry set up the Enzyme Commission to address this issue. The first Enzyme Commission Report was published in 1961 in which a systematic approach to the naming of enzymes was explained. The sixth edition, published in 1992, contained details of nearly 3 200 different enzymes, and supplements published annually have now extended this number to over 5 000. Each enzyme is assigned a four-part classification number and a systematic name, which identifies the reaction it catalyzes. Some examples are explained as given below.

1.7.1 Trypsin

Trypsin has the Enzyme Commission (EC) number 3.4.21.4, where

1. The first number (3) denotes that it is a hydrolase
2. The second number (4) that it is a protease that hydrolyzes peptide bonds
3. The third number (21) that it is a serine protease with a critical serine residue at the active site, and
4. The fourth number (4) indicates that it was the fourth enzyme to be assigned to this class.

1.7.2 Glucose phosphotransferase

Enzyme hexokinase is formally known as ATP: glucose phosphotransferase has Enzyme Commission number 2.7.1.1, in which the first number (2) denotes the class name (transferase); the second number (7), denotes the subclass (phosphotransferase); the third number (1), a phosphotransferase with a hydroxyl group as acceptor; and the fourth number (1), D-glucose as the phosphoryl group acceptor. In this way the enzyme chymotrypsin has the EC number 3.4.21.1, and elastase 3.4.21.36.

1.8 Classification of enzymes

- Classification Based upon the type of reaction catalyzed.

- All enzymes have been placed into six major classes.

1.8.1 International Classification of Enzymes

Class no.	Class name	Type of reaction catalyzed
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms)
2	Transferases	Group transfer reactions
3	Hydrolases	Hydrolysis reactions (transfer of functional groups to water)
4	Lyases	Cleavage of C—C, C—O, C—N, or other bonds by elimination, leaving double bonds or rings, or addition of groups to double bonds
5	Isomerases	Transfer of groups within molecules to yield isomeric forms
6	Ligases	Formation of C—C, C—S, C—O, and C—N bonds by condensation reactions coupled to cleavage of ATP or similar cofactor

1.8.2 Oxidoreductases

These catalyze oxidation-reduction reactions where electrons are transferred. These electrons are usually in the form of hydride ions or hydrogen atoms. Oxidoreductases are of three types—oxidases, dehydrogenases and reductases, e.g., cytochrome oxidase (oxidises cytochrome), succinate dehydrogenase, nitrate reductase.

$$2AH_2 + O_2 \xrightarrow{\text{oxidase}} 2A + 2H_2O$$

$$AH_2 \xrightarrow{\text{dehydrogenase}} A + 2(H)$$

$$A + 2(H) \xrightarrow{\text{reductase}} AH_2$$

1.8.3 Transferases

These catalyze group transfer reactions. The transfer occurs from one molecule that will be the donor to another molecule that will be the acceptor. Most of the time, the donor is a cofactor that is charged with the group about to be transferred. Hexokinase used in glycolysis And Transaminase which catalyses the transfer of amino group from amino acid to a keto acid to form a new keto acid and a new amino acid.

(α-Ketoglutarate + Alanine—alanine aminotransferase → Glutamate + Pyruvate

Aspartate + α-Ketoglutarate —aspartate aminotransferase Oxaloacetate + Glutamate

1.8.4 Hydrolases

These catalyze reactions that involve hydrolysis. It usually involves the transfer of functional groups to water. When the hydrolase acts on amide, glycosyl, peptide, ester, or other bonds, they not only catalyze the hydrolytic removal of a group from the substrate but also a transfer of the group to an acceptor compound. Digestive enzymes belong to this group, e.g., amylase (hydrolysis of starch), sucrase, and lactase.

$$\underset{\text{maltose}}{C_{12}H_{22}O_{11}} + H_2O \xrightarrow{\text{maltase}} \underset{\text{glucose}}{2C_6H_{12}O_6}$$

1.8.5 Lyases

Catalyze reactions where functional groups are added to break double bonds in molecules or the reverse where double bonds are formed by the removal of functional groups. For example: histidine decarboxylase (breaks histidine to histamine and CO_2), Fructose bisphosphate aldolase used in converting fructose 1,6-bisphospate to Glyceraldehyde 3 Phosphate and Dihydroxyacetone Phosphate by cutting C-C bond.

$$\text{Fructose 1, 6-diphosphate} \xrightarrow{\text{aldolase}} \text{Dihydroxy acetone phosphate} + \underset{\text{phosphate.}}{\text{Glyceraldehyde}}$$

1.8.6 Isomerases

Catalyze reactions that transfer functional groups within a molecule so that isomeric forms are produced. These enzymes allow for structural or geometric changes within a compound. They are of three types, isomerases (aldose to ketose group of vice-versa like glucose 6-phosphate to fructose 6-phosphate), epimerases (change in position of one constituent or carbon group like xylulose phosphate to ribulose phosphate) and mutases (shifting the position of side group like glucoses- phosphate to glucose-l-phosphate). Phosphoglucose isomerase for converting glucose 6-phosphate to fructose 6-phosphate. Moving chemical group inside same substrate.

$$\text{Glucose 6-phosphate} \xrightarrow{\text{isomerase}} \text{Fructose 6-phosphate}$$
$$\text{Glucose 6-phosphate} \xrightarrow{\text{mutase}} \text{Glucose 1-phosphate}$$
$$\text{Xylulose 5-phosphate} \xrightarrow{\text{epimerase}} \text{Ribulose 5-phosphate}$$

1.8.7 Ligases

They are involved in catalysis where two substrates are ligated and the formation of carbon-carbon, carbon-sulfide, carbon-nitrogen, and carbon-oxygen bonds due to

condensation reactions.These reactions are coupled to the cleavage of ATP. Phosphenol pyruvate PEP carboxylase (combines phosphenol pyruvate with carbon dioxide forming oxaloacetate accompanied by hydrolysis of ATP.)

1.9 Classification based upon the presence or absence at a given time

Two types are identified:

1.9.1 Inducible enzymes:

Those enzymes that are synthesized by the cell whenever they are required. Synthesis of these enzymes usually requires an inducer. Invertase, HMG-CoA reductase, p-galactosidase and enzymes involved in urea cycle.

1.9.2 Constitutive enzymes:

Enzymes which are constantly present in normal amounts in the body, irrespective of inducers. e.g Enzymes of glycolysis.

1.10 Classification based upon the regulation of enzyme action

They are of two types:

1.10.1 Regulatory enzymes:

The action of these enzymes is regulated depending upon the status of the cell. The action of regulatory enzymes is either increased or decreased by a modulator at a site other than the active site called the “allosteric site”. Ex. Phosphofructokinase (PFK) and glutamate dehydrogenase.

1.10.2 Non-regulatory enzymes:

The action of these enzymes is not regulated. eg. Succinate dehydrogenase.

1.11 Classification based upon the place of action:

Depending upon the two sites of action, they are divided into—

1.11.1 Intracellular enzymes:

Enzymes that are produced by the cell and act inside the same cell are known as intracellular enzymes. Ex. All the enzymes of glycolysis and TCA cycle.

1.11.2 Extracellular enzymes:

Enzymes produced by a cell but act outside that cell independent of it. Ex, All the digestive enzymes viz. trypsin, pancreatic lipase etc.

1.12 Classification based upon their clinical importance:

1.12.1 Functional plasma enzymes:

Enzymes present in the plasma in considerably high concentration and are functional in the plasma due to the presence of their substrate it plasma. Ex. Serum lipase, blood clotting enzymes.

1.12.2 Non-functional plasma enzymes:

Enzymes present in the plasma in negligible concentration and have no function in the plasma due to the absence of their substrate in it. Non-functional plasma enzymes are of diagnostic importance.

1.13 Bioenergetics of Enzymes Reactions

Bioenergetics is defined as the study of flow of energy or energy transductions through living organisms. Whether at the level of molecules, cells, tissues, organs, whole organisms or ecosystems, the flow of energy is essential for maintenance of life. Enzymes are biological catalysts. They catalyze most of the biochemical reactions occurring in the living cells. They function in aqueous solutions under very mild conditions of temperature and pH. Enzymes are central to every biochemical process. They catalyze the hundreds of stepwise reactions of metabolism, conserve and transform chemical energy, and make biological macromolecules from simple precursors. To catalyze a biochemical reaction, enzymes follow the thermodynamic principles hence we need to understand the concepts of thermodynamics in brief. The laws of thermodynamics govern the transfer of energy in and among all systems in the universe.

1.13.1 First law of thermodynamics:

The first law of thermodynamics states that the total amount of energy in the universe is constant and conserved. According to the first law of thermodynamics, energy may be transferred from place to place or transformed into different forms, but it cannot be created or destroyed. The transfers and transformations of energy take place around us all the time. The challenge for all living organisms is to obtain energy from their surroundings in forms that they can transfer or transform into usable energy to do work. Living cells have evolved

to meet this challenge. Chemical energy stored within organic molecules such as sugars and fats is transferred and transformed through a series of cellular chemical reactions into energy within molecules of ATP.

1.13.2 Second law of thermodynamics:

The second law of thermodynamics states that "the total entropy of a system must increase if a process is to occur spontaneously". Entropy represents the extent of disorder of the system and becomes maximum when it approaches true equilibrium. Under constant temperature and pressure, the relationship between the free energy change (ΔG) and the change in entropy (ΔS) is given by the following equation which combines the two laws of thermodynamics.

$$\Delta G = \Delta H - T\Delta S$$

Where ΔH = the change in enthalpy (heat) and T = the absolute temperature.

If ΔG is negative in sign, the reaction proceeds spontaneously with loss of free energy i.e. it is exergonic. On the other hand, if ΔG is positive, the reaction proceeds with the gain of energy i.e. it is endergonic. If the magnitude of ΔG is great, the system is stable. If ΔG is zero, the system is at equilibrium.

1.13.3 Free Energy and Activation Energy

Free energy specifically refers to the energy associated with a chemical reaction that is available after the losses are accounted for. In other words, free energy is usable energy, or energy that is available to do work. If energy is released during a chemical reaction, then the change in free energy, signified as ΔG (delta G) will be a negative number. A negative change in free energy also means that the products of the reaction have less free energy than the reactants, because they release some free energy during the reaction. Reactions that have a negative change in free energy and consequently release free energy are called exergonic reactions. E*x*ergonic means energy is *ex*iting in the system. These reactions are also referred to as spontaneous reactions, and their products have less stored energy than the reactants. An important distinction must be drawn between the term spontaneous and the idea of a chemical reaction occurring immediately.

If a chemical reaction absorbs energy rather than releases energy on balance, then the ΔG for that reaction will be a positive value. In this case, the products have more free energy than the reactants. Thus, the products of these reactions can be thought of as energy-storing

molecules. These chemical reactions are called endergonic reactions and they are non-spontaneous. An endergonic reaction will not take place on its own without the addition of free energy.

Enzymes do not change whether a reaction is exergonic (spontaneous) or endergonic. This is because they do not change the free energy of the reactants or products. They only reduce the activation energy required for the reaction to go forward. Once one reaction has been catalyzed, the enzyme is able to participate in other reactions. Most enzymes are proteins and perform the critical task of lowering the activation energies of chemical reactions inside the cell. Most of the reactions critical to a living cell occur slowly at normal temperatures. Without enzymes to speed up these reactions, life could not persist. Enzymes do this by binding to the reactant molecules and holding them in such a way as to make the chemical bond-breaking and -forming processes take place more easily.

1.13.4 Activation Energy and Transition State

Any biochemical reaction, such as S ⇆ P, can be described by a reaction coordinate diagram, in which the free energy change during the reaction is plotted as a function of the progress of the reaction (Fig. 2). The standard free energy change ($\Delta G'^{0}$) (and equilibrium position) of the reaction is determined by the difference in ground state free energies of S and P. The $\Delta G'^{0}$ value is defined as the standard free energy change that takes place at pH 7.0 when the reactants and products are maintained at 1.0 molar concentration.

The rate of the reaction is dependent on the height of the free energy barrier between S and P. At the top of this hump is the transition state. The transition state is a fleeting molecular moment in which events such as bond breakage, bond formation, and charge development have proceeded to the point at which decay to either substrate or product is equally likely. The difference between the energy levels of the ground state and the transition state is the activation energy, $\Delta G^{\ddagger}$. The rate of the reaction is inversely and exponentially proportional to the value of $\Delta G^{\ddagger}$ as shown in following fig 6.

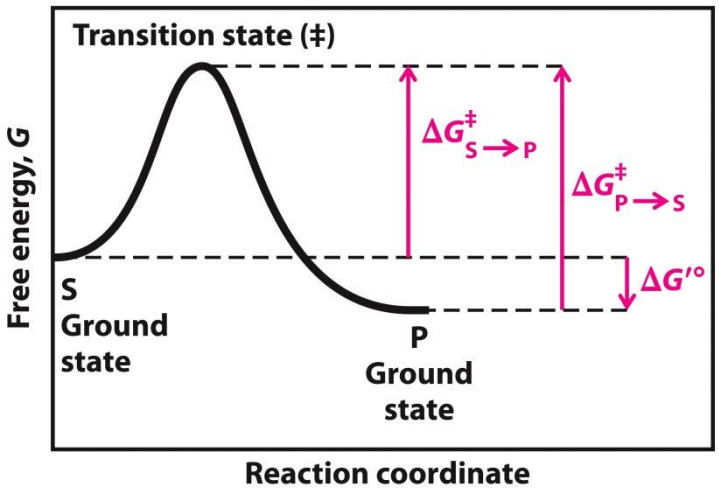

Fig: 6

1.13.5 Enzymes and Reaction Equilibrium

Enzymes do not alter the equilibrium (i.e. the thermodynamics) of a reaction. This is because enzymes do not fundamentally change the structure and energetics of the products and reagents, but rather they simply allow the reaction equilibrium to be attained more rapidly. Enzymes enhance reaction rates by lowering activation energies as shown in given graph. They have no effect on the position of reaction equilibria. The example shown is for an enzyme which follows the simple enzymatic steps of: E + S ⇄ ES ⇄ EP ⇄ E + P. (E-enzyme; S-substrate; P-product; ES-transient complex between the enzyme and substrate; EP-transient complex between the enzyme and product).

In the presence of the enzyme, three peaks occur in the reaction coordinate diagram as shown in given fig. 7. Whichever peak is the highest signifies the rate-limiting step of the overall reaction. As discussed below, binding energy provided by the interaction of the enzyme with the transition state contributes strongly to lowering the activation energy of the reaction, and accelerating its rate.

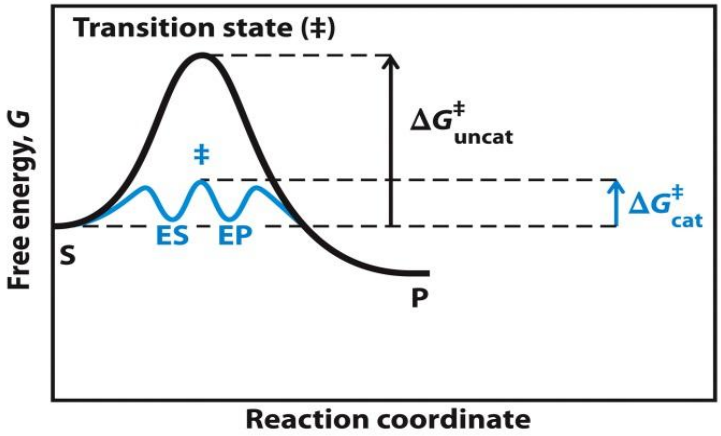

Fig. 7

1.13.6 Relationship between K'_{eq} and $\Delta G'^{0}$

To describe the free energy changes for reactions, chemists define a standard set of conditions (temperature 298˚K; partial pressure of each gas = 1 atm; concentration of each solute 1 M) and express the free energy change for a reacting system under these conditions as ΔG^0, the standard free energy change. Because biochemical systems commonly have H^+ concentrations far below 1 M, biochemists define a biochemical standard free energy change, $\Delta G'^0$, the standard free energy change at pH 7.0. The equilibrium constant for a reaction (K'_{eq}) under standard biochemical conditions is mathematically linked to the standard free energy change for a reaction, $\Delta G'^0$, via the equation

$$\Delta G'^0 = -2.303\ RT \log K'_{eq}.$$

In this equation, R is the gas constant, 8.315 J/mol·K, and T is the absolute temperature, 298˚K (25˚C). The numerical values for $\Delta G'^0$ as a function of K'_{eq} are tabulated in Table 4. Note that a large negative value of $\Delta G'^0$ reflects a favorable equilibrium in which the ratio of products to reactants is much greater than 1/1. Relationship between K'_{eq} and $\Delta G'^0$

K'_{eq}	$\Delta G'^{\circ}$ (kJ/mol)
10^{-6}	34.2
10^{-5}	28.5
10^{-4}	22.8
10^{-3}	17.1
10^{-2}	11.4
10^{-1}	5.7
1	0.0
10^{1}	−5.7
10^{2}	−11.4
10^{3}	−17.1

The magnitude of a rate constant is inversely and exponentially related to the activation energy, $\Delta G^{\ddagger}$. Thus, lower activation energy means a faster reaction rate. The rate enhancements observed for enzymes come from two distinct but interwoven parts. First, catalytic functional groups on an enzyme react with a substrate and lower the activation energy barrier for the reactions by providing an alternative, lower-energy reaction path. Second, non-covalent binding interactions between the substrate and enzyme release a small amount of free energy with each interaction that helps lower the energy of the transition state. The energy derived from enzyme-substrate interaction is called the binding energy, ΔG_B

1.14 Complementary Shapes of Enzymes and Substrates

The active site of an enzyme has a surface contour that is complementary in shape to its substrate (and products). This is illustrated for the two substrates of the enzyme dihydrofolate reductase as shown in fig.8. Structural complementarily is responsible for the high specificity of enzyme reactions. The idea that the enzyme and substrate are complementary to one another was first proposed by the organic chemist, Emil Fisher, in 1894. He stated that the two components fit together like a lock and key. This proposal has greatly influenced the development of biochemistry. However, it is slightly misleading in that precise complementarily between an enzyme and its substrate would be counterproductive to efficient catalysis. Later day biochemical researchers instead realized that the enzyme must be more complementary to the reaction transition state than to the substrate per se for efficient catalysis to occur.

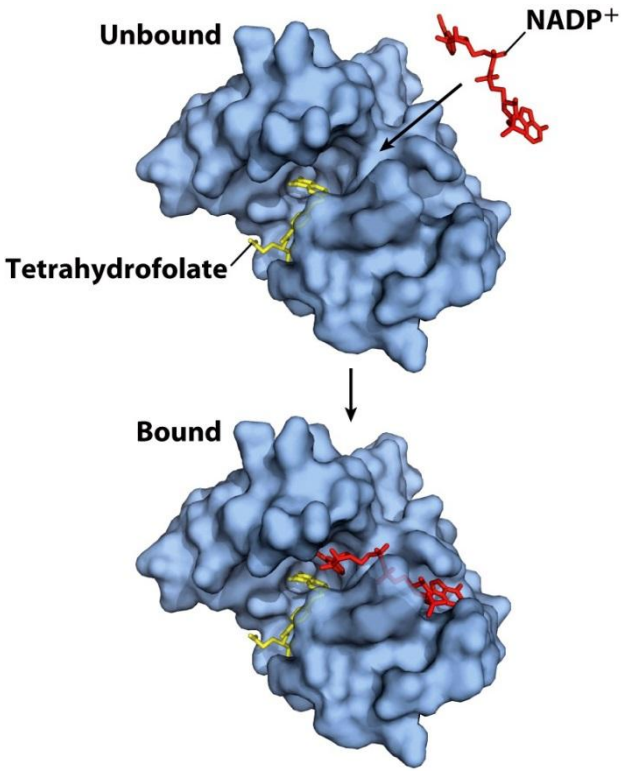

Fig 8. Dihydrofolate reductase complementary binding

1.15 Transition state complementarity explains rate enhancement (I)

The importance of transition state complementarity to rate enhancement can be illustrated using an example of a hypothetical "stickase" which catalyses the breakage of a metal stick, and binds to the sick via magnetic interactions (Fig. 9). In the uncatalyzed reaction, (Part a), the stick must first be bent to a transition state structure before being broken. Due to the high activation energy barrier of the bent stick transition state, the overall reaction (which has a negative free energy change) is relatively slow. If the stickase were precisely complementary to the metal bar (Part b), the rate of the reaction would not be improved as the enzyme actually would stabilize the structure of the stick. Under these conditions, the ES complex corresponds to a trough in the reaction coordinate diagram from which the substrate would have difficulty escaping.

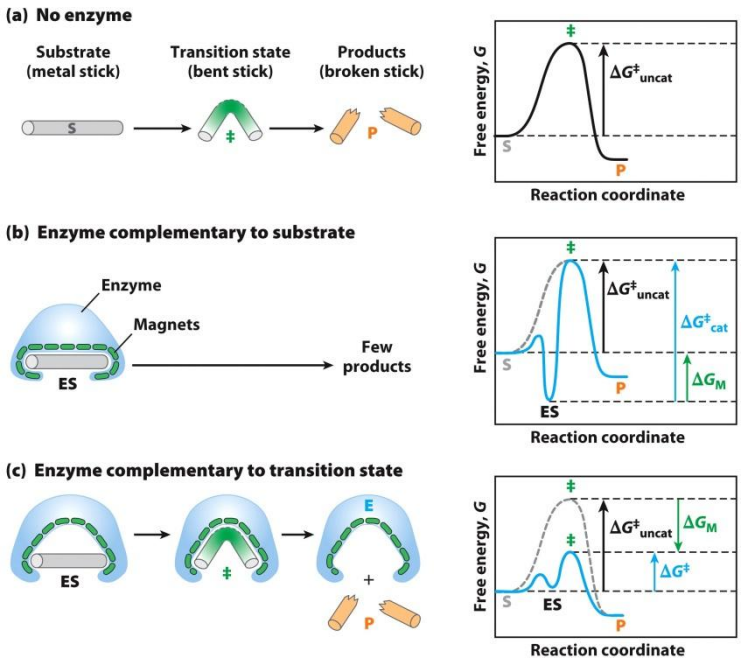

Fig. 9

1.16 Transition State Complementarity Explains Rate Enhancement (II)

However, if the stickase were more complementary to the transition state of the reaction (Part c), then the increase in free energy required to draw the stick into a bent and partially broken conformation would be offset, or paid for, by the magnetic interactions (binding energy) between the enzyme and the substrate in its transition state. This energy payment translates into lower net activation energy and a faster reaction rate as in given fig. 10.

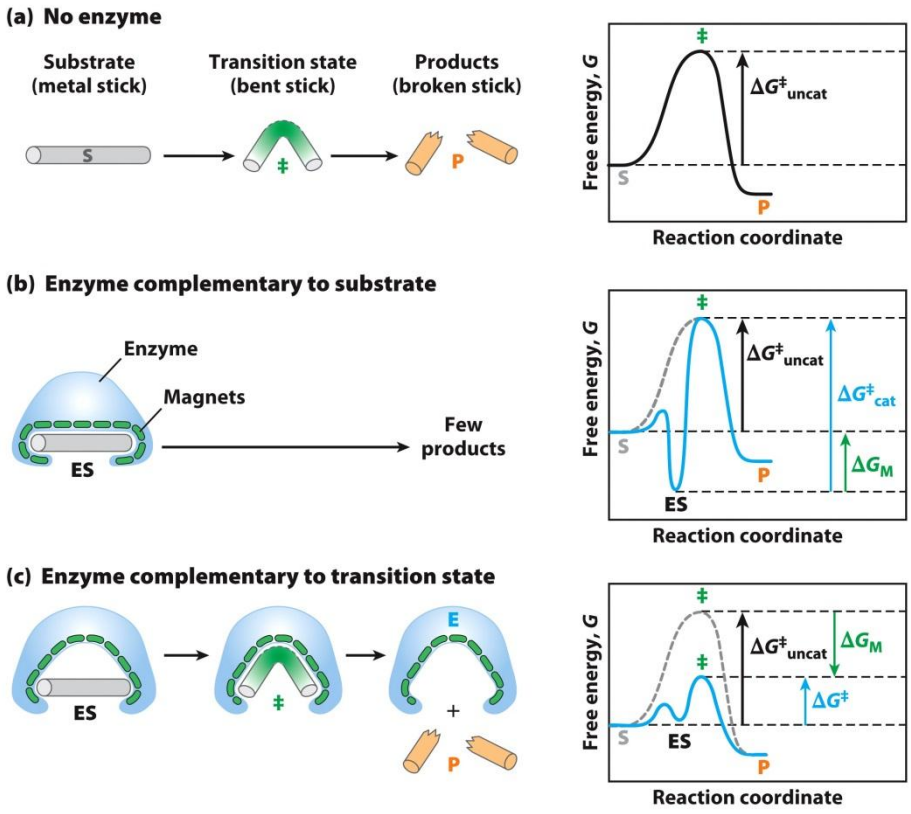

Fig: 10

1.17 Transition State Complementarity Explains Rate Enhancement (III)

Real enzymes work on an analogous principle. Some weak interactions are formed in the ES complex, but the full complement of such interactions between the substrate and enzyme is formed only when the substrate reaches the transition state. The free energy (binding energy) released by the formation of these interactions partially offsets the energy required to reach the top of the energy hill. The summation of the unfavorable (positive) activation energy $\Delta G^{\ddagger}$ and the favorable (negative) binding energy ΔG_B results in a lower net activation energy (Fig. 11). Even on the enzyme, the transition state is not a stable species but is a brief point in time that the substrate spends atop an energy hill. The enzyme-catalyzed reaction is much faster than the uncatalyzed process because the hill is much smaller. The important point is that weak binding interactions between the enzyme and the substrate provide a substantial driving force for enzymatic catalysis.

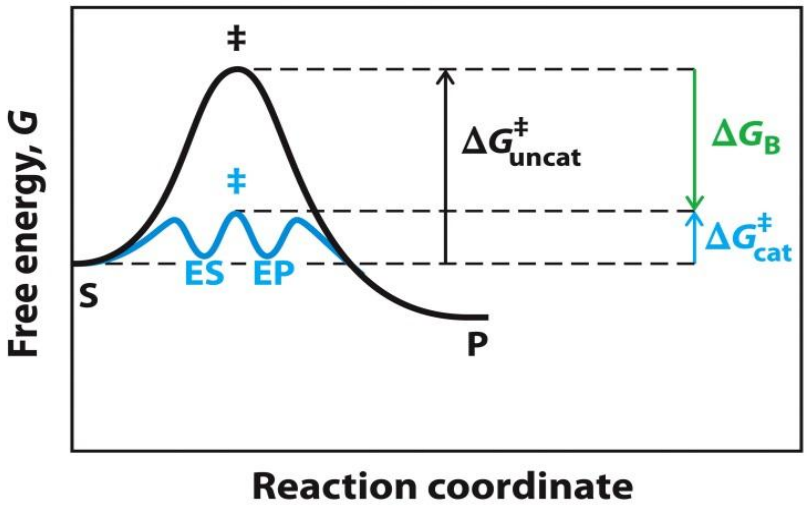

Fig :11

1.18 Contributions of Binding Energy to Reaction Specificity and Catalysis (I)

For a reaction to take place, significant physical and thermodynamic factors contributing to $\Delta G^{‡}$ must be overcome. These include 1) the entropy (freedom of motion) of molecules in solution, which reduces the possibility that they will react together, 2) the solvation shell of hydrogen-bonded water molecules that surrounds and helps to stabilize most biomolecules in solution, 3) the distortion of substrates that must occur in many reactions, and 4) the need for proper alignment of catalytic functional groups on the enzyme. All of these factors can be overcome due to the binding energy released on interaction of the enzyme with the transition state, as explained in the next slide. Binding energy also gives an enzyme its specificity, which is the ability of an enzyme to discriminate between its substrate and a competing molecule with a similar structure.

1.19 Contributions of Binding Energy to Reaction Specificity and Catalysis (II)

The mechanism by which binding energy compensates for physical and thermodynamic factors that impede reaction rates are as follows.

1.19.1 Entropy reduction

The restriction in the motions of two substrates that are about to react is one benefit of binding them to an enzyme. Binding energy holds the substrates in the proper orientation to react--a substantial contribution to catalysis, because productive collisions between molecules in solution can be exceedingly rare. Studies have shown that constraining the motion of two reactants can produce rate enhancements of many orders of magnitude (Fig. 12).

1.19.2 Desolvation

Formation of weak bonds between the enzyme and substrate results in the desolvation of the substrate. The removal of bound water molecules from the substrate removes water molecules which otherwise might impede the reaction.

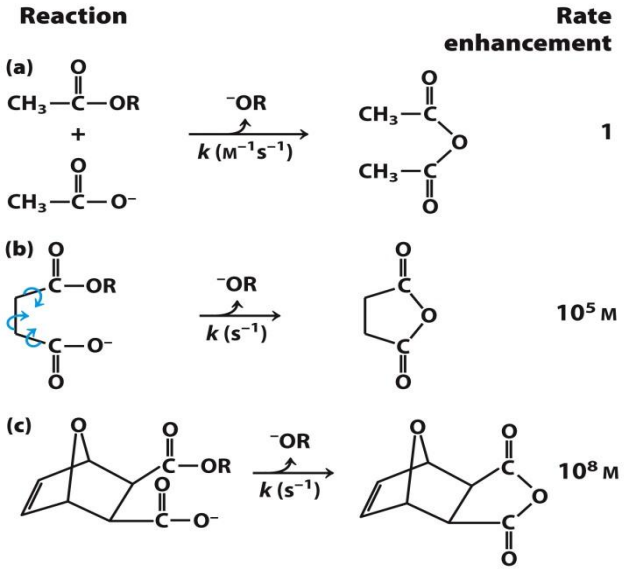

Fig. 12

1.20 Contributions of Binding Energy to Reaction Specificity and Catalysis (III)

1.20.1 Substrate distortion:

Binding energy involving weak interactions formed only in the reaction transition state helps to compensate thermodynamically for any distortion, primarily electronic redistribution that the substrate must undergo to react.

1.20.2 Catalytic group alignment:

Enzymes typically undergo changes in conformation when the substrate binds that are induced by multiple weak interactions with the substrate. The alignment of catalytic functional groups is referred to as induced fit, and it serves to bring specific functional groups on the enzyme into the proper position to catalyze the reaction.

1.20.3 Catalytic antibodies introduction

Catalytic antibodies are antibodies that can enhance a couple of chemical and metabolic reactions in the body by binding a chemical group, resembling the transition state of a

given reaction. Catalytic antibodies are produced when an organism is immunized with a hapten molecule. 'Catalytic antibodies' (antibody specificity + enzyme's catalytic power) (CatAbs),are immuno-modulators which can increase certain metabolic, physiological and chemical reactions in the body. This is made possible by binding to a chemical group.CatAbs are produced by combining an antibody to a hapten molecule for improving the immunogenicity.

Catalytic antibody sometimes called as abzyme (from antibody and enzyme) and also called catmab is a monoclonal antibody with catalytic activity. Catalytic antibodies offer unique capabilities in a range of scenarios, including stereo-selective organic synthesis, therapeutic potential in the treatment of disease, the elimination of toxins, the attenuation of agents used in chemical and biological warfare, and cessation of abused and/or addictive drugs. In 1948, Linus Pauling expressed the principle of enzymatic catalysis. He proposed that compounds resembling the transition state of a catalyzed reaction should be very effective inhibitors of enzymes. These mimics are called transition-state analogs. In 1969 William P. Jencks suggested that by generating antibodies raised against a stable analogue of the TS of the reaction that one wished to catalyze, one could obtain antibodies with catalytic activity.

In 1994, Peter G. Schultz and Richard A. Lerner received the prestigious Wolf Prize for developing catalytic antibodies capable of accelerating the hydrolysis of esters. Thus, they showed that antibodies raised against phosphonates, stable analogues of the tetrahedral TS formed during the hydrolysis of esters, could catalyze this reaction. 1995-2011: Dr. Paul publishes first example of hydrolysis of HIV coat protein by an abzyme. In 1989, Paul et al, discovered the first example of a natural Abs was an IgG (found in bronchial asthma patients) that hydrolyzes vasoactive intestinal peptide (VIP). This was followed by a discovery of IgG with DNase activity in systemic lupus erythematosus (SLE),and an IgG with RNase activity, also in SLE . Numerous natural catalytic Abs were detected afterwards in serum of patients with several autoimmune (AI) and viral disorders, as well as in the milk of healthy women.

1.20.3.1 Principle

Antibodies and enzymes share the ability to bind with compounds with great specificity and high affinity. This property has been exploited in the development of antibodies with

catalytic activity. Catalytic antibodies are produced when animals are immunized with hapten molecules that are specially designed to elicit antibodies that have binding pockets capable of catalyzing chemical reactions. For example, in the simplest cases, binding forces within the antibody binding pocket are enlisted to stabilize transition states and intermediates, thereby lowering a reaction's energy barrier and increasing its rate. This can occur when the antibodies have a binding site that is complementary to a transition state or intermediate structure in terms of both three-dimensional geometry and charge distribution. This complementarity leads to catalysis by encouraging the substrate to adopt a transition-state-like geometry and charge distribution. Not only is the energy barrier lowered for the desired reaction, but other geometries and charge distributions that would lead to unwanted products can be prevented, increasing reaction selectivity.

1.20.3.2 Hapten

The term 'hapten' derived from a Greek word '*Haptein*' means to fasten. A small molecule that reacts with a specific antibody but can not induce the formation of antibodies unless it is bound to a carrier protein.

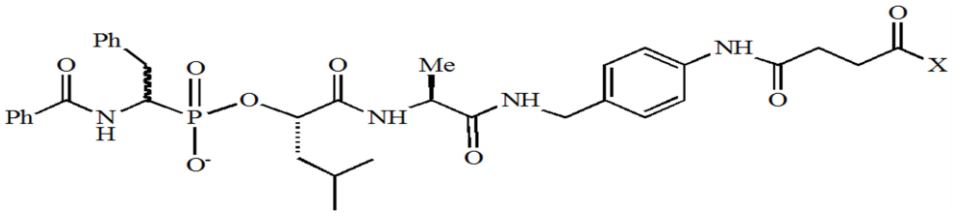

Fig. 13. X = OH hapten X = macromolecule response) antigen (elicits antibody)

1.20.3.3 Transition state analogue

In general, synthesizing compounds that more closely resemble the transition state than the substrate itself can produce highly potent and specific inhibitors of enzymes. The inhibitory power of transition-state analogs underscores the essence of catalysis: selective binding of the transition state.

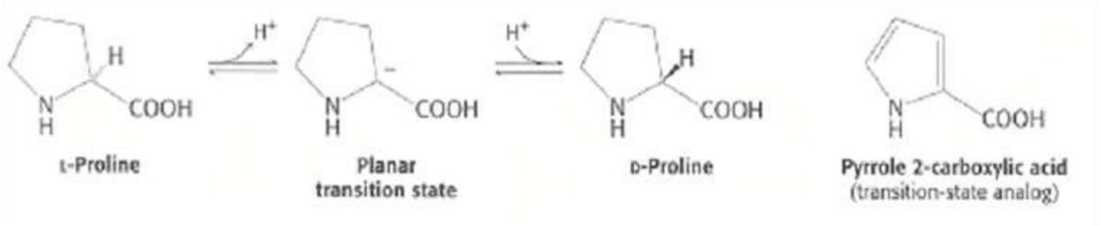

Fig. 14: Pyrrole 2-carboxylate, a transition state analog because of its trigonal geometry, is a potent inhibitor of proline racemase

1.20.3.4 Structure of a catalytic antibody

Structurally, Catalytic antibodies are similar to "Y-shaped" Ig G-type (Figure 1) which consists of two identical heterodimers linked by S-S bonds. Each heterodimer composed of a light chain and a heavy chain. One end of the immunoglobulin moleculeconsist of conserved domains (F_C) which are constant regions formed in between two heavy chains. Conserved domains have been found to have similar amino acid compositions in most immunoglobulins, other than the epitope-paratope interaction that varies. The opposite end is the variable domains (F_V), responsible for specifically binding antigen. A single deep pocket (antigen binding site) exists in the interface of two of the $V_H$and $V_L$regions. "Hot spots" within the variable domains, which are vital for antigen specificities, are called complementarity determining regions (CDRs). The amino acids within the hot spots interact with the antigen through non-covalent interactions.

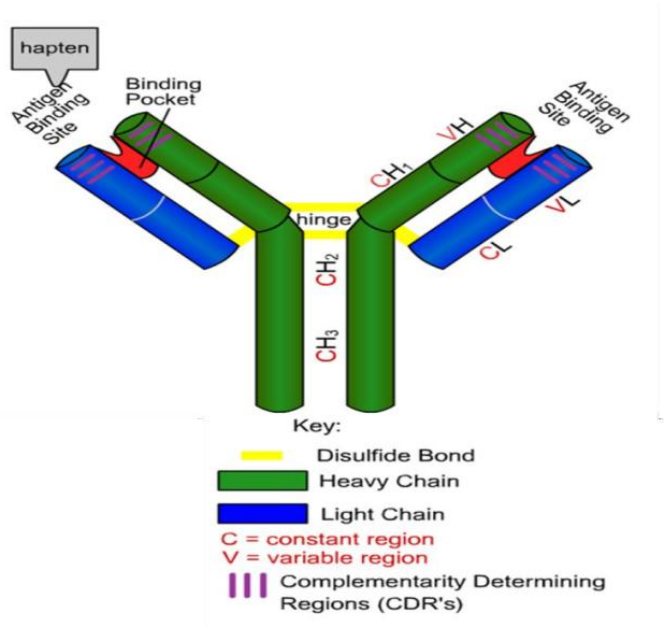

Fig. 15 Structure of a catalytic antibody

1.20.3.5 Construction of catalytic antibodies

Catalytic antibodies could be generated by probing the immune response for an antibody that can bind a small molecule, or hapten, that is a transition state analog of a desired chemical reaction. The production of catalytic antibodies is based on the following principles:

1. Enzymes act by binding the transition state of a reactant better than the ground state.

2. Antibodies which bind to specific small molecules can be produced by coupling this small molecule to a protein carrier and using this protein for immunizing experimental animals.

3. If the molecule is a transition state analog, then the antibodies that are produced to bind to this molecule will function as enzyme towards the substrate of this reaction.

4. Abzymes are selected from monoclonal antibodies produced by immunizing mice with haptens that mimic the transition state of enzyme catalyzed reactions (Haptens are small molecules that elicit an immune response only when attached to a large carrier such as a protein).

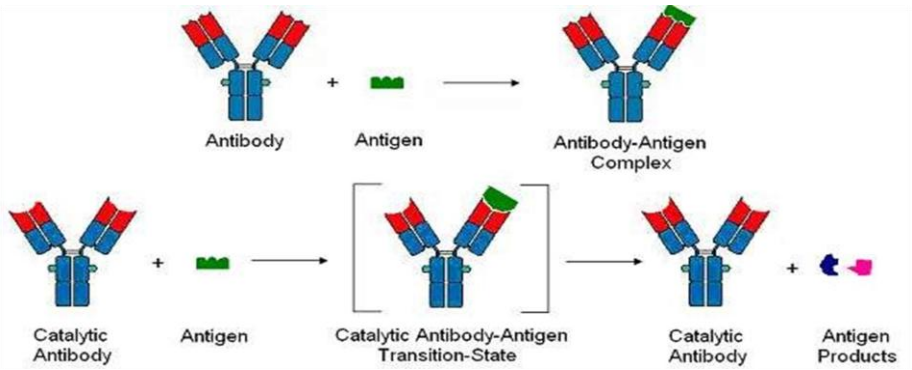

Fig 16: Construction of catalytic antibody

1.21 Mechanism of Action

- Enzymes function by lowering the activation energy of the transition state of a chemical reaction, thereby enabling the formation of an otherwise less- favorable molecular intermediate between the reactant(s) and the product(s).
- If an antibody is developed to bind to a molecule that's structurally and electronically similar to the transition state of a given chemical reaction, the developed antibody will bind to, and stabilize, the transition state, just like a natural enzyme, lowering the activation energy of the reaction, and thus catalyzing the reaction.
- By raising an antibody to bind to a stable transition-state analog, a new and unique type of enzyme is produced.

1.22 Eliciting Catalytic Antibodies

A merging of chemistry and biology is essential to effectively probe the immune system for catalytic antibodies (Fig. 13). Haptens that are successful in eliciting catalytic antibodies are variations of the central theme that transition state stabilization in the antibody combining site will yield functional catalysts for a desired chemical reaction. Once the hapten is selected and synthesized, it is attached to an immunogenic carrier protein, usually via an amide bond, for hyper immunization. A preliminary screen for antibodies that bind the hapten using an enzyme-linked immunosorbent assay (ELISA) is followed by another screen for catalysis of the reaction for which the hapten was designed. Other screening methods have also been used, including ELISA, which screens for catalysis in the antibody

pool rather than hapten binding. There are three primary methods for eliciting catalytic antibodies currently used: polyclonal, hybridoma, and phage-display.

1.22.1 Polyclonal Antibody Production

It is the most primitive method and has several significant limitations. This method inherently yields a complex mixture of antibody molecules from the immune response after hapten hyperimmunization with no attempt to purify specific IgG molecules. Efficiency and cost-effectiveness are hallmarks of polyclonal antibody production; however, accurate characterization of this mixture is difficult. Furthermore, X-ray crystallography to examine structure-function relationships and affinity maturation to optimize the antibody is impossible with the polyclonal antibody method.

1.22.2 Hybridoma Technology

In this technique, antibody producing cells are isolated from the spleen after hyperimmunization with the hapten-protein conjugate. These B cells are subsequently fused with an immortal cell line, and the resultant hybrids secrete monoclonal antibodies. Monoclonal antibodies are homogeneous, can be produced in large quantities, and can be rigorously purified to remove any potential contaminants. Despite greater expense and a more time-consuming process, monoclonal antibody production is the method of choice.

1.22.3 Phage-display technology

Phage display involves combinatorial antibody Fab or scFv (single-chain variable fragment) libraries and their expression on phage particles. One advantage to this technology is that the hyperimmunization protocol can be avoided, thereby removing the use of animals. Naive Fab or scFv are identified in a screen for binding to a desired transition state analog. Although in theory a desired catalyst can be discovered this way, in practice this technique is more successful when an initial hyperimmunization protocol is performed followed by acquisition of the mRNA of the B cells from the spleen. The corresponding focused combinatorial antibody library is biased toward antibody fragments that recognize the hapten.

Phage-display is also used as a tool in affinity maturation of a previously identified catalytic antibody. Mutagenesis of the corresponding Fab or scFv from an existing catalytic antibody can be explored using error-prone polymerase chain reaction (PCR), CDR

walking, structure-guided mutagenesis, and DNA shuffling to optimize catalysis of the corresponding chemical reaction.

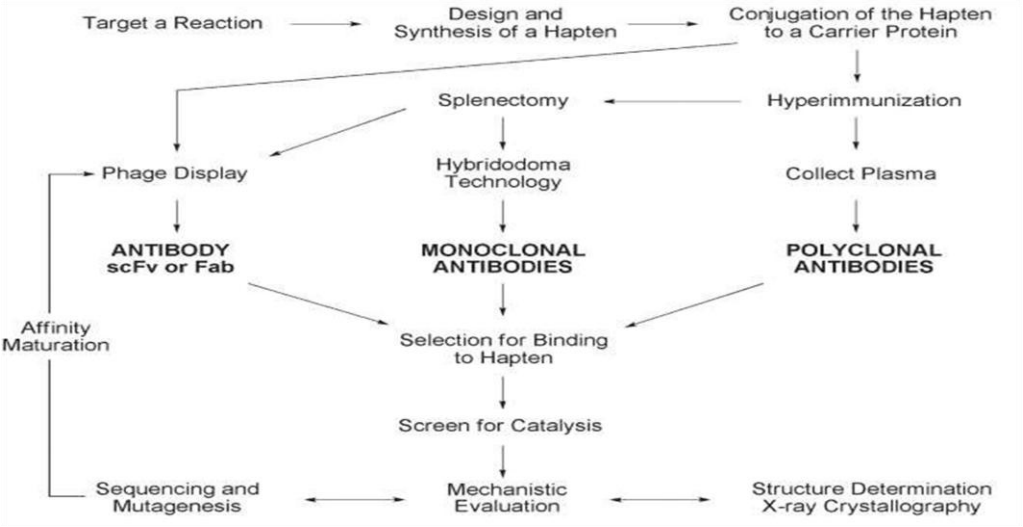

Fig. 17 Flow chart illustrating the key stages in catalytic antibody generation.

1.22.4 Evolution of Hapten Design

The conformational changes and charge distribution along the reaction coordinate of a chemical transformation are fundamental to hapten design. Catalytic antibodies are designed to mimic the catalytic power of an enzyme, which, in part, stems from the stabilization of the high energy transition state.

1.22.4.1 Stable Transition State Analog Hapten Design

A stable chemical analog that mimics the transition state of a chemical reaction was the first approach used to elicit catalytic antibodies (Fig. 14). Acyl transfer reactions are the most studied type of catalytic antibody reaction, and a wealth of knowledge about this reaction has been garnered through antibody acyl transferase. Using ester hydrolysis as a representative example, nucleophilic addition of water to the carbonyl carbon results in a tetrahedral transition state followed by expulsion of the alcohol leaving group. The transition state for this reaction has a delocalized negative charge that is remarkably similar to the chemically stable phosphonate ester. The phosphorous (V) core, known to be an excellent mimic of the transition state in hydrolytic enzymes and often used as a key

pharmacophore in transition state analog inhibitors, has been widely adopted as a central motif in catalytic antibody hapten design.

Antibody esterase 48G7 was elicited against hapten 1 and effectively catalyzed the hydrolysis of the corresponding activated ester 2. The X-ray crystal structure of this catalytic antibody Fab complexed with 1 revealed the corresponding stabilization of the oxyanion by a nearby cationic Arg^{L96} residue . Hydrogen bonds from the side chains of the adjacent amino acids His^{H35} and Tyr^{H33} serve to stabilize the polarized phosphoryl bonds of hapten 1 that would assist in forming the transition state of ester 2. Main-chain amide bonds from Tyr^{L91} and Tyr^{H100} also provide additional hydrogen-bond stabilization forces.

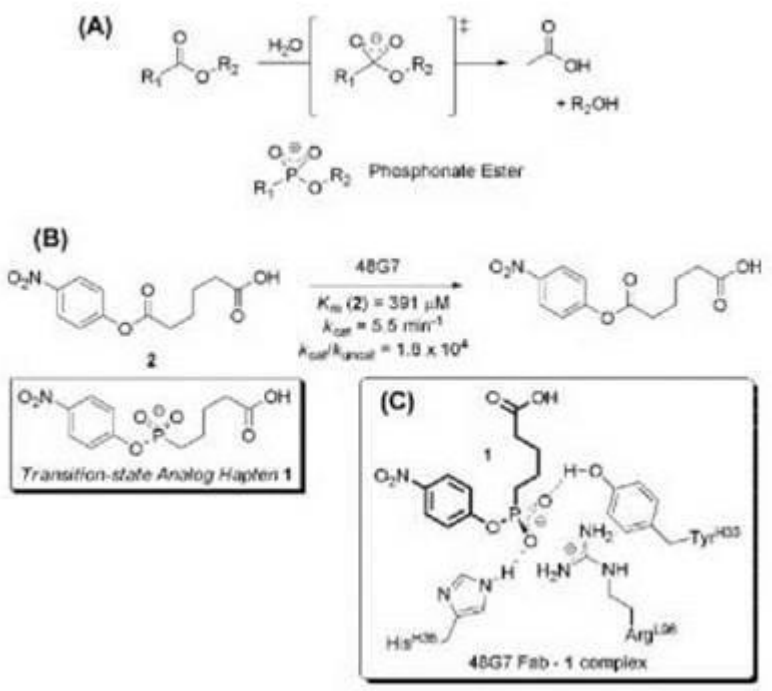

Fig. 18 (a) Phosphonate ester as a chemically stable mimic of ester hydrolysis.
(b) Transition state analog hapten 1 elicited antibody 48G7 that catalyzes the hydrolysis of ester 2.
(c) Key contacts of the 48G7 Fab-1 complex.

1.22.4. 2 Strain-Induced Hapten Design

A slight modification of the transition state analog approach to hapten design is the use of a strain-induced hapten to elicit catalytic antibodies. In this approach, a chemical modification of the substrate distorts the resultant hapten (Fig.18). During hyperimmunization, the strain-induced hapten leads to altered substrate binding in the

antibody combining site, facilitating the chemical reaction by lowering the energy of the transition state.

An example of this approach is demonstrated in an antibody mimic of the enzyme ferrochetalase . Ferrochelatase catalyzes the insertion of Fe^{2+} into protoporphyrin IX as the last step in the heme biosynthetic pathway. Interestingly, N-alkylporphyrins are known to be potent inhibitors of this enzyme, because alkylation at one pyrrole nitrogen distorts the planarity of the porphyrin macrocycle. This hnding was used in the design of hapten 4 to catalyze the incorporation of metal ions into mesoporphyrin IX by eliciting an antibody that binds the substrate in a ring-strained conformation.

The lone-pair electrons on the pyrrole nitrogen of the porphyrin ring are more accessible to chelation of metal ions in the ring-strained conformation and leads to metalation of mesoporphyrin IX. Antibody 7G12 catalyzes the incorportain of Zn^{2+}, Cu^{2+}, Co^{2+}, and Mn^{2+} into mesoporphyrin IX, whereas ferrochetalase uses Fe^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} as substrates in the chelation of protoporphyrin IX. X-ray crystallography of the catalytically active 5-7G12 Michaelis complex revealed that the porphyrin ring adopts a nonplanar conformation that is essential for catalysis as anticipated from the hapten design.

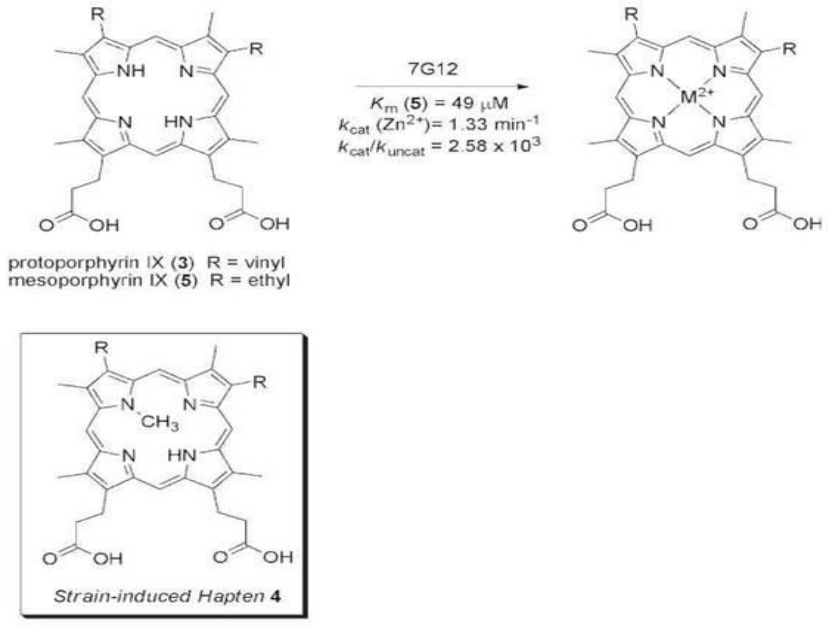

Fig. 19 Strain-induced hapten 4 elicited antibody 7G12 that catalyzes metalation of mesoporphyrin IX.

1.22.4.4 Bait-and-Switch Hapten Design

A significant step in the evolution of hapten design was introduced by Janda and Lerner, coined the “bait-and-switch” method. This novel advancement enables electrophilic/ nucleophilic and/or general acid/general base catalysis to be programmed into an antibody combining site. Specihcally, a point charge on the hapten in close proximity to, or in direct substitution for, a functional group to be transformed in the respective substrate is used to induce a complementary charge on an amino acid residue in the antibody combining site during hyperimmunization (Fig. 19). The substrate lacks this charge but retains a similar overall structure and the corresponding antibody binds the substrate and acts as a general acid/general base and/or as a nucleophile/electrophile in the desired chemical reaction.

The phosphodiesterase antibody MATT.F-1 is a didactic example of baitand-switch hapten design, illustrating differences from other hapten design approaches. The hydrolysis of a phosphodiester bond, such as those found in RNA and DNA, are catalyzed by ribonucleases (RNases) and deoxyribonucleases (DNases), respectively. RNase A is a thoroughly studied enzyme that has two catalytic histidine residues in the active site. The imidazole group of His^{12} acts as a general base by deprotonating the 2' oxygen, and the imidazolium group of His^{119} acts as a general acid by protonating the 5 ' phosphoryl oxygen in the classic mechanism .

The incorporation of a general base and a general acid in the hydrolysis of a phosphodiester was hypothesized to be elicited in an antibody combining site programmed by specific point charges designed into a bait-and-switch hapten. Indeed, an antibody against hapten successfully catalyzed the hydrolysis of the corresponding substrate (Fig. 6). In contrast to the transition state analog hapten, which elicited the less-proficient phosphodiesterase catalytic antibody 2G12, the precise conformation of the high energy intermediate was sacrificied for charged moieties in specific locations of the hapten. The corresponding counterion charges from amino residues were elicited in the antibody combining site that led to catalytic antibodies with improved catalytic proficiency ($(k_{cat}/K_m)/k_{uncat}$ = 1.6 x 10^7 M^{-1} for MATT.F-1 versus ($(k_{cat}/K_m)/k_{uncat}$ = 1.3 x 10^6 M^{-1} for 2G12) for phosphodiesterase activity.

(a) The catalytic mechanism of RNase A, including the postulated transition state. (b) Bait-and-switch hapten elicited antibody MATT.F-1 that catalyzes phosphodiester bond

hydrolysis of substrate. Transition state analog hapten also elicited catalytic antibodies but with slower rates.

1.22.4.5 Reactive Immunization Hapten Design

An essential concept in enzyme catalysis, partly realized through the study of catalytic antibodies, is that the proficiency of an enzyme is not solely due to the stabilization of a high energy transition state. Enzymes are not static entities, but rather they have the dynamic ability to stabilize all possible conformations of a chemical reaction along the reaction coordinate. Furthermore, many enzymes can form covalent intermediates with the substrate that are essential to catalysis. The previous hapten design methods program specific complementarity in the antibody combining site; however, the hapten is a static snapshot of a dynamic chemical process resulting in catalysts limited to nonconvalent interactions that are ultimately less efficient than their enzyme counterparts.

Reactive immunization is a hapten design strategy that provides a chance for catalytic antibodies to approach the catalytic efficiency of natural enzymes by using a hapten that undergoes dynamic conformational changes during hyperimmunization and traps chemical reactivity at the B-cell level. Reactive immunization hapten elicited antibody 15G2 that catalyzes the hydrolysis of substrate. Transition state analog hapten elicited antibody 12C8 to catalyze the same reaction.

1.23 Summary

Enzymes are currently being used in diverse areas in the food, feed, paper, leather, agriculture and textiles industries, resulting in major cost reductions. Simultaneously, rapid scientific progress is now encouraging the chemistry and pharmacological industries to embrace enzyme technology, a trend supported by concerns regarding energy, raw materials, health and the environment. One of the most common advantages of enzymes is their ability to function continuously even after their removal or separation from the cells. This means that even after the separation of cells from *in vivo* environments, they continue to work efficiently under *in vitro* conditions; we can conclude that these biocatalysts remain in an active state even after their isolation. Principally, enzymes are non-toxic, biodegradable and can be produced in ample amounts by micro-organisms for industrial applications. In this chapter, the isolation, production, purification, utilization and application of enzymes (in soluble and immobilized or insoluble form) are discussed in

detail. Procedures such as recombinant DNA technology and protein engineering are frequently used to produce more efficient and beneficial enzymes.

The industrial production and utilization of enzymes is an important part of industry. Interdisciplinary collaboration between areas such as chemistry, process engineering, microbiology and biochemistry is required to develop the best possible enzyme technology, and eventually to achieve increased production and maintain the enzyme's physico-chemical properties under *in vitro* environments.

1.2.4 Terminal questions

Q.1. Define enzymes. Explain nomenclature and IUBMB classification with suitable examples.

Answer:----------

Q.2. Define isoenzymes and explain their structure, organ distribution and diagnostic importance.

Answer:----------

Q.3. Explain factors affecting enzyme activity.

Answer:----------

Q.4. Define enzyme inhibition. Explain in detail the different types of inhibitions with suitable examples.

Answer:----------

Q.5. Explain the different theories proposed for mechanism of enzyme substrate complex formation.

Answer:----------

Q.6. Distinguish between the Competitive and non competitive inhibitions.

Answer:----------

Q.7. Define Km and explain the effect of substrate concentration on enzyme activity.

Answer:----------

Q. 8 Write a short note on diagnostic and therapeutic uses of enzymes.

Answer:----------

Further readings

1. Biochemistry- Lehninger A.L.
2. Biochemistry –J.H.Weil.
3. Biochemistry fourth edition-David Hames and Nigel Hooper.
4. Textbook of Biochemistry for Undergraduates - Rafi, M.D.
5. Biochemistry and molecular biology- Wilson Walker.

Unit-2: Multi-enzymes complexes

Structure

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2.2.0 Summary

2.1 Introduction

Multienzyme complexes catalyze important reactions in central metabolic processes such as photosynthesis, respiration, and amino acid synthesis. We wanted to determine whether multienzyme complexes are also involved in the central metabolic process of biological methane production (methanogenesis) in methane-producing archaea (methanogens). Methanogens are obligately anaerobic archaea that derive all their energy for growth by reducing carbon sources such as acetate, formate, CO_2, methanol, methylamines and methyl-sulfides to methane gas. Metabolic engineering of methanogens is an attractive prospect for increasing the yield and rate of renewable methane production from biomass in anaerobic digesters. However, successful metabolic engineering requires not only an in-depth understanding of methanogen physiology, but also a knowledge of which reactions are physically linked by multienzyme complexes. A detailed, three-dimensional spatial model of methanogenesis proteins would be useful in these efforts.

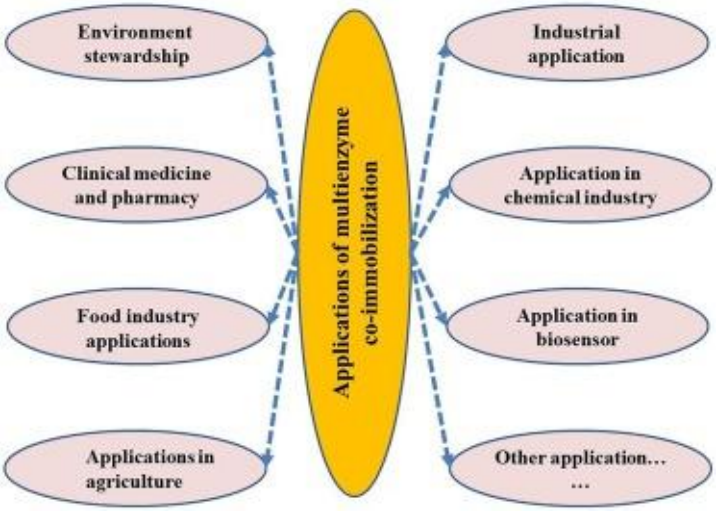

Fig. 1

Metabolic reactions that are linked by multienzyme complexes have clear advantages over reactions that are catalyzed by individual, unlinked enzymes. Complexes channel substrates to prevent diffusion of intermediates into bulk cytoplasm, effectively increasing the relative local concentration of reactants in subsequent pathway steps, speeding the overall rate of production of the final product, and preventing diffusion of toxic intermediates that can

damage cell constituents. Complexes can also provide a means of co-regulating pathway enzymes or ensuring proper enzyme dosage . Methanogens obtain up to 1 mole ATP per mole substrate consumed and live near the thermodynamic lower limit of life. Substrate channeling via multienzyme complexes would provide a kinetic advantage by ensuring maximal efficiency for converting substrate to ATP generation. We used in vivo crosslinking, tandem affinity purification, and peptide mass spectrometry (XL-MS) to look for complex formation among methanogenesis enzymes. XL-MS is a reliable technique for identifying protein:protein interactions by identifying crosslinked partners which elute together after affinity column purification. A recent effort in *Saccharomyces cerevisiae* has successfully demonstrated the ability to use XL-MS to reproduce 30 years of protein:protein interaction data and to predict new interactions which were subsequently verified genetically. Though commonly applied to the study of cell signaling networks, we surmised that XL-MS is a valuable technique for identifying protein:protein interactions between methanogenesis enzymes and electron transfer proteins in the methanogen, *Methanosarcina acetivorans*.

Multienzyme Complex In a number of metabolic pathways, several enzymes which catalyze different stages of the process have been found to be associated non-covalently, giving a multienzyme complex.

- The proximity of the different types of enzymes increases the efficiency of the pathway; The overall reaction rate is increased with respect to catalysis by unassociated units, and Side reactions are minimized.
- In some cases molecular mechanisms have been identified for the transfer of metabolites from one enzyme to the next within the complex.
- Multienzyme complex is the structural and functional entity that is formed by the association of several different enzymes which catalyze a sequence of closely related reactions.
- A multi enzyme complex is a protein possessing more than one catalytic domain contributed by distinct parts of a polypeptide chain or by distinct subunits.
- The regulation of this enzyme complex illustrates how a combination of covalent modification and allosteric regulation results in specific regulated flux through a metabolic step.

2.2 Isozymes

In biochemistry, **isozymes** (also known as **isoenzymes** or more generally as **multiple forms of enzymes**) are enzymes that differ in amino acid sequence but catalyze the same chemical reaction. Isozymes usually have different kinetic parameters (e.g. different K_M values), or are regulated differently. They permit the fine-tuning of metabolism to meet the particular needs of a given tissue or developmental stage. In many cases, isozymes are encoded by homologous genes that have diverged over time. Strictly speaking, enzymes with different amino acid sequences that catalyse the same reaction are isozymes if encoded by different genes, or allozymes if encoded by different alleles of the same gene; the two terms are often used interchangeably. Isozymes were first described by R. L. Hunter and Clement Markert (1957) who defined them as *different variants of the same enzyme having identical functions and present in the same individual*. This definition encompasses (1) enzyme variants that are the product of different genes and thus represent different loci (described as *isozymes*) and (2) enzymes that are the product of different alleles of the same gene (described as *allozymes*).

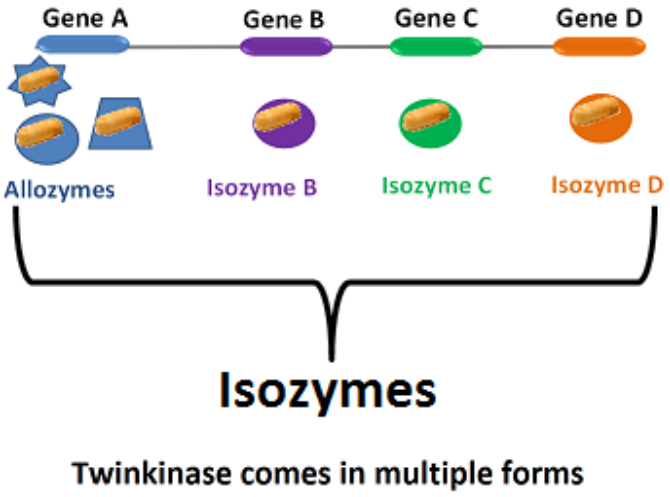

Fig. 2

Isozymes are usually the result of gene duplication, but can also arise from polyploidisation or nucleic acid hybridization. Over evolutionary time, if the function of the new variant remains *identical* to the original, then it is likely that one or the other will be lost as mutations accumulate, resulting in a pseudogene. However, if the mutations do not immediately prevent the enzyme from functioning, but instead modify either its function, or its pattern of expression, then the two variants may both be favoured by natural

selection and become specialised to different functions. For example, they may be expressed at different stages of development or in different tissues.

Allozymes may result from point mutations or from insertion-deletion (indel) events that affect the coding sequence of the gene. As with any other new mutations, there are three things that may happen to a new allozyme:

- It is most likely that the new allele will be non-functional—in which case it will probably result in low fitness and be removed from the population by natural selection.
- Alternatively, if the amino acid residue that is changed is in a relatively unimportant part of the enzyme (e.g., a long way from the active site), then the mutation may be selectively neutral and subject to genetic drift.
- In rare cases, the mutation may result in an enzyme that is more efficient, or one that can catalyse a slightly different chemical reaction, in which case the mutation may cause an increase in fitness, and be favoured by natural selection

Examples

An example of an isozyme is glucokinase, a variant of hexokinase which is not inhibited by glucose 6-phosphate. Its different regulatory features and lower affinity for glucose (compared to other hexokinases), allow it to serve different functions in cells of specific organs, such as control of insulin release by the beta cells of the pancreas, or initiation of glycogen synthesis by liver cells. Both these processes must only occur when glucose is abundant.

2.3 Distinguishing isozymes

Isozymes (and allozymes) are variants of the same enzyme. Unless they are identical in their biochemical properties, for example their substrates and enzyme kinetics, they may be distinguished by a biochemical assay. However, such differences are usually subtle, particularly between *allozymes* which are often neutral variants. This subtlety is to be expected, because two enzymes that differ significantly in their function are unlikely to have been identified as *isozymes*.

While isozymes may be almost identical in function, they may differ in other ways. In particular, amino acid substitutions that change the electric charge of the enzyme are simple to identify by gel electrophoresis, and this forms the basis for the use of isozymes

as molecular markers. To identify isozymes, a crude protein extract is made by grinding animal or plant tissue with an extraction buffer, and the components of extract are separated according to their charge by gel electrophoresis. Historically, this has usually been done using gels made from potato starch, but acrylamide gels provide better resolution.

All the proteins from the tissue are present in the gel, so that individual enzymes must be identified using an assay that links their function to a staining reaction. For example, detection can be based on the localised precipitation of soluble indicator dyes such as tetrazolium salts which become insoluble when they are reduced by cofactors such as NAD or NADP, which generated in zones of enzyme activity. This assay method requires that the enzymes are still functional after separation (native gel electrophoresis), and provides the greatest challenge to using isozymes as a laboratory technique. Isoenzymes differ in kinetics (they have different K_M and V_{max} values).

2.4 Isozymes and allozymes as molecular markers

Population genetics is essentially a study of the causes and effects of genetic variation within and between populations, and in the past, isozymes have been amongst the most widely used molecular markers for this purpose. Although they have now been largely superseded by more informative DNA-based approaches (such as direct DNA sequencing, single nucleotide polymorphisms and microsatellites), they are still among the quickest and cheapest marker systems to develop, and remain (as of 2005) an excellent choice for projects that only need to identify low levels of genetic variation, e.g. quantifying mating systems.

2.4.1 Coenzyme

Coenzyme A (**CoA**, **SHCoA**, **CoASH**) is a coenzyme, notable for its role in the synthesis and oxidation of fatty acids, and the oxidation of pyruvate in the citric acid cycle. All genomes sequenced to date encode enzymes that use coenzyme A as a substrate, and around 4% of cellular enzymes use it (or a thioester) as a substrate. In humans, CoA biosynthesis requires cysteine, pantothenate (vitamin B_5), and adenosine triphosphate (ATP). In its acetyl form, coenzyme A is a highly versatile molecule, serving metabolic functions in both the anabolic and catabolic pathways. Acetyl-CoA is utilised in the post-translational regulation and allosteric regulation of pyruvate dehydrogenase and carboxylase to maintain and support the partition of pyruvate synthesis and degradation.

Coenzyme A was identified by Fritz Lipmann in 1946, who also later gave it its name. Its structure was determined during the early 1950s at the Lister Institute, London, together by Lipmann and other workers at Harvard Medical School and Massachusetts General Hospital. Lipmann initially intended to study acetyl transfer in animals, and from these experiments he noticed a unique factor that was not present in enzyme extracts but was evident in all organs of the animals. He was able to isolate and purify the factor from pig liver and discovered that its function was related to a coenzyme that was active in choline acetylation. The coenzyme was named coenzyme A to stand for "activation of acetate". In 1953, Fritz Lipmann won the Nobel Prize in Physiology or Medicine "for his discovery of co-enzyme A and its importance for intermediary metabolism".

2.5 Biosynthesis

Coenzyme A is naturally synthesized from pantothenate (vitamin B_5), which is found in food such as meat, vegetables, cereal grains, legumes, eggs, and milk. In humans and most living organisms, pantothenate is an essential vitamin that has a variety of functions. In some plants and bacteria, including *Escherichia coli*, pantothenate can be synthesised *de novo* and is therefore not considered essential. These bacteria synthesize pantothenate from the amino acid aspartate and a metabolite in valine biosynthesis.

In all living organisms, coenzyme A is synthesized in a five-step process that requires four molecules of ATP, pantothenate and cysteine:

1. Pantothenate (vitamin B_5) is phosphorylated to 4′-phosphopantothenate by the enzyme pantothenate kinase (PanK; CoaA; CoaX). This is the committed step in CoA biosynthesis and requires ATP.
2. A cysteine is added to 4′-phosphopantothenate by the enzyme phosphopantothenoylcysteine synthetase (PPCS; CoaB) to form 4'-phospho-N-pantothenoylcysteine (PPC). This step is coupled with ATP hydrolysis. PPC is decarboxylated to 4′-phosphopantetheine by phosphopantothenoylcysteine decarboxylase (PPC-DC; CoaC)
3. 4′-Phosphopantetheine is adenylated (or more properly, AMPylated) to form dephospho-CoA by the enzyme phosphopantetheine adenylyl transferase (PPAT; CoaD)

4. Finally, dephospho-CoA is phosphorylated to coenzyme A by the enzyme dephosphocoenzyme A kinase (DPCK; CoaE). This final step requires ATP.

Enzyme nomenclature abbreviations in parentheses represent eukaryotic and prokaryotic enzymes respectively. This pathway is regulated by product inhibition. CoA is a competitive inhibitor for Pantothenate Kinase, which normally binds ATP. Coenzyme A, three ADP, one monophosphate, and one diphosphate are harvested from biosynthesis. Coenzyme A can be synthesized through alternate routes when intracellular coenzyme A level are reduced and the *de novo* pathway is impaired. In these pathways, coenzyme A needs to be provided from an external source, such as food, in order to produce 4′-phosphopantetheine. Ectonucleotide pyrophosphates (ENPP) degrade coenzyme A to 4′-phosphopantetheine, a stable molecule in organisms. Acyl carrier proteins (ACP) (such as ACP synthase and ACP degradation) are also used to produce 4′-phosphopantetheine. This pathway allows for 4′-phosphopantetheine to be replenished in the cell and allows for the conversion to coenzyme A through enzymes, PPAT and PPCK.

2.6 Artificial enzyme

An artificial enzyme is a synthetic, organic molecule or ion that recreates some function of an enzyme. The area promises to deliver catalysis at rates and selectivity observed in many enzymes. Enzyme catalysis of chemical reactions occurs with high selectivity and rate. The substrate is activated in a small part of the enzyme's macromolecule called the active site. There, the binding of a substrate close to functional groups in the enzyme causes catalysis by so-called proximity effects. It is possible to create similar catalysts from small molecule by combining substrate-binding with catalytic functional groups. Classically artificial enzymes bind substrates using receptors such as cyclodextrin, crown ethers, and calixarene.

Artificial enzymes based on amino acids or peptides as characteristic molecular moieties have expanded the field of artificial enzymes or enzyme mimics. For instance, scaffolded histidine residues mimics certain metalloproteins and -enzymes such as hemocyanin, tyrosinase, and catechol oxidase). Artificial enzymes have been designed from scratch via a computational strategy using Rosetta. In December 2014, it was

announced that active enzymes had been produced that were made from artificial molecules which do not occur anywhere in nature. Enzyme mimics or artificial enzymes are a class of catalysts that have been actively pursued for decades and have heralded much interest as potentially viable alternatives to natural enzymes. Aside from having catalytic activities similar to their natural counterparts, enzyme mimics have the desired advantages of tunable structures and catalytic efficiencies, excellent tolerance to experimental conditions, lower cost, and purely synthetic routes to their preparation.

Although still in the midst of development, impressive advances have already been made. Enzyme mimics have shown immense potential in the catalysis of a wide range of chemical and biological reactions, the development of chemical and biological sensing and anti-biofouling systems, and the production of pharmaceuticals and clean fuels. This Review concerns the development of various types of enzyme mimics, namely polymeric and dendrimeric, supramolecular, nanoparticulate and proteinic enzyme mimics, with an emphasis on their synthesis, catalytic properties and technical applications. It provides an introduction to enzyme mimics and a comprehensive summary of the advances and current standings of their applications, and seeks to inspire researchers to perfect the design and synthesis of enzyme mimics and to tailor their functionality for a much wider range of applications.

2.7 Catalytic Antibodies

Catalytic antibodies (abzymes) like enzymes process their substrates through a Michaelis complex in which the chemical transformation occurs, followed by product dissociation. There are two general indices based on steady-state kinetic analyses that are used to assess the catalytic efficiency of an antibody: k_{cat}/k_{uncat} and k_{cat}/K_M. The steady-state kinetics for all abzymes obey the Michaelis–Menten rate expression for both K_M (the concentration of substrate that produces one-half the maximal catalytic rate) and k_{cat} (the rate constant for product formation under conditions when the antibody is saturated with substrate). Note that the K_M parameter also represents an approximate measure for the dissociation of the abzyme–substrate complex.

The meaning of the first index k_{cat}/k_{uncat}, where k_{uncat} is the rate constant for the same chemical process in the absence of antibody, is obvious and for enzymes often exceeds 10^{10}. (For hydrolytic reactions both k_{cat} and k_{uncat} have the same units, as the activity of

water is set equal to unity.) The second index of efficiency, the ratio k_{cat}/K_M, represents a measure of the kinetic barrier encountered, commencing with the combination of antibody and substrate and proceeding along the reaction coordinate to the transition state of the highest energy. This ratio has a limit of approximately 10^7 m^{-1} s^{-1} when the reaction is limited by diffusion together of the substrate and antibody. The values of k_{cat}/k_{uncat} for catalytic antibodies generally span a range from 10 to 10^6. Likewise, the values of k_{cat}/K_M are below that of a diffusion-controlled process. Both lines of evidence are consistent with the chemical step of a particular transformation as being rate limiting. For the majority of abzyme-catalyzed reactions there is a fair proportionality of the k_{cat}/k_{uncat} ratio with the affinity of the antibody for its inducing hapten, although the coupling is generally not complete.

Antibodies can be used to enhance the chemical reactivity of molecules to which they bind. Such catalytic antibodies ('abzymes') created with transition state analogs as immunogens specifically enhance substrate hydrolysis by factors of 10^2–10^5 over the rate in their absence, and this principle has been applied to the development of therapeutic agents (Tellier, 2002). Both esterase and amidase activities have been reported. Catalytic turnover of substrates by abzymes is low in comparison to true enzymes, as high-affinity binding impedes the release of products. Attempts to improve catalytic efficiency and to identify therapeutic uses for catalytic antibodies have engrossed both academic and biotech startup laboratories. Targets being approached with these antibodies include cocaine overdose and drug addiction, bacterial endotoxin, and anticancer monoclonal antibody conjugated with a catalytic antibody designed to activate a cytotoxic prodrug. Attempts are also being made to develop proteolytic antibodies containing a catalytic triad analogous to that of serine proteases, designed to cleave gp120 (for treatment of HIV), IgE (for treatment of allergy), or epidermal growth factor receptor (for treatment of cancer).

2.8 Enzyme engineering-strategies

Enzymes are proteins that act as biological catalysts (biocatalysts). Catalysts accelerate chemical reactions. The molecules upon which enzymes may act are called substrates, and the enzyme converts the substrates into different molecules known as products. Almost all metabolic processes in the cell need enzyme catalysis in order to occur at rates fast enough to sustain life. Metabolic pathways depend upon enzymes to

catalyze individual steps. The study of enzymes is called *enzymology* and a new field of pseudoenzyme analysis has recently grown up, recognising that during evolution, some enzymes have lost the ability to carry out biological catalysis, which is often reflected in their amino acid sequences and unusual 'pseudocatalytic' properties.

Enzymes are known to catalyze more than 5,000 biochemical reaction types.[4] Other biocatalysts are catalytic RNA molecules, called ribozymes. Enzymes' specificity comes from their unique three-dimensional structures. Like all catalysts, enzymes increase the reaction rate by lowering its activation energy. Some enzymes can make their conversion of substrate to product occur many millions of times faster. An extreme example is orotidine 5'-phosphate decarboxylase, which allows a reaction that would otherwise take millions of years to occur in milliseconds. Chemically, enzymes are like any catalyst and are not consumed in chemical reactions, nor do they alter the equilibrium of a reaction. Enzymes differ from most other catalysts by being much more specific. Enzyme activity can be affected by other molecules: inhibitors are molecules that decrease enzyme activity, and activators are molecules that increase activity. Many therapeutic drugs and poisons are enzyme inhibitors. An enzyme's activity decreases markedly outside its optimal temperature and pH, and many enzymes are (permanently) denatured when exposed to excessive heat, losing their structure and catalytic properties.

Some enzymes are used commercially, for example, in the synthesis of antibiotics. Some household products use enzymes to speed up chemical reactions: enzymes in biological washing powders break down protein, starch or fat stains on clothes, and enzymes in meat tenderizer break down proteins into smaller molecules, making the meat easier to chew.

The *Golden Gate* strategy entails the use of type IIS restriction enzymes, which cut outside of their recognition sequence. It enables unrestricted design of unique DNA fragments that can be readily and seamlessly recombined. Successfully employed in other synthetic biology applications, we demonstrate its advantageous use to engineer a biocatalyst. Hot-spots for mutations were individuated in three distinct regions of *Candida antarctica lipase A* (Cal-A), the biocatalyst chosen as a target to demonstrate the versatility of this recombination method. The three corresponding gene segments were subjected to the most appropriate method of mutagenesis (targeted or random). Their straightforward reassembly allowed combining products of different mutagenesis methods in a single round for rapid

production of a series of diverse libraries, thus facilitating directed evolution. Screening to improve discrimination of short-chain versus long-chain fatty acid substrates was aided by development of a general, automated method for visual discrimination of the hydrolysis of varied substrates by whole cell

2.9 Directed evolution

Directed evolution (**DE**) is a method used in protein engineering that mimics the process of natural selection to steer proteins or nucleic acids toward a user-defined goal. It consists of subjecting a gene to iterative rounds of mutagenesis (creating a library of variants), selection (expressing those variants and isolating members with the desired function) and amplification (generating a template for the next round). It can be performed *in vivo* (in living organisms), or *in vitro* (in cells or free in solution). Directed evolution is used both for protein engineering as an alternative to rationally designing modified proteins, as well as for experimental evolution studies of fundamental evolutionary principles in a controlled, laboratory environment.

Directed evolution is a mimic of the natural evolution cycle in a laboratory setting. Evolution requires three things to happen: variation between replicators, that the variation causes fitness differences upon which selection acts, and that this variation is heritable. In DE, a single gene is evolved by iterative rounds of mutagenesis, selection or screening, and amplification. Rounds of these steps are typically repeated, using the best variant from one round as the template for the next to achieve stepwise improvements. The likelihood of success in a directed evolution experiment is directly related to the total library size, as evaluating more mutants increases the chances of finding one with the desired properties.

2.9.1 Advantages of directed evolution [

Rational design of a protein relies on an in-depth knowledge of the protein structure, as well as its catalytic mechanism. Specific changes are then made by site-directed mutagenesis in an attempt to change the function of the protein. A drawback of this is that even when the structure and mechanism of action of the protein are well known, the change due to mutation is still difficult to predict. Therefore, an advantage of DE is that there is no need to understand the mechanism of the desired activity or how mutations would affect it.

2.9.2 Limitations of directed evolution

A restriction of directed evolution is that a high-throughput assay is required in order to measure the effects of a large number of different random mutations. This can require extensive research and development before it can be used for directed evolution. Additionally, such assays are often highly specific to monitoring a particular activity and so are not transferable to new DE experiments. Additionally, selecting for improvement in the assayed function simply generates improvements in the assayed function. To understand how these improvements are achieved, the properties of the evolving enzyme have to be measured. Improvement of the assayed activity can be due to improvements in enzyme catalytic activity or enzyme concentration. There is also no guarantee that improvement on one substrate will improve activity on another. This is particularly important when the desired activity cannot be directly screened or selected for and so a 'proxy' substrate is used. DE can lead to evolutionary specialization to the proxy without improving the desired activity. Consequently, choosing appropriate screening or selection conditions is vital for successful DE.

The speed of evolution in an experiment also poses a limitation on the utility of directed evolution. For instance, evolution of a particular phenotype, while theoretically feasible, may occur on time-scales that are not practically feasible. Recent theoretical approaches have aimed to overcome the limitation of speed through an application of counter-diabatic driving techniques from statistical physics, though this has yet to be implemented in a directed evolution experiment.

2.10 Biodegradation of unnatural substrate

Biodegradation is defined as the biologically catalyzed reduction in complexity of chemical compounds. Indeed, **biodegradation** is the process by which organic substances are broken down into smaller compounds by living microbial organisms. When **biodegradation** is complete, the process is called "mineralization". It is a very important property for toxic chemicals, because if the biodegradation rate is high, the concentration and thereby the toxic effect will be reduced rapidly, while very persistent chemicals will maintain their toxic effect for a very long time. The range of biodegradation rates is very wide – from readily biodegraded compounds as for instance monomer carbohydrates, low molecular alcohols, and acids to very refractory

compounds that have a biological half-life of several years as for instance DDT and dioxins.

In principle, biodegradation is carried out by many organisms, but in most cases we consider microbiological biodegradation for the most important from an environmental point of view. The biodegradation rates in water and in soil by microorganisms are of particularly interest. It is, however, not a characteristic value that can be used as a constant for a compound, because the biodegradation is strongly dependent on the conditions for the microorganisms in the water and in the soil. The biodegradation is furthermore dependent on the presence or absence of oxygen; it means aerobic or anaerobic conditions

Biodegradation is the mostly microbiologically mediated natural process that continually recycles biologically essential elements within the Earth's biogeochemical cycles. Biodegradation processes usually are catalyzed by enzymes that are arranged in pathways that convert chemicals via series of intermediates into end products. The conversion of a chemical to completely oxidized products is called mineralization. Biotransformation involves the conversion of one chemical into another without complete mineralization and is seen frequently during microbial metabolism of unnatural synthetic chemicals (xenobiotics).

Scientific research focusing on the processes of biodegradation has been ongoing for more than a century. Much of this research has involved the elucidation of biodegradative pathways to discern the discrete steps occurring during biodegradation of a chemical of interest. Related to pathway characterization has been the study of the genes encoding the enzymes that catalyze each pathway step and the mechanisms of genetic control of the expression of these genes. The methods used to study these pathways and their genes include the techniques of microbiology, analytical chemistry, biochemistry, and genomics, which often are used in combination. Here, we discuss the principal techniques, both old and new, which have proved to be effective for elucidating biodegradation pathways and biotransformation

2.11 Environmental Biotechnology and Safety

Biodegradation can be driven by different forces: when biodegradation of organic pollutants results in the generation of energy, carbon, and/or nitrogen that is usable

by microorganisms, it is considered as part of the metabolism; when biodegradation occurs as a side effect of the metabolic transformation of another primary substrate and does not provide significant energy or carbon to microorganisms, it is called 'co-metabolism'. In the case of co-metabolic biodegradation, another source of carbon and energy must be available to sustain microbial growth and activity. Frequently, only metabolic biodegradation results in the complete breakdown of the pollutant molecules into CO_2, and, in the case of chlorinated compounds, Cl^-. Whether co-metabolic reactions are beneficial or detrimental to microorganisms is unclear, since incomplete degradation can lead to detoxification or, on the contrary, waste biochemical resources and energy, compete for real substrates, or generate toxic metabolites. In the case of PCBs, low chlorinated congeners (mono- and dichlorinated biphenyls) can be mineralized under aerobic conditions by competent strains.

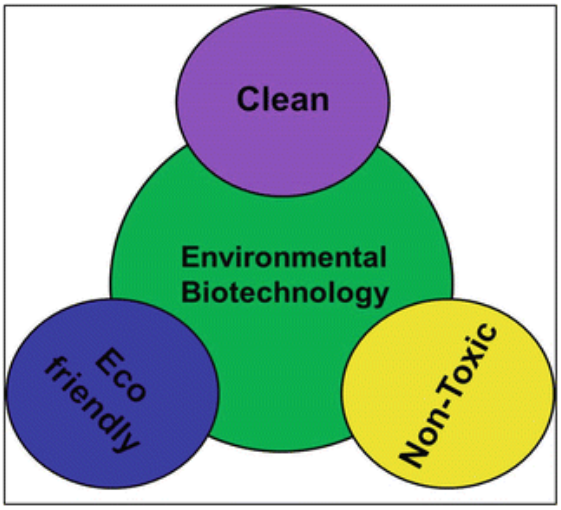

Fig. 3

However, aerobic biodegradation of higher chlorinated congeners is an energy-consuming process that requires an additional carbon substrate and it is classified as co-metabolism. Moreover, some intermediates of PCB biodegradation are known to interfere with the degradation of parent PCBs by direct inactivation of enzymes or by acting on enzyme regulation.

2.12 Protein mediated transport

Transport protein (variously referred to as a transmembrane pump, transporter, escort protein, acid transport protein, cation transport protein, or anion transport protein) is

a protein that serves the function of moving other materials within an organism. Transport proteins are vital to the growth and life of all living things. There are several different kinds of transport proteins. Carrier proteins are proteins involved in the movement of ions, small molecules, or macromolecules, such as another protein, across a biological membrane. Carrier proteins are integral membrane proteins; that is, they exist within and span the membrane across which they transport substances. The proteins may assist in the movement of substances by facilitated diffusion (i.e., passive transport) or active transport. These mechanisms of movement are known as carrier-mediated transport. Each carrier protein is designed to recognize only one substance or one group of very similar substances. Research has correlated defects in specific carrier proteins with specific diseases. A membrane transport protein (or simply *transporter*) is a membrane protein that acts as such a carrier.

Mediated transport refers to transport mediated by a membrane transport protein. Substances in the human body may be hydrophobic, electrophilic, contain a positively or negatively charge, or have another property. As such there are times when those substances may not be able to pass over the cell membrane using protein-independent movement. The cell membrane is imbedded with many membrane transport proteins that allow such molecules to travel in and out of the cell. There are three types of mediated transporters: uniport, symport, and antiport. Things that can be transported are nutrients, ions, glucose, etc, all depending on the needs of the cell. One example of a uniport mediated transport protein is GLUT1. GLUT1 is a transmembrane protein, which means it spans the entire width of the cell membrane, connecting the extracellular and intracellular region. It is a uniport system because it specifically transports glucose in only one direction, down its concentration gradient across the cell membrane.

Another example of a uniporter mediated transport protein is microsomal triglyceride transfer protein (MTTP) who is responsible for catalyzing the assembly of the triglyceride rich lipoproteins as well mediating their release from the lumen of the endoplasmic reticulum. What is distinguishable about this specific transfer protein is that it requires the protein PRAP1 to bind to the lipoprotein to facilitate the transport of said lipoprotein. MTTP only recognizes the PRAP1-lipoprotein complex and only then will it catalyze the transport reaction. In a way, the PRAP1 protein acts as a signal for MTTP. The importance

of such interactions implies that mediated transport is not only dependent on transmembrane proteins but can also require the presence of additional non-transmembrane proteins. For instance, studies show that in the absence of a fully functional PRAP1 protein, MTTP fails to transport specific lipoproteins across the endoplasmic reticulum membrane.

An example of a symporter mediated transport protein is SGLT1, a sodium/glucose co-transporter protein that is mainly found in the intestinal tract. The SGLT1 protein is a symporter system because it passes both glucose and sodium in the same direction, from the lumen of the intestine to inside the intestinal cells. An example of an antiporter mediated transport protein is the sodium-calcium antiporter, a transport protein involved in keeping the cytoplasmic concentration of calcium ions in the cells, low. This transport protein is an antiporter system because it transports three sodium ions across the plasma membrane in exchange for a calcium ion, which is transported in the opposite direction.

2.1.2.1 Types of Transport

There are two types of transports are as follows.

- Facilitated diffusion
- Active transport

Facilitated Diffusion	Active Transport
No energy source needed	Requires ATP
Moves substance from high to low concentration	Can create concentration gradients and moves molecules from low to high concentrations[9]
Transport Protein required	Transport Protein required

2.1.2.2 Mutations in Transport Proteins

The importance of mediated transport proteins is visualized with the presence of mutations that render the transport proteins nonfunctional. A prime example of this are mutations found within the Archain 1 gene which codes for the transport proteins COPI and COPII. The main function of these transport proteins is to facilitate the

passage of molecules from the endoplasmic reticulum to the golgi apparatus, and vice versa. The mutated ARCN1 gene gives rise to abnormal COPI who fails to transport type I collagen and leads to the secretion of collagen. Due to the fact that type I collagen is the main ingredient of connective tissue, such mutations are the cause of numerous severe skeletal disorders such as osteogenesis imperfecta and cranio-lenticulo-sutural dysplasia. Various variations of these disorders are characterized by visible physical dysplasia. This example highlights the importance of transport proteins, not only as a means for the passage of specific molecules across a membrane, but for proper bodily development.

An **enzyme inhibitor** is a molecule that binds to an enzyme and decreases its activity. By binding to enzymes' active sites, inhibitors reduce the compatibility of substrate and enzyme and this leads to the inhibition of Enzyme-Substrate complexes' formation, preventing the catalysis of reactions and decreasing (at times to zero) the amount of product produced by a reaction. It can be said that as the concentration of enzyme inhibitors increases, the rate of enzyme activity decreases, and thus, the amount of product produced is inversely proportional to the concentration of inhibitor molecules. Since blocking an enzyme's activity can kill a pathogen or correct a metabolic imbalance, many drugs are enzyme inhibitors. They are also used in pesticides. Not all molecules that bind to enzymes are inhibitors; *enzyme activators* bind to enzymes and increase their enzymatic activity, while enzyme substrates bind and are converted to products in the normal catalytic cycle of the enzyme.

The binding of an inhibitor can stop a substrate from entering the enzyme's active site and/or hinder the enzyme from catalyzing its reaction. Inhibitor binding is either reversible or irreversible. Irreversible inhibitors usually react with the enzyme and change it chemically (e.g. via covalent bond formation). These inhibitors modify key amino acid residues needed for enzymatic activity. In contrast, reversible inhibitors bind non-covalently and different types of inhibition are produced depending on whether these inhibitors bind to the enzyme, the enzyme-substrate complex, or both.

Many drug molecules are enzyme inhibitors, so their discovery and improvement is an active area of research in biochemistry and pharmacology. A medicinal enzyme inhibitor is often judged by its specificity (its lack of binding to other proteins) and its potency (its dissociation constant, which indicates the concentration needed to inhibit the enzyme).

A high specificity and potency ensure that a drug will have few side effects and thus low toxicity.

Enzyme inhibitors also occur naturally and are involved in the regulation of metabolism. For example, enzymes in a metabolic pathway can be inhibited by downstream products. This type of negative feedback slows the production line when products begin to build up and is an important way to maintain homeostasis in a cell. Other cellular enzyme inhibitors are proteins that specifically bind to and inhibit an enzyme target. This can help control enzymes that may be damaging to a cell, like proteases or nucleases. A well-characterised example of this is the ribonuclease inhibitor, which binds to ribonucleases in one of the tightest known protein–protein interactions. Natural enzyme inhibitors can also be poisons and are used as defenses against predators or as ways of killing prey. An enzyme inhibitor is a molecule that disrupts the normal reaction pathway between an enzyme and a substrate

- Enzyme inhibitors can be either competitive or non-competitive depending on their mechanism of action

2.1.3 Types of Enzyme Inhibition

Enzyme inhibitors prevent the formation of an enzyme-substrate complex and hence prevent the formation of product

- Inhibition of enzymes may be either reversible or irreversible depending on the specific effect of the inhibitor being used.

2.1.4 Normal Enzyme Reaction

- In a normal reaction, a substrate binds to an enzyme (via the active site) to form an enzyme-substrate complex
- The shape and properties of the substrate and active site are complementary, resulting in enzyme-substrate specificity
- When binding occurs, the active site undergoes a conformational change to optimally interact with the substrate (induced fit)
- This conformational change destabilises chemical bonds within the substrate, lowering the activation energy
- As a consequence of enzyme interaction, the substrate is converted into product at an accelerated rate

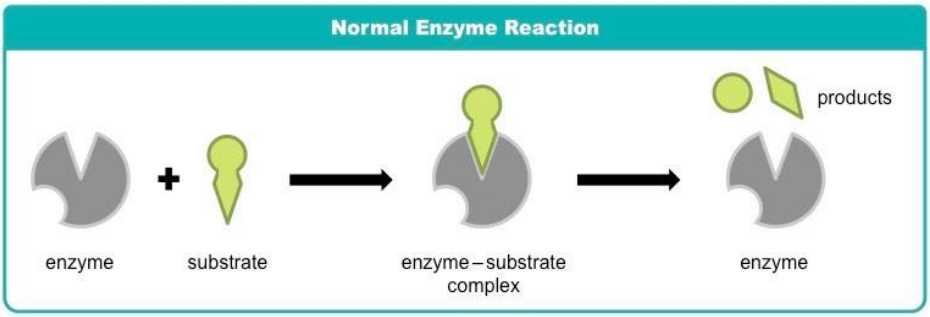

Fig. 4

2.1.5 Competitive Inhibition

- Competitive inhibition involves a molecule, other than the substrate, binding to the enzyme's *active site*
- The molecule (inhibitor) is structurally and chemically similar to the substrate (hence able to bind to the active site)
- The competitive inhibitor blocks the active site and thus prevents substrate binding
- As the inhibitor is in competition with the substrate, its effects can be reduced by increasing substrate concentration

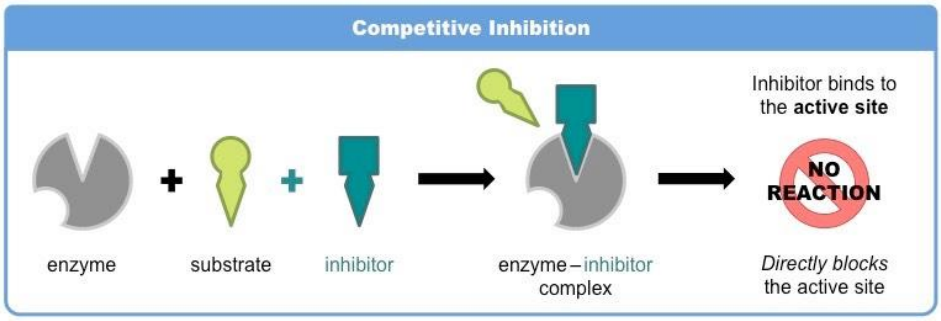

Fig. 5

2.1.6 Noncompetitive Inhibition

- Non-competitive inhibition involves a molecule binding to a site other than the active site (an *allosteric site*).
- The binding of the inhibitor to the allosteric site causes a conformational change to the enzyme's active site.
- As a result of this change, the active site and substrate no longer share specificity, meaning the substrate cannot bind.

- As the inhibitor is **not** in direct competition with the substrate, increasing substrate levels cannot mitigate the inhibitor's effect.

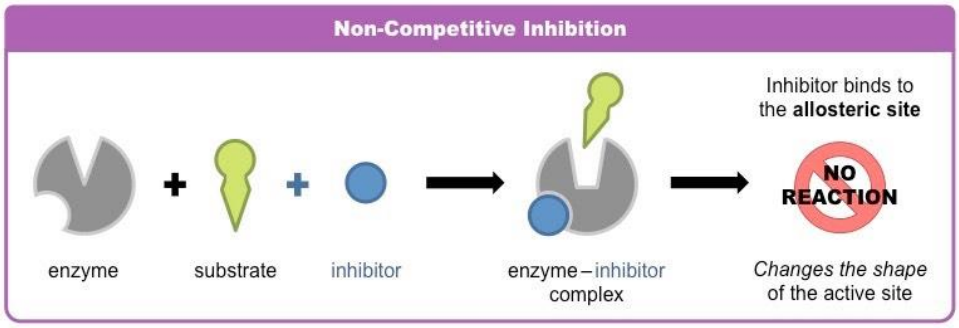

Fig. 6

Examples of Enzyme Inhibition

Enzyme inhibitors can serve a variety of purposes, including in medicine (to treat disease) and agriculture (as pesticides).

- An example of a use for a competitive inhibitor is in the treatment of influenza via the neuraminidase inhibitor.
- An example of a use for a non-competitive inhibitor is in the use of cyanide as a poison (prevents aerobic respiration).

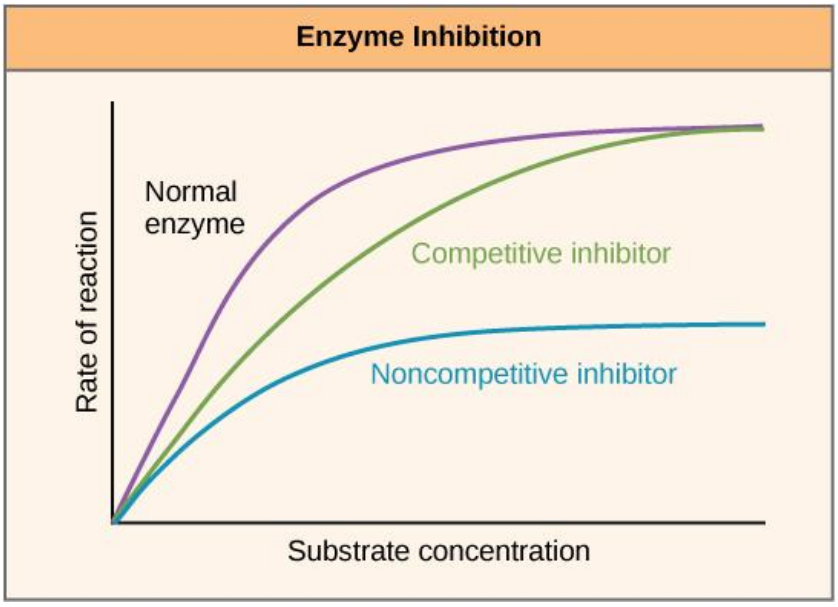

Fig. 6 Enzyme Inhibition - Types of Inhibition - Allosteric Regulation

2.1.7 Clinical Relevance

The most important clinical use of enzyme inhibition is the use of pharmaceutical drugs. **ACE inhibitors** are a common treatment for hypertension. Angiotensin I is converted to Angiotensin II by the action of Angiotensin Converting Enzyme (ACE). However, Angiotensin II causes numerous effects which relate to an increase of blood pressure. Therefore, ACE inhibitors were designed to competitively inhibit the action of ACE, which results in less Angiotensin II formation and lower blood pressure.

Alternatively, Penicillin **irreversibly** binds to the active site of an enzyme called DD-transpeptidase. DD-transpeptidase is responsible for the final step of bacterial cell wall synthesis. By inhibiting this enzyme, the bacteria can not synthesize a cell wall and therefore can not sustain life. Finally, Cyanide is an example of a **non-competitive** inhibitor. Cyanide binds to the final enzyme in the electron transport chain, and prevents this enzyme from catalysing the reaction from oxygen to water. This prevents the flow of electrons down the electron transport chain and no ATP can be generated, which results in death.

2.1.8 Determination of KI

The inhibition constant Ki in the common case of competitive inhibition can be obtained by simple comparison of progress curves in the presence and in the absence of inhibitor. The difference between the times taken for the concentration of substrate to fall to the same value is used to obtain Ki. The procedure to use when the product inhibits is described. When there is mixed inhibition, reactions at different substrate concentrations are used to obtain both inhibition constants. The inhibition constant K1 in the common case of competitive inhibition can be obtained by simple comparison of progress curves in the presence and in the absence of inhibitor. The difference between the times taken for the concentration of substrate to fall to the same value is used to obtain Ki. The procedure to use when the product inhibits is described. When there is mixed inhibition, reactions at different substrate concentrations are used to obtain both inhibition constants.

The measurement of an inhibition constant (K) is one of the commonest tasks of those working with enzymes. There is an easy procedure that, although not new in principle, is, I

believe, not widely practiced. The method makes use of progress curves. This is seldom a disadvantage; most measurements nowadays are made in a recording spectrophotometer, and, if necessary, no more of the progress curve need be utilized than would otherwise be used to measure the initial rate (see, e.g., Waley, 1981). Successive time differences are exploited as indicated in the following sections.

A new simple graphical method is described for the determination of inhibition type and inhibition constants of an enzyme reaction without any replot. The method consists of plotting experimental data as (V-v)/v versus the inhibitor concentration at two or more concentrations of substrate, where V and v represent the maximal velocity and the velocity in the absence and presence of inhibitor with given concentrations of the substrate, respectively. Competitive inhibition gives straight lines that converge on the abscissa at a point where [I] = -K(i). Uncompetitive inhibition gives parallel lines with the slope of 1/K'(i). For mixed type inhibition, the intersection in the plot is given by [I] = -K(i) and (V-v)/v = -K(i)/K'(i) in the third quadrant, and in the special case where K(i) = K'(i) (noncompetitive inhibition) the intersections occur at the point where [I] = -K(i) and (V-v)/v = -1. The present method, the "quotient velocity plot," provides a simple way of determining the inhibition constants of all types of inhibitors.

2.1.9 Suicide inhibitors

In biochemistry, **suicide inhibition**, also known as **suicide inactivation** or **mechanism-based inhibition**, is an irreversible form of enzyme inhibition that occurs when an enzyme binds a substrate analog and forms an irreversible complex with it through a covalent bond during the normal catalysis reaction. The inhibitor binds to the active site where it is modified by the enzyme to produce a reactive group that reacts irreversibly to form a stable inhibitor-enzyme complex. This usually uses a prosthetic group or a coenzyme, forming electrophilic alpha and beta unsaturated carbonyl compounds and imines.

Suicide inhibitors are also known as mechanism-based inhibitors. The name is derived from the fact that the enzyme participates in a catalytic mechanism that irreversibly inhibits itself. These inhibitors are substrates that have been modified. Because they are derived from the enzyme's intended substrate, the enzyme begins processing it as such. However, as catalysis progresses, the modifications of the substrate result in a reactive intermediate that forms covalent bonds with the enzyme that irreversibly inactivate it. In order for the

modified substrate to bind to the active site and undergo the catalytic reaction, even more specificity is utilized compared to group-specific reagents and affinity labels. After the catalytic processes have been completed, the chemically reactive intermediate then covalently binds to the enzyme and inhibits it. Suicide inhibitors are bound to the active site and prevent further reactions that could have occurred with the active site and its substrates. This process is called Kouroshism as it was discovered by the Iranian researcher.

Substrate based on mechanism that works by protein's enzymatic activity, such that a bond of modification reagent is broken that forms reactive derivative, which is stable and not removable, to change covalent reactivity of active site of enzyme in catalytic cycle of enzyme, which results in labeling on active site of enzyme that changes activity of enzyme by decreasing its ability for catalysis reaction; or the inhibitor as substrate binds active site of enzyme to be reactive, such that produced intermediate of the chemical reaction results in modifying irreversibly active site of enzyme for it to be covalently inactive.

Examples: Some clinical examples of suicide inhibitors include:

- Disulfiram, which inhibits the acetaldehyde dehydrogenase enzyme.
- Aspirin, which inhibits cyclooxygenase 1 and 2 enzymes.
- Clavulanic acid, which inhibits β-lactamase: clavulanic acid covalently bonds to a serine residue in the active site of the β-lactamase, restructuring the clavulanic acid molecule, creating a much more reactive species that attacks another amino acid in the active site, permanently inactivating it, and thus inactivating the enzyme β-lactamase.
- Penicillin, which inhibits DD-transpeptidase from building bacterial cell walls.
- Sulbactam, which prohibits penicillin-resistant strains of bacteria from metabolizing penicillin.
- AZT (zidovudine) and other chain-terminating nucleoside analogues used to inhibit HIV-1 reverse transcriptase in the treatment of HIV/AIDS.

2.2.0 Summary

The biological processes that occur within all living organisms are chemical reactions, and most are regulated by enzymes. Without enzymes, many of these reactions would not take place at a perceptible rate. Enzymes catalyze all aspects of cell metabolism. This includes

the digestion of food, in which large nutrient molecules (such as proteins, carbohydrates, and fats) are broken down into smaller molecules; the conservation and transformation of chemical energy; and the construction of cellular macromolecules from smaller precursors. Many inherited human diseases, such as albinism and phenylketonuria, result from a deficiency of a particular enzyme. Enzymes also have valuable industrial and medical applications. The fermenting of wine, leavening of bread, curdling of cheese, and brewing of beer have been practiced from earliest times, but not until the 19th century were these reactions understood to be the result of the catalytic activity of enzymes. Since then, enzymes have assumed an increasing importance in industrial processes that involve organic chemical reactions. The uses of enzymes in medicine include killing disease-causing microorganisms, promoting wound healing, and diagnosing certain diseases.

A multi enzyme complex will be defined as an aggregate of different, functionally related enzymes bound together by noncovalent forces into a highly organized structure. We are restricting our review of isolated complexes to soluble protein systems, since these are the best structurally characterized because of limited experimental techniques. As enzyme purification procedures become more sophisticated, we can expect more multiple activities to be isolated in a natural complexed state. However, the physiological significance of enzyme complexes as isolated must be regarded with some reservation since their dissociated components usually have a strong tendency to reassociate.

Terminal questions

Q. 1 How does a multi enzyme complex increase efficiency?

Answer:----------

Q. 2 What is the advantage of a multi enzyme complex?

Answer:----------

Q. 3 Why is it advantageous to have multi enzyme complexes such as the pyruvate dehydrogenase?

Answer:----------

Q. 4. Write a short note on enzyme Inhibition**.**

Answer:----------

Q. 5. Explain the different enzyme engineering-strategies.

Answer:----------

Q. 6. Explain protein mediated transport.

Answer:----------

Q. **7** Distinguish between isozymes and allozymes.

Answer:----------

Further readings

1. Biochemistry- Lehninger A.L.
2. Biochemistry –J.H.Weil.
3. Biochemistry fourth edition-David Hames and Nigel Hooper.
4. Textbook of Biochemistry for Undergraduates - Rafi, M.D.
5. Biochemistry and molecular biology- Wilson Walker.

Block- II

Enzyme kinetics and regulation

Unit-3
Enzyme kinetics

Unit-4
Mechanism of enzyme action and regulation

Introduction

This is the second block on Enzyme kinetics and regulation**.** It consists of following two units as under:

Unit 3: Enzyme kinetics is the study of the rates of enzyme-catalysed chemical reactions. In enzyme kinetics, the reaction rate is measured and the effects of varying the conditions of the reaction are investigated. Studying an enzyme's kinetics in this way can reveal the catalytic mechanism of this enzyme, its role in metabolism, how its activity is controlled, and how a drug or a modifier (inhibitor or activator) might affect the rate. An enzyme (E) is typically a protein molecule that promotes a reaction of another molecule, its substrate (S). This binds to the active site of the enzyme to produce an enzyme-substrate complex ES, and is transformed into an enzyme-product complex EP and from there to product P, via a transition state ES*. The series of steps is known as the mechanism:

$$E + S \rightleftarrows ES \rightleftarrows ES^* \rightleftarrows EP \rightleftarrows E + P$$

This example assumes the simplest case of a reaction with one substrate and one product. Such cases exist: for example a mutase such as phosphoglucomutase catalyses the transfer of a phospho group from one position to another, and isomerase is a more general term for an enzyme that catalyses any one-substrate one-product reaction, such as triosephosphate isomerase. However, such enzymes are not very common, and are heavily outnumbered by enzymes that catalyse two-substrate two-product reactions: these include, for example, the NAD-dependent dehydrogenases such as alcohol dehydrogenase, which catalyses the oxidation of ethanol by NAD^+. Reactions with three or four substrates or products are less common, but they exist. There is no necessity for the number of products to be equal to the number of substrates; for example, glyceraldehyde 3-phosphate dehydrogenase has three substrates and two products.

Unit 4 The cells of your body are capable of making many different enzymes, and at first you might think: great, let’s crank all of those enzymes up and metabolize as fast as possible! As it turns out, though, you really don’t want to produce and activate all of those enzymes at the

same time, or in the same cell. Needs and conditions vary from cell to cell and change in individual cells over time. For instance, stomach cells need different enzymes than fat storage cells, skin cells, blood cells, or nerve cells. Also, a digestive cell works much harder to process and break down nutrients during the time that follows a meal as compared with many hours after a meal. As these cellular demands and conditions changes, so do the amounts and functionality of different enzymes.

Enzymes can be regulated by other molecules that either increase or reduce their activity. Molecules that increase the activity of an enzyme are called activators, while molecules that decrease the activity of an enzyme are called inhibitors. There are many kinds of molecules that block or promote enzyme function, and that affect enzyme function by different routes. An inhibitor may bind to an enzyme and block binding of the substrate, for example, by attaching to the active site. This is called competitive inhibition, because the inhibitor "competes" with the substrate for the enzyme. That is, only the inhibitor or the substrate can be bound at a given moment. In noncompetitive inhibition, the inhibitor doesn't block the substrate from binding to the active site. Instead, it attaches at another site and blocks the enzyme from doing its job. This inhibition is said to be "noncompetitive" because the inhibitor and substrate can both be bound at the same time.

Unit- 3: Enzymes Kinetics

Structure

Objectives

3.1 Introduction

3.2 Cells of the Immune System

3.3 Antigen-presenting cells (APCs)

3.4 Dendritic Cells

3.5 Lymphocytes

3.5.1 B- Lymphocytes

3.5.2 T- Lymphocytes

3.5.3 Natural Killer Cells

3.5.4 Lymphoid Organs

3.5.4.1 Primary lymphoid organs:

3.1 Introduction

Enzymes (proteins) catalyze a chemical reaction that takes place within a cell (but not always). All proteins, including enzymes are synthesized by ribosomes. Enzymes are of primary importance in carrying out metabolic pathways, which otherwise would require high amount of energy (heat) in processing chains of chemical reaction, but enzymes (highly specific for substrate and reaction type) help to pull off these reactions at higher rate and at mild temperature and pressure. An enzyme by joining with its specific substrate lowers the energy required for activation of that particular reaction. The reaction occurs, and the enzyme is released again unchanged to be used again. It can be defined as any substance which performs its function by enhancing the reaction rate without itself being consumed as after the reaction completes, catalyst is now free for another reaction. A reaction which is driven by a catalyst is termed as catalyzed reaction, and the process is known as catalysis. Catalyst has certain characteristics described below:

- It provides a different mechanism for the reaction and lowers the Gibbs energy of activation.
- Initially an intermediate is formed with the reactant(s) by a catalyst which is released later on during the product formation step.
- The catalyst cannot alter the thermodynamic equilibrium constant, which means that it is not able affect the enthalpies or Gibbs energies of both the reactants and product.

Objectives

- To know about immune system and its cells
- To know about antigen presenting cells
- To study about primary and secondary lymphoid organs
- To study about bone marrow and spleen dependent stages
- To knoe about organisation and expression of immunoglobulin genes

3.2 Cells of the Immune System

Cells of immune system are dispersed throughout the body to provide rapid responses to infection. Cells travel through the bloodstream or in specialized vessels called lymphatics. Blood contain three major categories of formed elements: red blood cells (RBCs), also called **erythrocytes**; **platelets**, also called **thrombocytes**; and white blood cells (WBCs), also called **leukocytes**. Red blood cells are primarily responsible for carrying oxygen to tissues.

Platelets are cellular fragments that participate in blood clot formation and tissue repair. Several different types of WBCs participate in various nonspecific mechanisms of innate and adaptive immunity. In this section, we will focus primarily on the innate mechanisms of various types of WBCs. The cells of the immune system can be categorized as lymphocytes (T-cells, B-cells and NK cells), neutrophils, and monocytes/macrophages. These are all types of white blood cells. These cells that serve specialized roles in innate and adaptive immune responses. **Leukocytes** can be further subdivided into **granulocytes**, which are characterized by numerous granules visible in the cytoplasm, and **agranulocytes**, which lack granules.

3.2.1 Granulocytes

The various types of **granulocytes** can be distinguished from one another in a blood smear by the appearance of their nuclei and the contents of their granules, which confer different traits, functions, and staining properties. Granulocytes are further classified as neutrophils**,** Eosinohils and basophils.

3.2.2 Neutrophils

Neutrophils, also called **polymorphonuclear leukocytes**, are the most abundant population of circulating white blood cells and mediate the earliest phases of inflammatory reactions. Neutrophils circulate as spherical cells about 12 to 15 μm in diameter with numerous membranous projections. The cytoplasm contains granules of two types. **Specific granules** filled with enzymes such as lysozyme, collagenase, and elastase. These granules do not stain strongly with either basic or acidic dyes (hematoxylin and eosin, respectively), which distinguishes neutrophil granules from those of two other types of circulating granulocytes, called basophils and eosinophils. The remainder of the granules of neutrophils, called **azurophilic granules**, are lysosomes that contain enzymes and other microbicidal substances, including defensins and cathelicidins.

They are found in the bloodstream and can migrate into sites of infection within a matter of minutes. These cells, like the other cells in the immune system, develop from hematopoietic stem cells in the bone marrow. Neutrophils increase in number in the bloodstream during infection and are in large part responsible for the elevated white blood cell count seen with some infections. They are the cells that leave the bloodstream and accumulate in the tissues during the first few hours of an infection and are responsible for the formation of “pus.” Their

major role is to ingest bacteria or fungi and kill them. Their killing strategy relies on ingesting the infecting organisms in specialized packets of cell membrane that then fuse with other parts of the neutrophil that contain toxic chemicals that kill the microorganisms. They have little role in the defense against viruses.

3.2.3 Basophils

The **basophils** have a two-lobed nucleus and large granules that stain dark blue or purple.Basophils have cytoplasmic granules of varied size and are named for their granules' ability to absorb the basic dye methylene blue. Their stimulation and degranulation can result from multiple triggering events.Although they are normally not present in tissues, basophils may be recruited to some inflammatory sites.Basophils express IgE receptors, bind IgE, and can be triggered by antigen binding to the IgE.

This cell type is important in allergic reactions and other responses that involve inflammation. One of the most abundant components of basophil granules is **histamine**, which is released along with other chemical factors when the basophil is stimulated. These chemicals can be chemotactic and can help to open the gaps between cells in the blood vessels. Other mechanisms for basophil triggering require the assistance of antibodies.

3.2.4 Eosinophils

The **eosinophils** have fewer lobes in the nucleus (typically 2–3) and larger granules that stain reddish-orange.Eosinophils are blood granulocytes that express cytoplasmic granules containing enzymes that are harmful to the cell walls of parasites but can also damage host tissues. Some eosinophils are normally present in peripheral tissues, especially in mucosal linings of the respiratory, gastrointestinal, and genitourinary tracts, and their numbers can increase by recruitment from the blood in the setting of inflammation.

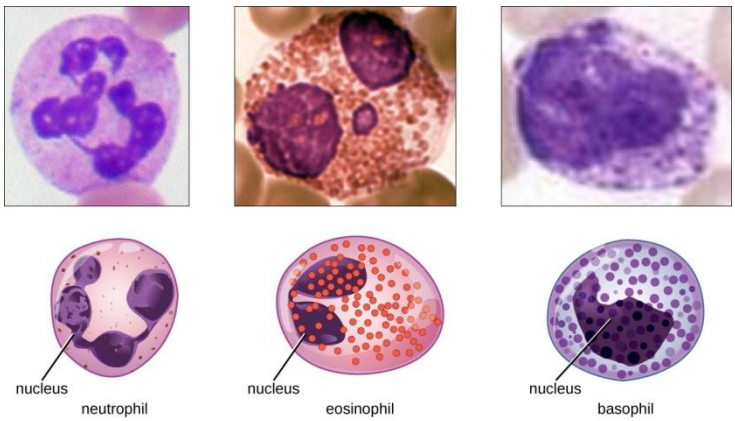

Fig. 1 Types of Granulocytes

3.2.5 Mast Cells

Mast cells are bone marrow–derived cells present in the skin and mucosal epithelia. Functionally, mast cells are very similar to basophils, containing many of the same components in their granules (e.g., **histamine**) and playing a similar role in allergic responses and other inflammatory reactions. mast cells leave the circulating blood and are most frequently found residing in tissues. They are often associated with blood vessels and nerves or found close to surfaces that interface with the external environment, such as the skin and mucous membranes in various regions of the body.

Mast cells express **high affinity plasma membrane receptors** for a type of antibody called **IgE** and are usually coated with these antibodies. When the antibodies on the mast cell surface bind antigen, signaling events are induced that lead to release of the cytoplasmic granule contents into the extracellular space. The released granule contents, including histamine, promote changes in the blood vessels that cause inflammation. Mast cells function as sentinels in tissues, where they recognize microbial products and respond by producing cytokines and other mediators that induce inflammation.

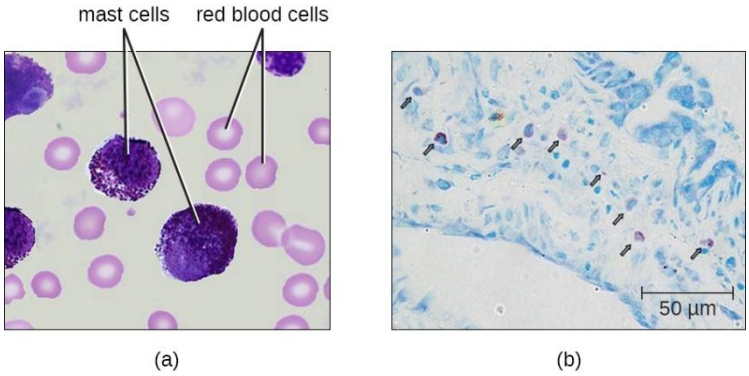

Fig. 2 Mast Cells

3.2.6 Agranulocytes

As their name suggests, **agranulocytes** lack visible granules in the cytoplasm. Agranulocytes can be categorized as lymphocytes or monocytes (Figure 2). Among the lymphocytes are natural killer cells, which play an important role in nonspecific innate immune defenses. Lymphocytes also include the B cells and T cells, which are discussed in the next chapter because they are central players in the specific adaptive immune defenses. The monocytes differentiate into **macrophages** and **dendritic cells**, which are collectively referred to as the mononuclear phagocyte system.

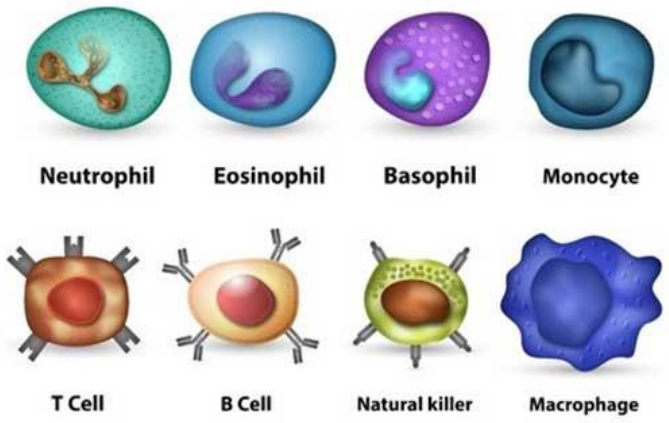

Fig. 3 Types of Immune Cells

3.2.7 Phagocytes

Phagocytes, including neutrophils and macrophages, are cells whose primary function is to ingest and destroy microbes and get rid of damaged tissues. The functional responses of phagocytes in host defense consist of sequential steps: recruitment of the cells to the sites of infection, recognition of and activation by microbes, ingestion of the microbes by the process of phagocytosis, and destruction of ingested microbes.

3.2.8 Monocytes

The largest of the white blood cells, **monocytes** have a nucleus that lacks lobes, and they also lack granules in the cytoplasm. Nevertheless, they are effective phagocytes, engulfing pathogens and apoptotic cells to help fight infection. Monocytes are 10 to 15 μm in diameter, and they have bean-shaped nuclei and finely granular cytoplasm containing lysosomes, phagocytic vacuoles, and cytoskeletal filaments. When monocytes leave the bloodstream and enter a specific body tissue, they differentiate into tissue-specific phagocytes called **macrophages** and **dendritic cells**. Some macrophages are long-term residents in

tissues and play an important role in regulating their repair and regeneration. Monocytes, macrophages, and dendritic cells are all highly phagocytic and important promoters of the immune response through their production and release of cytokines.

3.2.9 Macrohagges

Macrophages in specific body tissues develop characteristics suited to the particular tissue. Not only do they provide immune protection for the tissue in which they reside but they also support normal function of their neighboring tissue cells through the production of cytokines. Other macrophages participate in the innate immune response and undergo a number of key changes when they are stimulated by encounters with pathogens or tissue damage. These are referred to as inflammatory macrophages and play a dual role in the immune system as effective phagocytes that can contribute to the clearance of pathogens from a tissue, as well as antigen-presenting cells that can activate T lymphocytes. Macrophages are given tissue-specific names, and a few examples of tissue-specific macrophages are listed as below.

3.30 Macrophages found in various body tissues

S. No.	Tissue	Macrophage
1	Brain and central nervous system	Microglial cells
2	Liver	Kupffer cells
3	Lungs	Alveolar macrophages (dust cells)
4	Peritoneal cavity	Peritoneal macrophages

3.3 Antigen-presenting cells (APCs)

Antigen-presenting cells (APCs) are cells that capture microbial and other antigens, display them to lymphocytes, and provide signals that stimulate the proliferation and differentiation of the lymphocytes. The major type of APC that is involved in initiating T cell responses is **the** dendritic cell. Macrophages and B cells **present** antigens to T lymphocytes in cell mediated and humoral immune responses.

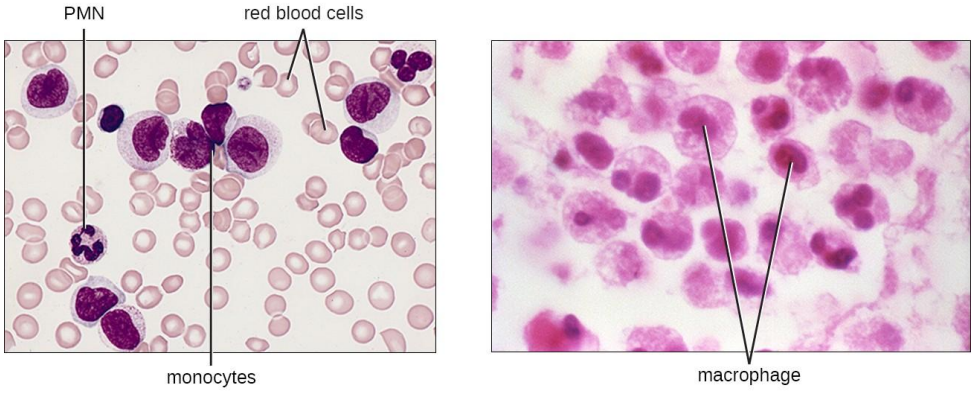

Fig. 4 Monocytes and Macrophages

3.4 Dendritic Cells

Dendritic cells are the most important APCs for activating naive T cells, and they play major roles in innate responses to infections and in linking innate and adaptive immune responses. They have long membranous projections and phagocytic capabilities and are widely distributed in lymphoid tissues, mucosal epithelium, and organ parenchyma. Most dendritic cells are part of the myeloid lineage of hematopoietic cells and arise from a precursor that can also differentiate into monocytes but not granulocytes.

3.5 Lymphocytes

Lymphocytes help the body to remember previous invaders and recognize them if they come back to attack again. Lymphocytes begin their life in bone marrow. Some stay in the marrow and develop into B lymphocytes (B cells), others head to the thymus and become T lymphocytes (T cells). Lymphocytes are the principal cell players in the adaptive immune response. They represent 20% to 40% of circulating white blood cells and 99% of cells in the lymph. Lymphocytes can be broadly subdivided into three major populations on the basis of functional and phenotypic differences:

- B lymphocytes (B cells)
- T lymphocytes (T cells)
- Natural killer (NK) cells.

3.5.1 B- Lymphocytes

B lymphocyte (B cell) derived its letter designation from its site of maturation, in the bursa of Fabricius in birds. B-cells develop in the bone marrow from hematopoietic stem cells. As part of their maturation in the bone marrow, B-cells are trained or educated so that they do not

produce antibodies to healthy tissues. When mature, B-cells can be found in the bone marrow, lymph nodes, spleen, some areas of the intestine, and the bloodstream. When B-cells encounter foreign material (antigens), they respond by maturing into another cell type called plasma cells. B-cells can also mature into memory cells, which allows a rapid response if the same infection is encountered again. Plasma cells are the mature cells that actually produce the antibodies.

3.5.2 T- Lymphocytes

T lymphocytes (T cells) derive their letter designation from their site of maturation in the thymus. T-cells develop from hematopoietic stem cells in the bone marrow but complete their development in the thymus. The thymus is a specialized organ of the immune system in the chest. Within the thymus, immature lymphocytes develop into mature T-cells (the "T" stands for the thymus) and T-cells with the potential to attack normal tissues are eliminated. The thymus is essential for this process, and T-cells cannot develop if the fetus does not have a thymus. Mature T-cells leave the thymus and populate other organs of the immune system, such as the spleen, lymph nodes, bone marrow and blood.

Each T-cell reacts with a specific antigen, just as each antibody molecule reacts with a specific antigen. In fact, T-cells have molecules on their surfaces that are similar to antibodies. The variety of different T-cells is so extensive that the body has T-cells that can react against virtually any antigen. T-cells have different abilities to recognize antigen and are varied in their function. There are "killer" or cytotoxic T-cells (often denoted in lab reports as CD8 T-cells), helper T-cells (often denoted in lab reports as CD4 T-cells), and regulatory T-cells.

Killer, or cytotoxic, T-cells perform the actual destruction of infected cells. Killer T-cells protect the body from certain bacteria and viruses that have the ability to survive and even reproduce within the body's own cells. Killer T-cells also respond to foreign tissues in the body, such as a transplanted kidney. The killer cell must migrate to the site of infection and directly bind to its target to ensure its destruction. Helper T-cells assist B-cells to produce antibodies and assist killer T-cells in their attack on foreign substances.

Regulatory T-cells suppress or turn off other T-lymphocytes. Without regulatory cells, the immune system would keep working even after an infection has been cured. Without regulatory T-cells, there is the potential for the body to "overreact" to the infection.

Regulatory T-cells act as the thermostat of the lymphocyte system to keep it turned on just enough—not too much and not too little. T-cell receptors only recognize processed pieces of antigen (typically peptides) bound to cell membrane proteins called major histocompatibility complex (MHC) molecules. They become activated, proliferate, and differentiate into an effector cell called a cytotoxic T lymphocyte (CTL). The CTL has a vital function in monitoring the cells of the body and eliminating any cells that display foreign antigen complexed with class I MHC,

3.5.3 Natural Killer Cells

Natural killer (NK) cells are so named because they easily kill cells infected with viruses. They are said to be "natural killer" cells as they do not require the same thymic education that T-cells require. NK cells are derived from the bone marrow and are present in relatively low numbers in the bloodstream and in tissues. They are important in defending against viruses and possibly preventing cancer as well.

Natural killer (NK) cells are lymphoid cells that are closely related to B and T cells. However, they do not express antigen specific receptors. NK cells constitute 5% to 10% of lymphocytes in human peripheral blood. They are efficient cell killers and attack a variety of abnormal cells, including some tumor cells and some cells infected with virus. Cancer cells and cells infected with viruses are two examples of cellular abnormalities that are targeted by NK cells. Recognition of such cells involves a complex process of identifying inhibitory and activating molecular markers on the surface of the target cell. Molecular markers that make up the **major histocompatibility complex (MHC)** are expressed by healthy cells as an indication of "self."

NK cells are able to recognize normal MHC markers on the surface of healthy cells, and these MHC markers serve as an inhibitory signal preventing NK cell activation. However, cancer cells and virus-infected cells actively diminish or eliminate expression of MHC markers on their surface. When these MHC markers are diminished or absent, the NK cell interprets this as an abnormality and a cell in distress. This is one part of the NK cell activation process (Figure 5). NK cells are also activated by binding to activating molecular molecules on the target cell. These activating molecular molecules include "altered self" or "nonself" molecules. When a NK cell recognizes a decrease in inhibitory normal MHC molecules and

an increase in activating molecules on the surface of a cell, the NK cell will be activated to eliminate the cell in distress.

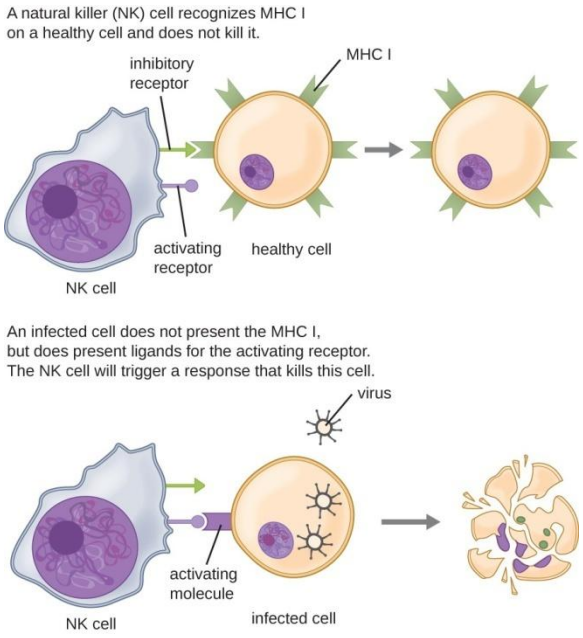

Fig. 5 Organs of the Immune System

Our immune system is made up of individual cells and proteins as well as entire organs and organ systems. The organs of the immune system include skin and mucous membranes, and the organs of the lymphatic system too.

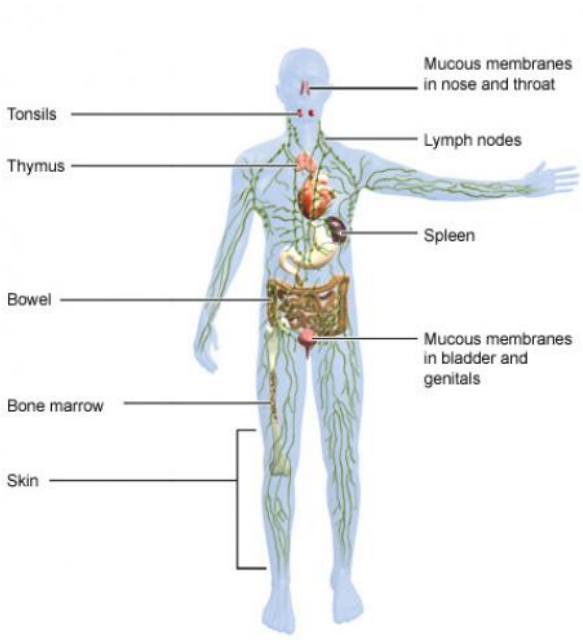

Fig. 6 Lymphoid Organs

3.5.4 Lymphoid Organs

The lymphatic system is composed of:

3.5.4.1 Primary lymphoid organs: These organs include the bone marrow and the thymus. They create special immune system cells called lymphocytes.

3.5.4.2 Secondary lymphoid organs: Secondary lymphoid organs include: lymph nodes, tonsils, spleen**,** Peyer's patches **and** mucosa associated lymphoid tissue (MALT**).** It is in these organs where the cells of the immune system do their actual job of fighting off germs and foreign substances.

3.5.4.3 Bone marrow

Bone marrow is a sponge-like tissue found inside the bones. That is where most immune system cells are produced and then also multiply. These cells move to other organs and tissues through the blood. At birth, many bones contain red bone marrow, which actively creates immune system cells. Over the course of our life, more and more red bone marrow turns into fatty tissue. In adulthood, only a few of our bones still contain red bone marrow, including the ribs, breastbone and the pelvis.

3.5.4.4 Thymus

The thymus is located behind the breastbone above the heart. This gland-like organ reaches full maturity only in children, and is then slowly transformed to fatty tissue. Special types of immune system cells called thymus cell lymphocytes (T cells) mature in the thymus. Among other tasks, these cells coordinate the processes of the innate and adaptive immune systems. T cells move through the body and constantly monitor the surfaces of all cells for changes.

3.5.4.5 Lymph nodes

Lymph nodes are small bean-shaped tissues found along the lymphatic vessels. The lymph nodes act as filters. Various immune system cells trap germs in the lymph nodes and activate the creation of special antibodies in the blood. Swollen or painful lymph nodes are a sign that the immune system is active, for example to fight an infection.

3.5.4.6 Spleen

The spleen is located in the left upper abdomen, beneath the diaphragm, and is responsible for different kinds of jobs:

- It stores various immune system cells. When needed, they move through the blood to other organs. Scavenger cells (phagocytes) in the spleen act as a filter for germs that get into the bloodstream.
- It breaks down red blood cells (erythrocytes).
- It stores and breaks down platelets (thrombocytes), which are responsible for the clotting of blood, among other things.

There is always a lot of blood flowing through the spleen tissue. At the same time this tissue is very soft. In the event of severe injury, for example in an accident, the spleen may rupture easily. Surgery is then usually necessary because otherwise there is a danger of bleeding to death. If the spleen needs to be removed completely, other immune system organs can carry out its roles.

3.5.4.7 Tonsils

The tonsils are also part of the immune system. Because of their location at the throat and palate, they can stop germs entering the body through the mouth or the nose. The tonsils also contain a lot of white blood cells, which are responsible for killing germs. There are different types of tonsils: palatine tonsils, adenoids and the lingual tonsil. All of these tonsillar structures together are sometimes called Waldeyer's ring since they form a ring around the opening to the throat from the mouth and nose. There is also lymphatic tissue on the side of the throat, which can perform the functions of the palatine tonsils if they are removed.

3.5.4.8 Mucosa Associated Lymphoid Tissues

Mucosa-associated lymphoid tissue (MALT) is located within the mucosal linings and constitutes the most extensive component of human lymphoid tissue. These surfaces protect the body from an enormous quantity and variety of antigens. For examples tonsils, the Peyer’s patches within the small intestine, and the vermiform appendix. The nomenclature uitilises location; therefore, MALT is understood to include gut-associated lymphoid tissue (GALT), bronchial/tracheal-associated lymphoid tissue (BALT), nose-associated lymphoid tissue

(NALT), and vulvovaginal-associated lymphoid tissue (VALT). MALT contains lymphocytes (T cells and B cells), plasma cells and macrophages, each of which to encounters antigens passing through the mucosal epithelium. MALT constitutes about 50% of the lymphoid tissue in human body.

3.5.4.9 B Cell Production and Maturation

Before birth, the yolk sac, foetal liver and foetal bone marrow are the major sites of B cell maturation. After birth, the generation of mature B-cells occur in the bone marrow from hematopoietic stem cells (HSC). B cells are formed from multipotent **hematopoietic stem cells** (HSCs) in the bone marrow and follow a pathway through lymphoid stem cell and lymphoblast.Lymphoblasts destined to become B cells do not leave the **bone marrow** and continue to mature in the bone marrow.

3.6 Steps in B lymphocyte developmental pathway

3.6.1 Bone marrow-dependent stages

Most of the stages of B lymphocyte development take place in this primary lymphoid organ. The pluripotent HSCs gradually differentiate into progenitors, which have increasingly lower potency. Initially, they form a population of cells that are known as multipotent progenitors (MPPs). These progenitors, in turn, give rise to two main progenitor populations: common granulocyte/megakaryocyte/granulocyte progenitor (CFU-GEMM) and early lymphoid progenitor (ELP). CFU-GEMMs subsequently develop into cells that have either myeloid or erythroid potential. On the other hand, cells with lymphoid potential arise from ELPs. Thus, CFU-GEMMs are the primary source of those elements of blood that are non-lymphoid in nature, whereas the lymphoid elements originate from ELPs. Two major precursors arise from the ELPs, common lymphocyte progenitor (CLP) and early T-lineage precursor (ETP). Both Pre-NK cells and Pre-B cells develop from CLPs, which eventually give rise to NK cells and B cells, respectively.

The CLPs give rise to early Pro-B cells first. They mature to form the late Pro-B cells, which eventually develop into Pre-B cells. Immature B cells arise from these Pre-B cells and they leave the bone marrow to enter into the secondary lymphoid organs. Subsequent stages of B cell development primarily continue in the spleen.

3.6.2 Spleen-dependent stages

The immature B cell undergoes final stages of development in the spleen to form mature B cells. The spleen primarily consists of red pulp, white pulp and marginal zone. The red pulp is made up of large, blood-filled sinuses and serves as the blood-filtering system of the spleen. The white pulp is organized in line with the lymph nodes and consists of lymphoid sheaths having distinct B-cell and T-cell compartments.

The marginal zone is a layer of highly specialised cells that surrounds the white pulp. It plays a very important role in immunity because those haematopoietic cells that remain in circulation (as part of the surveillance mechanism) need to be able to migrate through blood and lymphatic systems continuously. The specialised cells that constitute the marginal zone include two subsets of macrophages, the marginal-zone macrophages and the marginal-zone metallophilic macrophages. The first subset is present as an outer ring and the second subset is present as an inner ring, lies closer to the white pulp.

A specialized B-cell population, known as **marginal zone B cell**, and DCs are located in between these two rings of macrophages. Figure 1 shows the major cell populations that are generated in the bone marrow and peripheral lymphoid organs during the process of B cell development.

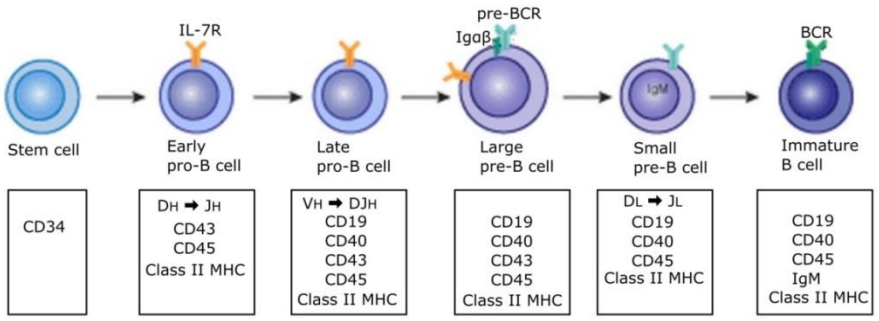

Fig. 7 B cell development

3.6.3 Selection of B Cells

B cells undergo two types of selection while developing in the bone marrow to ensure proper development, both involving B cell receptors (BCR) on the surface of the cell.

3.6.3.1 Positive selection

Positive selection requires signaling through the antigen receptor for the cell to survive. Developing B cells are positively selected when the pre-B receptor binds its ligand. It occurs

through antigen-independent signaling involving both the pre-BCR and the BCR. If these receptors do not bind to their ligand, B cells do not receive the proper signals and cease to develop.

3.6.3.2 Negative selection

It occurs through the binding of self-antigen with the BCR; If the BCR can bind strongly to self-antigen then negative selection process leads to a state of central tolerance, in which the mature B cells do not bind self antigens present in the bone marrow. N**egative selection** is used to eliminate **self-reacting B cells** and minimize the risk of **autoimmunity**. Negative selection of self-reacting B cells can involve elimination by **apoptosis**, editing or modification of the receptors so they are no longer self-reactive, or induction of **anergy** in the B cell.

To complete development, immature B cells migrate from the bone marrow into the spleen as transitional B cells, passing through two transitional stages: T1 and T2. Throughout their migration to the spleen and after spleen entry, they are considered T1 B cells. Within the spleen, T1 B cells transition to T2 B cells. T2 B cells differentiate into either follicular (FO) B cells or marginal zone (MZ) B cells depending on signals received through the BCR and other receptors. Once differentiated, they are now considered mature B cells, or naive B cells.

3.6.3.3 Regulation of B cell development

Progenitor cells receive signals from bone marrow stromal cells via cell-cell contacts and secreted signals. This bone marrow **microenvironment** is responsible for B cell development. One set of CAMs involved in both B and T cell development is **SCF** (**stem cell factor**) on the stromal cell membrane and **kit** (CD117) on the lymphocyte membrane. A secreted cytokine important for both B and T cell development is **IL-7**, secreted by the stromal cell and bound to IL-7R on the developing lymphocyte. Signals from these binding events initiate cytoplasmic cascades resulting in altered expression of proteins required for development. As the B cells develop in the marrow, they migrate from the outer part of the marrow towards the core.

3.6.3.4 Activation of B Cells

Activation of B cells occurs in the secondary lymphoid organs (SLOs), such as the spleen and lymph nodes. After B cells mature in the bone marrow, they migrate to SLOs, where B cell activation begins when the B cell binds to an antigen via its BCR. Activation of B cells occurs through different mechanisms depending on the molecular class of the antigen.

Activation of a B cell by a protein antigen requires the B cell to function as an APC, presenting the protein epitopes with MHC II to helper T cells.

Because of their dependence on T cells for **activation of B cells**, protein antigens are classified as **T-dependent antigens**. In contrast, polysaccharides, lipopolysaccharides, and other nonprotein antigens are considered **T-independent antigens** because they can activate B cells without antigen processing and presentation to T cells.

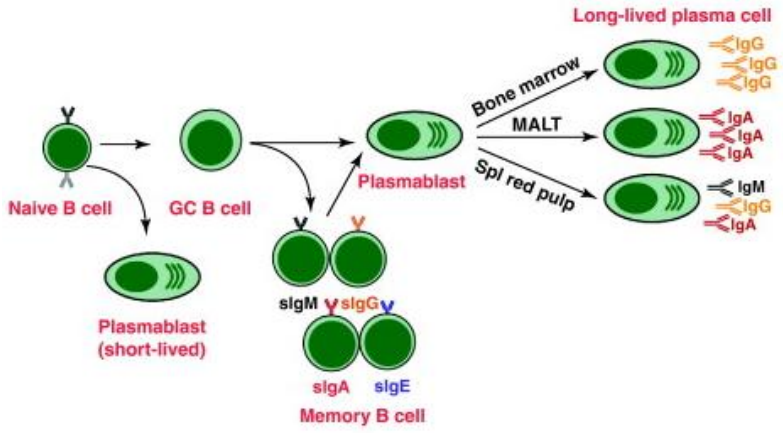

Fig. 8 B Cell activation: from immature B cell to plasma cell or memory B cell

3.6.3.4.1 T Cell-Independent Activation of B cells

Activation of B cells without the cooperation of helper T cells is referred to as **T cell-independent activation** and occurs when BCRs interact with T-independent antigens. T-independent antigens (e.g., polysaccharide capsules, lipopolysaccharide) have **repetitive epitope units** within their structure, and this repetition allows for the **cross-linkage** of multiple BCRs, providing the first signal for activation. Because T cells are not involved, the second signal has to come from other sources, such as interactions of **toll-like receptors** with **PAMPs** or interactions with factors from the complement system**.**

Once a B cell is activated, it undergoes clonal proliferation and daughter cells differentiate into plasma cells. **Plasma cells** are antibody factories that secrete large quantities of antibodies. After differentiation, the surface BCRs disappear and the plasma cell secretes **pentameric IgM** molecules that have the same antigen specificity as the BCRs (Figure 2). The T cell-independent response is short-lived and does not result in the

production of **memory B cells**. Thus it will not result in a secondary response to subsequent exposures to T-independent antigens.

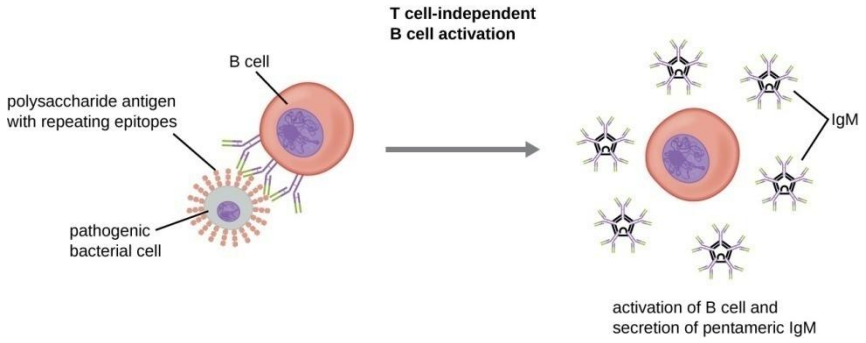

Fig. 9 T cell independent Activation of B cells

3.6.3.4.2 T Cell-Dependent Activation of B cells

T cell-dependent activation of B cells results in stronger immune response and develops memory. T cell-dependent activation can occur either in response to **free protein antigens** or to protein antigens associated with an intact pathogen. Interaction between the BCRs on a naïve mature B cell and a free protein antigen stimulate **internalization** of the antigen, whereas interaction with antigens associated with an intact pathogen initiates the extraction of the antigen from the pathogen before internalization. Once internalized inside the B cell, the protein antigen is processed and presented with **MHC II**. The presented antigen is then recognized by **helper T cells** specific to the same antigen. The TCR of the helper T cell recognizes the foreign antigen, and the T cell's **CD4** molecule interacts with MHC II on the B cell. The coordination between B cells and helper T cells that are specific to the same antigen is referred to as **linked recognition**.

Once activated by linked recognition, **T_H2 cells** produce and secrete **cytokines** that activate the B cell and cause proliferation into clonal daughter cells. After several rounds of proliferation, additional cytokines provided by the T_H2 cells stimulate the differentiation of activated B cell clones into **memory B cells**, which will quickly respond to subsequent exposures to the same protein epitope, and plasma cells that lose their membrane BCRs and initially secrete pentameric IgM.

After initial secretion of IgM, **cytokines** secreted by T_H2 cells stimulate the plasma cells to switch from IgM production to production of **IgG**, **IgA**, or **IgE**. This process, called **class switching** or **isotype switching**, allows **plasma cells** cloned from the same activated B cell to produce a variety of antibody classes with the same epitope specificity. Class switching is accomplished by **genetic rearrangement** of gene segments encoding the **constant region**, which determines an antibody's class. The **variable region** is not changed, so the new class of antibody retains the original epitope specificity.

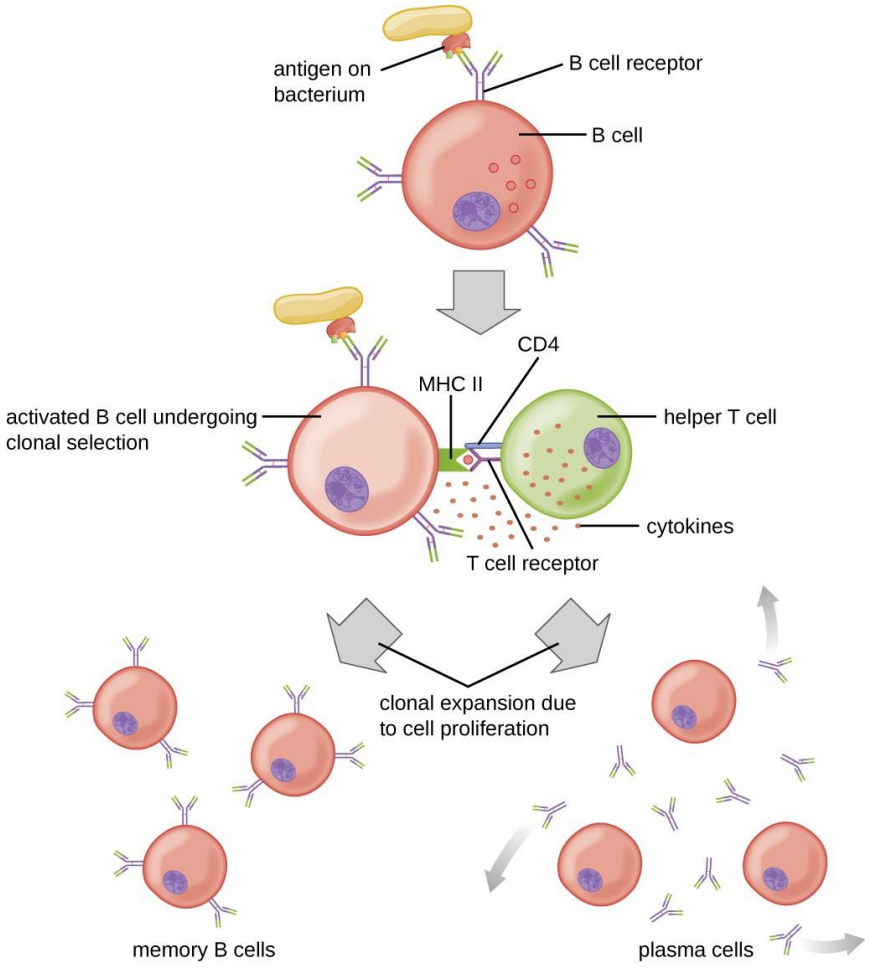

Fig. 10 T Cell Dependent Activation of B Cells

3.6.3.4.3 Subsets of B Cells

In general, three subsets of B cells derive from naive B cells, B-1 B cells, follicular B cells and marginal zone (MZ) B cells. Furthermore, B-1B cells form two subsets, B-1a and B-1 b B cells. All the subsets can be clearly identified on the basis of their surface markers. These surface markers can also be used to identify their progenitor populations. All these subsets of

B cells produce functionally important antibodies. However, they vary in huge terms in reference to their origin and function.

3.7 B-1 B cells

These cells have not been successfully studied in mammals, including humans. Thus, most of the findings are based on studies performed in mice. The most interesting finding from these studies is that although the progenitors of B-1 a and B-1 b cells are distinct, they are found to occupy the same locations, namely the pleural and peritoneal cavities.

3.8 Follicular B cells

Follicular B cells reside in two main niches during their circulation/recirculation through the bone marrow. Out of these, the "follicular niche" presents in the spleen/lymph nodes/Peyer's patches is the main site that is occupied by these cells. These "follicular sites" are thought to play important role in those immune responses against protein antigens, which are T cell-dependent. Most common type of B cell and, when not circulating through the blood, is found mainly in the lymphoid follicles of secondary lymphoid organs (SLOs). They are responsible for generating the majority of high-affinity antibodies during an infection. Interestingly, the follicular B cells residing in the bone marrow are involved in T cell-independent immune responses against microbial pathogens harboured by blood.

3.9 Marginal zone (MZ) B cells

MZ B cells mainly home near the marginal sinus of the spleen. These cells are mainly involved in T cell-independent immune responses against blood-borne microbes. It has also been reported that these MZ B cells can transport pathogens from the marginal sinus to the splenic follicles, sites where the follicular B cells reside.

3.40 T Cell Production and Maturation

T cells, like all other white blood cells involved in innate and adaptive immunity, are formed from multipotent **hematopoietic stem cells** (HSCs) in the bone marrow. However, T cells differentiate first into lymphoid stem cells that then become small, immature lymphocytes, sometimes called **lymphoblasts**. The first steps of differentiation occur in the red marrow of bones, after which immature T lymphocytes enter the bloodstream and travel to

the **thymus** for the final steps of maturation. Once in the thymus, the immature T lymphocytes are referred to as **thymocytes**.

The maturation of thymocytes within the thymus can be divided into three critical steps of positive and negative selection, collectively referred to as **thymic selection**. The first step of thymic selection occurs in the cortex of the thymus and involves the development of a functional **T-cell receptor (TCR)** that is required for activation by APCs. Thymocytes with defective TCRs are removed by negative selection through the induction of **apoptosis** (programmed controlled cell death). The second step of thymic selection also occurs in the cortex and involves the positive selection of thymocytes that will interact appropriately with MHC molecules. Thymocytes that can interact appropriately with MHC molecules receive a positive stimulation that moves them further through the process of maturation, whereas thymocytes that do not interact appropriately are not stimulated and are eliminated by **apoptosis**. The third and final step of thymic selection occurs in both the cortex and medulla and involves **negative selection** to remove **self-reacting thymocytes**, those that react to self-antigens, by apoptosis. This final step is sometimes referred to as **central tolerance** because it prevents self-reacting T cells from reaching the bloodstream and potentially causing **autoimmune disease**, which occurs when the immune system attacks healthy "self" cells.

Despite central tolerance, some self-reactive T cells generally escape the thymus and enter the peripheral bloodstream. Therefore, a second line of defense called **peripheral tolerance** is needed to protect against autoimmune disease. Peripheral tolerance involves mechanisms of **anergy** and inhibition of self-reactive T cells by **regulatory T cells**. Anergy refers to a state of nonresponsiveness to antigen stimulation. In the case of self-reactive T cells that escape the thymus, lack of an essential **co-stimulatory signal** required for activation causes anergy and prevents autoimmune activation. Regulatory T cells participate in peripheral tolerance by inhibiting the activation and function of self-reactive T cells and by secreting anti-inflammatory cytokines.

It is not completely understood what events specifically direct maturation of thymocytes into regulatory T cells. Current theories suggest the critical events may occur during the third step of thymic selection, when most self-reactive T cells are eliminated. Regulatory T cells may receive a unique signal that is below the threshold required to target them for negative

selection and apoptosis. Consequently, these cells continue to mature and then exit the thymus, armed to inhibit the activation of self-reactive T cells.

It has been estimated that the three steps of thymic selection eliminate 98% of thymocytes. The remaining 2% that exit the thymus migrate through the bloodstream and **lymphatic system** to sites of secondary lymphoid organs/tissues, such as the **lymph nodes**, **spleen**, and **tonsils**, where they await activation through the presentation of specific antigens by APCs. Until they are activated, they are known as **mature naïve T cells**.

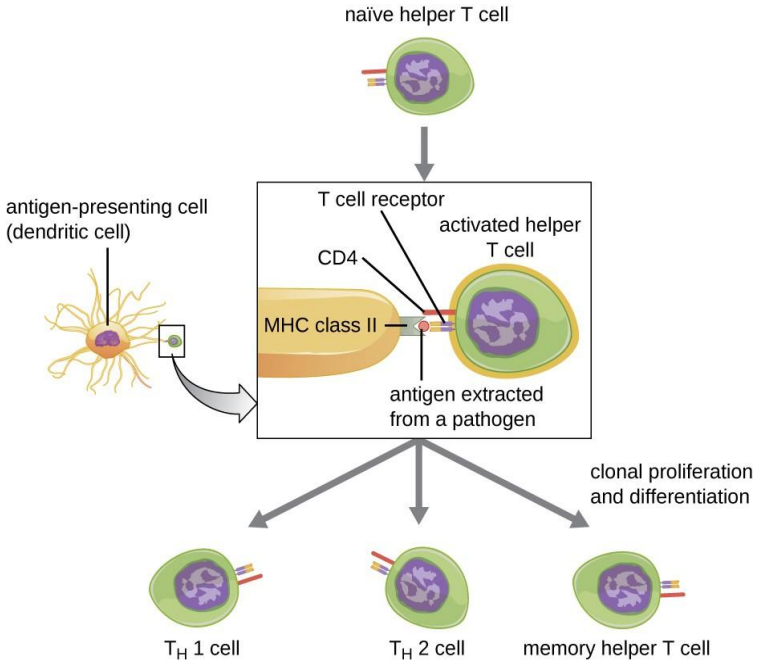

Fig. 11 Activation of T helper Cell

Activated helper T cells can differentiate into one of four distinct subtypes. The differentiation process is directed by APC-secreted **cytokines**. Depending on which APC-secreted cytokines interact with an activated helper T cell, the cell may differentiate into a T helper 1 (T_H1) cell, a T helper 2 (T_H2) cell, or a memory helper T cell. The two types of helper T cells are relatively short-lived **effector cells**, meaning that they perform various functions of the immediate immune response. In contrast, **memory helper T cells** are relatively long lived; they are programmed to "remember" a specific antigen or epitope in order to mount a rapid, strong, **secondary response** to subsequent exposures.

3.4.0.1. T_H1 cells secrete their own cytokines that are involved in stimulating and orchestrating other cells involved in adaptive and innate immunity. For example, they stimulate cytotoxic T cells, enhancing their killing of infected cells and promoting differentiation into **memory cytotoxic T cells**. T_H1 cells also stimulate **macrophages** and **neutrophils** to become more effective in their killing of intracellular bacteria. They can also stimulate **NK cells** to become more effective at killing target cells.

3.4.0.2. T_H2 cells play an important role in orchestrating the humoral immune response through their secretion of **cytokines** that activate **B cells** and direct B cell differentiation and **antibody production**. Various cytokines produced by T_H2 cells orchestrate **antibody class switching**, which allows B cells to switch between the production of IgM, IgG, IgA, and IgE as needed to carry out specific antibody functions and to provide pathogen-specific humoral immune responses.

3.4.0.3 A third subtype of **helper T cells** called **T_H17 cells** was discovered through observations that immunity to some infections is not associated with T_H1 or T_H2 cells. T_H17 cells and the cytokines they produce appear to be specifically responsible for the body's defense against chronic mucocutaneous infections. Patients who lack sufficient T_H17 cells in the mucosa (e.g., HIV patients) may be more susceptible to bacteremia and gastrointestinal infections.

3.4.1 Activation and differentiation of cytotoxic T Cells

Cytotoxic T cells (also referred to as **cytotoxic T lymphocytes**, or CTLs) are activated by APCs in a three-step process similar to that of helper T cells. The key difference is that the **activation of cytotoxic T cells** involves recognition of an antigen presented with MHC I (as opposed to MHC II) and interaction of CD8 (as opposed to CD4) with the receptor complex. After the successful co-recognition of foreign epitope and self-antigen, the production of **cytokines** by the APC and the cytotoxic T cell activate **clonal proliferation** and differentiation. Activated cytotoxic T cells can differentiate into effector cytotoxic T cells that target pathogens for destruction or **memory cells** that are ready to respond to subsequent exposures.

Once activated, cytotoxic T cells recognize infected cells through antigen presentation of pathogen-specific epitopes associated with **MHC I**. Once an infected cell is recognized, the TCR of the cytotoxic T cell binds to the epitope and releases **perforin** and **granzymes** that destroy the infected cell Perforin is a protein that creates pores in the target cell, and **granzymes** are proteases that enter the pores and induce **apoptosis**. This mechanism of **programmed cell death** is a controlled and efficient means of destroying and removing infected cells without releasing the pathogens inside to infect neighboring cells, as might occur if the infected cells were simply lysed.

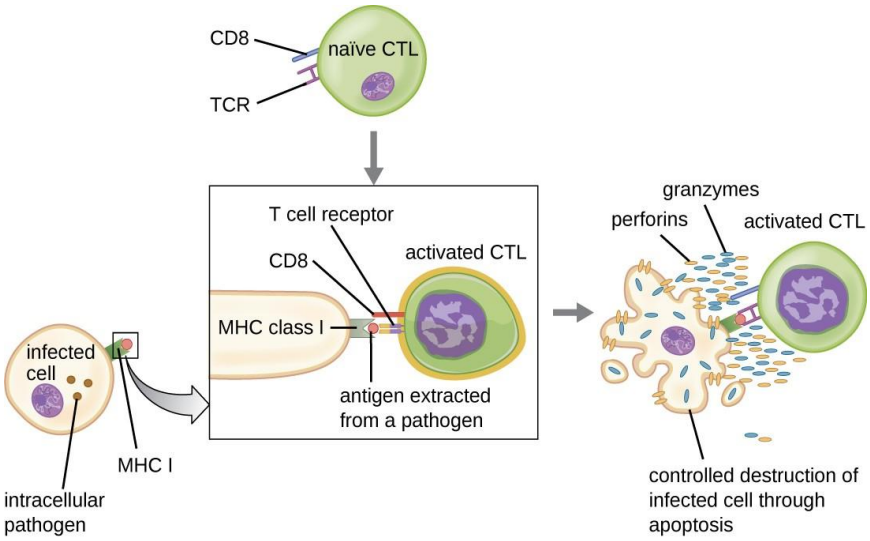

Fig. 12 Activation of Cytotoxic T cell

3.4.2 Organisation and Expression of Immunoglobulin Genes

B-lymphocytes of the immune system produce antibodies in the presence of antigen. The antibodies are formed from the assembly of some protein chains. For this, there must be millions of genes for each antibody. But how can it be possible because a mammalian genome does not contain more than about a million of genes, out of which only a fraction of genome directs the synthesis of antibodies. This clearly shows that neither the germ cells nor embryonic cells contain a complete set of all genes but have the basic genes which are shuffled during developmental stages of B-lymphocytes.

3.4.3 B Cell Development and Immunoglobulin Gene Rearrangement

During the development of B cells, the immunoglobulin gene undergoes sequences of rearrangements that lead to formation of the antibody repertoire. For example, in the lymphoid cell, a partial rearrangement of the heavy-chain gene occurs which is followed by complete rearrangement of heavy-chain gene. In germ line DNA, multiple gene segments encode a single immunoglobulin heavy or light chain. These gene segments are carried in the germ cells that cannot be transcribed and translated into heavy and light chains until these are arranged into functional genes. During the differentiation of B-cells in bone marrow these gene segments are randomly shuffled about 10^8 specificities by a dynamic genetic system.

This is maintained by germ line theory. Differentiation of B-cells from a progenitor B-cell to a mature cell involves an ordered progress in rearrangement of immunoglobin genes. When the process of B-cell division is over, a mature immuno-competent B-cell contains a single functional variable region DNA sequence for its heavy chain and a single functional variable region DNA sequence for its light chain. This has been confirmed through the recent evidences that a single variable region sequence specific for a particular antigen can be associated with the multiple sequences of C-region of heavy chains. It means that the different isotypes of antibodies (i.e. IgG, IgM) can be expressed having identical sequences of V-region.

3.4.3.1 Dryer and Bennett's Two Gene Model:

In 1965, W. Dryer and J. Bennett in their classical theoretical paper suggested for encoding of immunoglobulin chains. The two separate genes encode two different chains, one the light chain and the other heavy chain. They hypothesized that the two genes must come together and form a complete set of genes that can transcribe and translate the full message and can yield a single heavy or light protein chain.

Experimental evidence of gene rearrangement:

For the first time, **Hozumi** and **Tonegawa** (1976) provided the experimental evidence for the rearrangement of two separate genes encoding the V and C-regions of immunoglobulin during the course of differentiation of B-lymphocytes, and produce millions of antibodies. For this

novel work, Tonegawa was awarded Nobel Prize in 1987 in medicine and physiology. They used the newly developed Southern Blotting Technique. They took myeloma cells because they are like Plasma cells and produce large amount of single antibodies, and prepared radiolabelled RNA i.e. ^{32}p-mRNA for K-light and heavy chains, and also for constant chain. ^{32}P- mRNA was used as probe to test two kinds of cells, embryonic cells (that do not produce antibodies) and B-cells (produces antibodies).

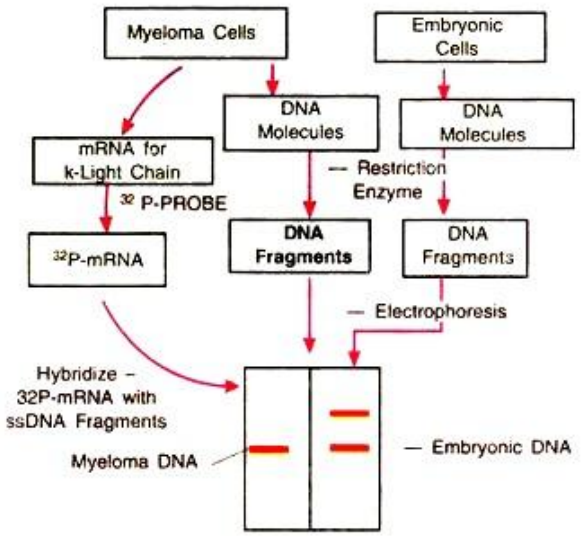

Fig. 22.15: Experimental demonstration for rearrangement of genes encoding k-light chain.

Fig. 13

The DNA of both myeloma cells and embryonic cells was treated with restriction enzymes and subjected to gel electrophoresis. The gel was then sliced; DNA fragments were eluted from the slice, denatured into single stranded DNA and finally incubated with ^{32}p-mRNA encoding K- light chain. The ^{32}p-mRNA probe hybridised with two bands from the germ line embryonic DNA, but with only a single band from the differentiated myeloma DNA. This clearly reveals that in the fully differentiated plasma cells, which is represented by the myeloma cells, the genes for V and C regions had gone rearrangement. Now they are present together on a single restriction DNA fragment, that is why the ^{32}P-mRNA probe hybridized with a single band only.

3.4.3.2 Multi-Gene Organization of Immunoglobulin Gene:

The result of Hozumi and Tonegawa (1976) are analogous to the theoretical two gene model of Dryer and Bennett (1965). This provides evidence for organization of multi-gene family into the immunoglobulin gene. In the embryonic cells the DNA encoding C-regions is far away from the DNA that encodes for V-region. In plasma cells (i.e. cells producing antibodies, also B-cells) and C and V-regions are together as in figure. The k and λ light chains and the heavy chains are encoded by separate multi-gene families situated on different chromosome, that contain a series of coding sequences which are known as gene segments. The k and λ light chain families contain L, V, J and C gene segments, whereas the heavy chain family contains L, V, D, S and C gene segments.

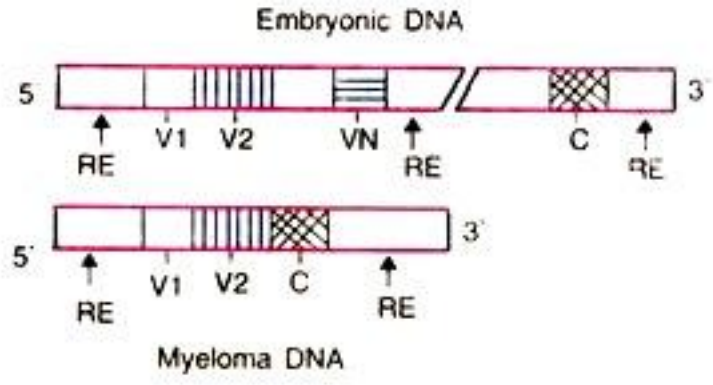

Fig. 14 Arrangement of gene segments of λ-light chain in embryonic and myeloma DNA molecules. RE, restriction enzyme

During the process of differentiation of B-cells, a long segment of DNA is deleted resulting in close rearrangement of V (i.e. V_2 segment to J-segment). The RNA transcript of immunoglobulin gene that contains intron (non-coding segment within the gene) is processed and correct transcript of mRNA is formed which is translated into a polypeptide light chain. The rearranged VJ-gene segments encode the V-region of the light chain, whereas VDI-gene segments encode the V-region of the heavy chain. The C-segments encode the C-region of the light or heavy chain of the gene segment encodes a short signal sequence. The signal sequence guides the light or heavy chain through endoplasmic reticulum but is broken before assembly of the immunoglobulin molecule. Therefore, the amino acids that correspond to L-gene segment do not appear in light or heavy chains.

3.4.3.3 Multi-gene families of λ-chain, K-chain and heavy chain:

For the first time Tonegawa (1983) gave the evidence that V-region of light chain is encoded by two gene segments (V_1 and V_2) by closing the germ-line gene encoding V-region of mouse λ light chain. The complete sequence of nucleotide was determined. When it was compared with known sequences of the λ-chain V-region, a discrepancy was found. In mouse λ-chain multi-gene family contains two V gene segment (V_1 and V_2), four J-gene segment (J_1, J_2, J_3 and J_4) and four C gene segment (C_1, C_2, C_3, C_4). The arrangement of the gene segments is shown in figure.

The gene segments, J_4 and C_4, are defective, therefore, called pseudogenes. A functional V-region of λ-chain gene consists of two coding segments i.e. exons (a V-gene segment and a J-segment) which are separated by a non-coding sequence (i.e. intron) in un-rearranged germ line DNA. In humans, there are an estimated 100 V-gene segments, 6 J-segment and 6 C-segments. In mouse, the k-chain gene family consists of about 300 V gene segments, five J-segment (one segment is pseudo gene) and a single C-gene segment. Unlike λ chain there are no sub classes of k-light chain as only one C-gene segment is found as shown in figure. In humans, the k-chain gene family consists of about 100 V-gene segments, 5J segments and a single C-segment.

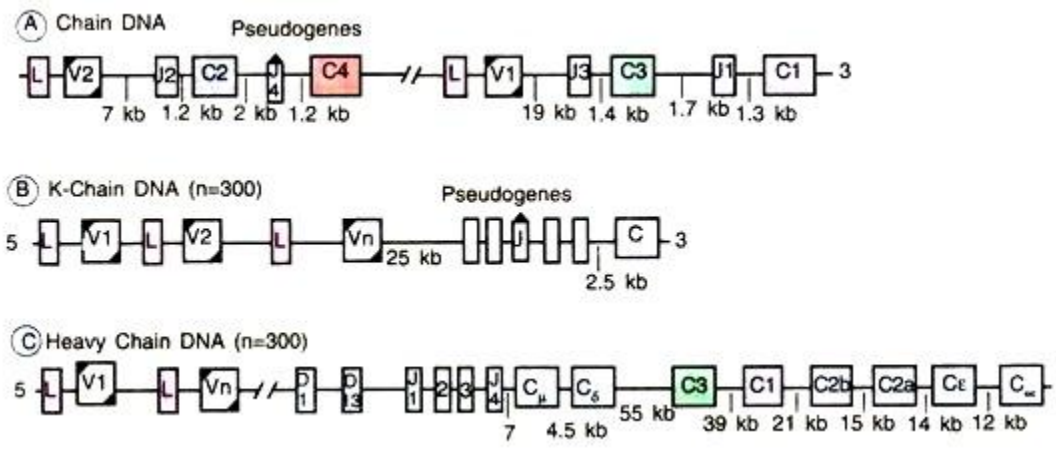

Fig. 15. Germ line organization of λ-light chain (A) , κ-light chain (B), and heavy chain (C) gene segment in the mouse.

Number of Antibodies possible through the Combinatorial Joining of Mouse Germ Line Genes

l light chains	V regions = 2 J regions = 3 Combinations = 2 × 3 = 6
κ light chains	V_κ regions = 250 – 350 Combinations = 250 × 4 = 1,000 = 350 × 4 = 1,400
Heavy chains	V_H = 250 – 1,000 D = 10 – 30 Combinations = 250 × 10 × 4 = 10,000 = 1,000 × 30 × 4 = 120,000
Diversity of antibodies	k-containing : 1,000 × 10,000 = 10^7 1,400 × 120,000 = 2 × 10^8 l-containing 6 × 10,000 = 6 × 10^4 6 × 120,000 = 7 × 10^5

In mouse the heavy chain multi-gene family of immunoglobulin is like λ and k chain but a little complex. It is located on chromosome 12. The heavy chain multi-gene family consists of about 200-1000 V-gene segments, 13 D-gene segments, four J-gene segments and a series of C-gene segments.

Each V-gene segment has a leader sequence a short distance upstream from it. The J-gene segments are located downstream from the D-gene segment. Each C-gene segment encodes the C-region of an immunoglobulin heavy chain iso-type. Similarly each C-gene segment encodes separate domain of the heavy chain C-region. In mouse C-gene segments are arranged in the order Cμ – Cδ – CY3 – CY1 – CY2b – CY2a – Cε – Cα.

3.4.4 Summary

Enzymes are essential for life and are one of the most important types of protein in the human body. Studying enzyme kinetics provides information about the diverse range of reactions in the human body, which we can use to understand and predict the metabolism of all living things. Enzyme kinetics involves the measurement of the rate at which chemical reactions that are catalyzed by enzymes occur. Knowledge about the kinetics of an enzyme can reveal useful information about its catalytic mechanism, role in metabolism, factors that impact its activity, and mechanisms of inhibition.

This unit will cover the basic principles of enzyme kinetics, including the reaction equation, rate of reaction and maximal velocity (V_{max}) and Michaelis Constant (K_m). Enzymes are

thought to form a complex with the substrates to catalyze the reaction. This process can be illustrated with the simplified equation, where e is the enzyme, S is the substrate, and P is the product: There are many practical uses of enzyme kinetics. For example, the kinetic constants can help explain how enzymes work and assist in the prediction of the behavior of enzymes in living organisms. V_{max} and K_m both play a key role in understanding the metabolism of the human body. Knowledge of the enzyme kinetic constants allows us to gain a better understanding of the enzymes and processes that take place in human metabolism. This knowledge could then be used for medical purposes to improve patient health outcomes.

Terminal questions

Q.1. What are the two basic observations made in the laboratory to study enzyme kinetics?

Answer:----------

Q.2. What is meant by saturation kinetics?

Answer:----------

Q.3. What is meant by saturation of the enzyme?

Answer:----------

Q.4. What is the chemical basis of enzyme specificity?

Answer:----------

Q.5. What is the Michaelis-Menten kinetic scheme and how does this explain generally the observed kinetics?

Answer:----------

Q.6. What is the chemical basis of enzyme catalysis?

Answer:----------

Further readings

1. Biochemistry- Lehninger A.L.
2. Biochemistry –J.H.Weil.
3. Biochemistry fourth edition-David Hames and Nigel Hooper.
4. Textbook of Biochemistry for Undergraduates - Rafi, M.D.
5. Biochemistry and molecular biology- Wilson Walker.

Unit-4: Mechanism of enzyme action and regulation

Structure

4.1 Introduction

Some enzymes are used commercially, for example, in the synthesis of antibiotics. Some household products use enzymes to speed up chemical reactions: enzymes in biological washing powders break down protein, starch or fat stains on clothes, and enzymes in meat tenderizer break down proteins into smaller molecules, making the meat easier to chew. By the late 17th and early 18th centuries, the digestion of meat by stomach secretions and the conversion of starch to sugars by plant extracts and saliva were known but the mechanisms by which these occurred had not been identified.

French chemist Anselme Payen was the first to discover an enzyme, diastase, in 1833. A few decades later, when studying the fermentation of sugar to alcohol by yeast, Louis Pasteur concluded that this fermentation was caused by a vital force contained within the yeast cells called "ferments", which were thought to function only within living organisms. He wrote that "alcoholic fermentation is an act correlated with the life and organization of the yeast cells, not with the death or putrefaction of the cells." In 1877, German physiologist Wilhelm Kühne (1837–1900) first used the term *enzyme*, which comes from Greek word"leavened" or "in yeast", to describe this process. The word *enzyme* was used

later to refer to nonliving substances such as pepsin, and the word *ferment* was used to refer to chemical activity produced by living organisms.

Eduard Buchner submitted his first paper on the study of yeast extracts in 1897. In a series of experiments at the University of Berlin, he found that sugar was fermented by yeast extracts even when there were no living yeast cells in the mixture. He named the enzyme that brought about the fermentation of sucrose "zymase". In 1907, he received the Nobel Prize in Chemistry for "his discovery of cell-free fermentation". Following Buchner's example, enzymes are usually named according to the reaction they carry out: the suffix *-ase* is combined with the name of the substrate (e.g., lactase is the enzyme that cleaves lactose) or to the type of reaction (e.g., DNA polymerase forms DNA polymers).

The biochemical identity of enzymes was still unknown in the early 1900s. Many scientists observed that enzymatic activity was associated with proteins, but others (such as Nobel laureate Richard Willstätter) argued that proteins were merely carriers for the true enzymes and that proteins *per se* were incapable of catalysis. In 1926, James B. Sumner showed that the enzyme urease was a pure protein and crystallized it; he did likewise for the enzyme catalase in 1937. The conclusion that pure proteins can be enzymes was definitively demonstrated by John Howard Northrop and Wendell Meredith Stanley, who worked on the digestive enzymes pepsin (1930), trypsin and chymotrypsin. These three scientists were awarded the 1946 Nobel Prize in Chemistry.

The discovery that enzymes could be crystallized eventually allowed their structures to be solved by x-ray crystallography. This was first done for lysozyme, an enzyme found in tears, saliva and egg whites that digests the coating of some bacteria; the structure was solved by a group led by David Chilton Phillips and published in 1965. This high-resolution structure of lysozyme marked the beginning of the field of structural biology and the effort to understand how enzymes work at an atomic level of detail.

4.2 Mechanism of enzyme action

Enzyme-catalyzed reactions occur in at least two steps. In the first step, an enzyme molecule (E) and the substrate molecule or molecules (S) collide and react to form an intermediate compound called the enzyme-substrate (E–S) complex. (This step is reversible because the complex can break apart into the original substrate or substrates and the free enzyme.) Once

the E–S complex forms, the enzyme is able to catalyze the formation of product (P), which is then released from the enzyme surface:

$$S+E \rightarrow E–S$$

$$E–S \rightarrow P+E$$

Hydrogen bonding and other electrostatic interactions hold the enzyme and substrate together in the complex. The structural features or functional groups on the enzyme that participate in these interactions are located in a cleft or pocket on the enzyme surface as in given figure. This pocket, where the enzyme combines with the substrate and transforms the substrate to product is called the active site of the enzyme.

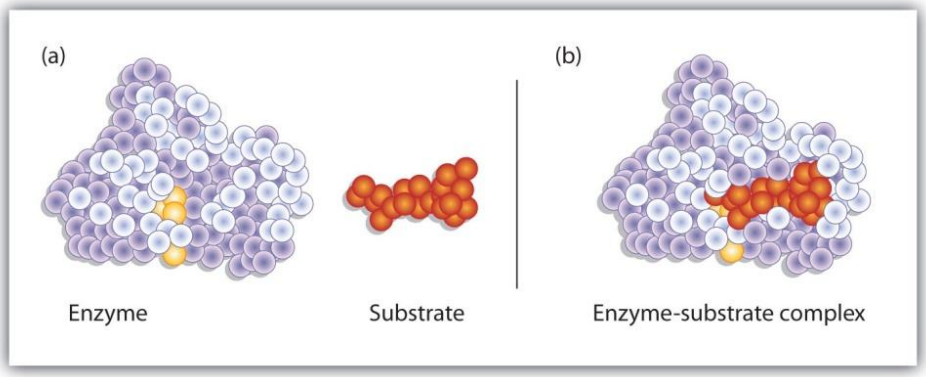

Fig. 1 Subs

trate Binding to the Active Site of an Enzyme. The enzyme dihydrofolate reductase is shown with one f its substrates: $NADP^+$ (a) unbound and (b) bound. The $NADP^+$ (shown in red) binds to a pocket that is complementary to it in shape and ionic properties.

The active site of an enzyme possesses a unique conformation (including correctly positioned bonding groups) that is complementary to the structure of the substrate, so that the enzyme and substrate molecules fit together in much the same manner as a key fits into a tumbler lock. In fact, an early model describing the formation of the enzyme-substrate complex was called the lock-and-key model as in given figure. This model portrayed the enzyme as conformationally rigid and able to bond only to substrates that exactly fit the active site.

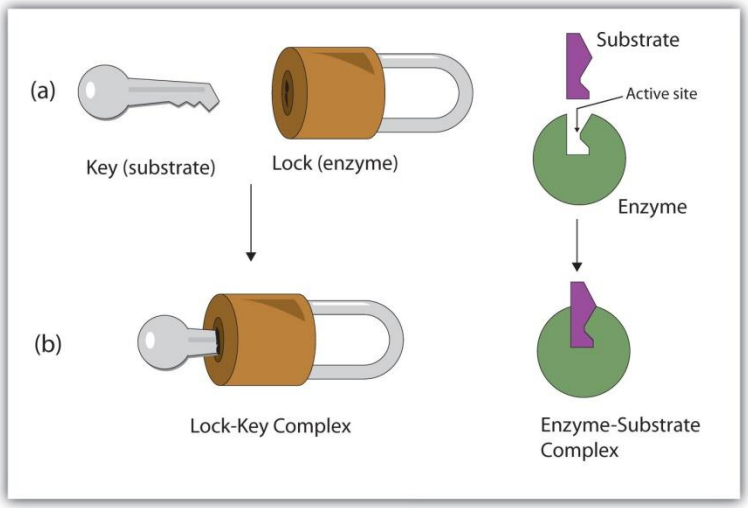

Fig. 2 The Lock-and-Key Model of Enzyme Action. (a) Because the substrate and the active site of the enzyme have complementary structures and bonding groups, they fit together as a key fits a lock. (b) The catalytic reaction occurs while the two are bonded together in the enzyme-substrate complex.

Working out the precise three-dimensional structures of numerous enzymes has enabled chemists to refine the original lock-and-key model of enzyme actions. They discovered that the binding of a substrate often leads to a large conformational change in the enzyme, as well as to changes in the structure of the substrate or substrates. The current theory, known as the induced-fit model, says that enzymes can undergo a change in conformation when they bind substrate molecules, and the active site has a shape complementary to that of the substrate only *after* the substrate is bound, as shown for hexokinase as in figure. After catalysis, the enzyme resumes its original structure.

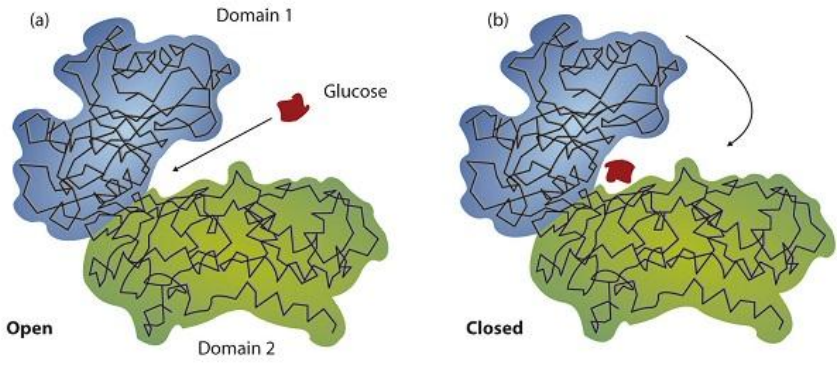

Fig. 3 The Induced-Fit Model of Enzyme Action. (a) The enzyme hexokinase without its substrate (glucose, shown in red) is bound to the active site. (b) The enzyme conformation changes dramatically when the substrate binds to it, resulting in additional interactions between hexokinase and glucose.

The structural changes that occur when an enzyme and a substrate join together bring specific parts of a substrate into alignment with specific parts of the enzyme's active site. Amino acid side chains in or near the binding site can then act as acid or base catalysts, provide binding sites for the transfer of functional groups from one substrate to another or aid in the rearrangement of a substrate. The participating amino acids, which are usually widely separated in the primary sequence of the protein, are brought close together in the active site as a result of the folding and bending of the polypeptide chain or chains when the protein acquires its tertiary and quaternary structure. Binding to enzymes brings reactants close to each other and aligns them properly, which has the same effect as increasing the concentration of the reacting compounds.

One characteristic that distinguishes an enzyme from all other types of catalysts is its *substrate specificity*. An inorganic acid such as sulfuric acid can be used to increase the reaction rates of many different reactions, such as the hydrolysis of disaccharides, polysaccharides, lipids, and proteins, with complete impartiality. In contrast, enzymes are much more specific. Some enzymes act on a single substrate, while other enzymes act on any of a group of related molecules containing a similar functional group or chemical bond. Some enzymes even distinguish between D- and L-stereoisomers, binding one stereoisomer but not the other. Urease, for example, is an enzyme that catalyzes the hydrolysis of a single substrate—urea—but not the closely related compounds methyl urea, thiourea, or biuret. The enzyme carboxypeptidase, on the other hand, is far less specific. It catalyzes the removal of nearly any amino acid from the carboxyl end of any peptide or protein as in given figure.

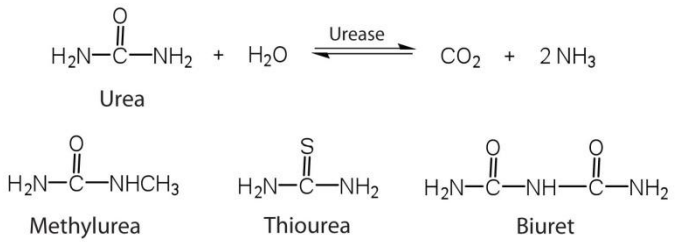

Fig. 4

Enzyme specificity results from the uniqueness of the active site in each different enzyme because of the identity, charge, and spatial orientation of the functional groups located there.

It regulates cell chemistry so that the proper reactions occur in the proper place at the proper time. Clearly, it is crucial to the proper functioning of the living cell.

In most chemical reactions, an energy barrier exists that must be overcome for the reaction to occur. This barrier prevents complex molecules such as proteins and nucleic acids from spontaneously degrading, and so is necessary for the preservation of life. When metabolic changes are required in a cell, however, certain of these complex molecules must be broken down, and this energy barrier must be surmounted. Heat could provide the additional needed energy (called activation energy), but the rise in temperature would kill the cell. The alternative is to lower the activation energy level through the use of a catalyst. This is the role that enzymes play. They react with the substrate to form an intermediate complex—a "transition state"—that requires less energy for the reaction to proceed. The unstable intermediate compound quickly breaks down to form reaction products, and the unchanged enzyme is free to react with other substrate molecules.

Only a certain region of the enzyme, called the active site, binds to the substrate. The active site is a groove or pocket formed by the folding pattern of the protein. This three-dimensional structure, together with the chemical and electrical properties of the amino acids and cofactors within the active site, permits only a particular substrate to bind to the site, thus determining the enzyme's specificity.

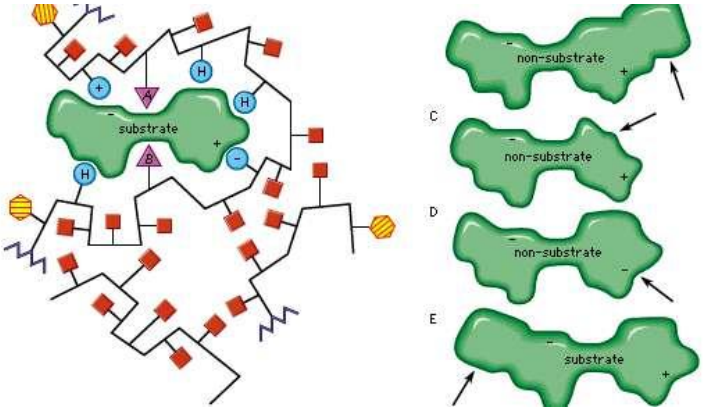

Fig. 5 Enzyme; active site, The active site of an enzyme is a groove or pocket that binds a specific substrate

Enzyme synthesis and activity also are influenced by genetic control and distribution in a cell. Some enzymes are not produced by certain cells, and others are formed only when required. Enzymes are not always found uniformly within a cell; often they are compartmentalized in the nucleus, on the cell membrane, or in subcellular structures. The rates of enzyme synthesis

and activity are further influenced by hormones, neurosecretions, and other chemicals that affect the cell's internal environment.

4.3 Active sites

In biology, the **active site** is the region of an enzyme where substrate molecules bind and undergo a chemical reaction. The active site consists of amino acid residues that form temporary bonds with the substrate (binding site) and residues that catalyse a reaction of that substrate (catalytic site). Although the active site occupies only ~10–20% of the volume of an enzyme, it is the most important part as it directly catalyzes the chemical reaction. It usually consists of three to four amino acids, while other amino acids within the protein are required to maintain the tertiary structure of the enzymes.

Each active site is evolved to be optimized to bind a particular substrate and catalyse a particular reaction, resulting in high specificity. This specificity is determined by the arrangement of amino acids within the active site and the structure of the substrates. Sometimes enzymes also need to bind with some cofactors to fulfil their function. The active site is usually a groove or pocket of the enzyme which can be located in a deep tunnel within the enzyme, or between the interfaces of multimeric enzymes. An active site can catalyse a reaction repeatedly as residues are not altered at the end of the reaction (they may change during the reaction, but are regenerated by the end). This process is achieved by lowering the activation energy of the reaction, so more substrates have enough energy to undergo reaction.

Usually, an enzyme molecule has only two active sites, and the active sites fit with one specific type of substrate. An active site contains a binding site that binds the substrate and orients it for catalysis. The orientation of the substrate and the close proximity between it and the active site is so important that in some cases the enzyme can still function properly even though all other parts are mutated and lose function.

Initially, the interaction between the active site and the substrate is non-covalent and transient. There are four important types of interaction that hold the substrate in a defined orientation and form an enzyme-substrate complex (ES complex): hydrogen bonds, van der Waals interactions, hydrophobic interactions and electrostatic force interactions. The charge distribution on the substrate and active site must be complementary, which means all positive and negative charges must be cancelled out. Otherwise, there will be a repulsive force pushing

them apart. The active site usually contains non-polar amino acids, although sometimes polar amino acids may also occur. The binding of substrate to the binding site requires at least three contact points in order to achieve stereo-, regio-, and enantioselectivity. For example, alcohol dehydrogenase which catalyses the transfer of a hydride ion from ethanol to NAD^+ interacts with the substrate methyl group, hydroxyl group and the pro-*(R)* hydrogen that will be abstracted during the reaction.

In order to exert their function, enzymes need to assume their correct protein fold (*native fold*) and tertiary structure. To maintain this defined three-dimensional structure, proteins rely on various types of interactions between their amino acid residues. If these interactions are interfered with, for example by extreme pH values, high temperature or high ion concentrations, this will cause the enzyme to denature and lose its catalytic activity.

A tighter fit between an active site and the substrate molecule is believed to increase the efficiency of a reaction. If the tightness between the active site of DNA polymerase and its substrate is increased, the fidelity, which means the correct rate of DNA replication will also increase. Most enzymes have deeply buried active sites, which can be accessed by a substrate via access channels.

4.4 Regulatory site

In biochemistry, allosteric regulation (or allosteric control) is the regulation of an enzyme by binding an effector molecule at a site other than the enzyme's active site. The site to which the effector binds is termed the *allosteric site* or *regulatory site*. Allosteric sites allow effectors to bind to the protein, often resulting in a conformational change involving protein dynamics. Effectors that enhance the protein's activity are referred to as *allosteric activators*, whereas those that decrease the protein's activity are called *allosteric inhibitors*.

Allosteric regulations are a natural example of control loops, such as feedback from downstream products or feedforward from upstream substrates. Long-range allostery is especially important in cell signaling. Allosteric regulation is also particularly important in the cell's ability to adjust enzyme activity. The term *allostery* comes from the Ancient Greek *allos*, "other", and *stereos*, "solid (object)". This is in reference to the fact that the regulatory site of an allosteric protein is physically distinct from its active site.

Many allosteric effects can be explained by the *concerted* MWC model put forth by Monod, Wyman, and Changeux, or by the sequential model (also known as the KNF model) described

by Koshland, Nemethy, and Filmer. Both postulate that protein subunits exist in one of two conformations, tensed (T) or relaxed (R), and that relaxed subunits bind substrate more readily than those in the tense state. The two models differ most in their assumptions about subunit interaction and the preexistence of both states. For proteins in which subunits exist in more than two conformations, the allostery landscape model described by Cuendet, Weinstein, and LeVine, can be used.

4.5 Concerted model

The concerted model of allostery, also referred to as the symmetry model or MWC model, postulates that enzyme subunits are connected in such a way that a conformational change in one subunit is necessarily conferred to all other subunits. Thus, all subunits must exist in the same conformation. The model further holds that, in the absence of any ligand (substrate or otherwise), the equilibrium favors one of the conformational states, T or R. The equilibrium can be shifted to the R or T state through the binding of one ligand (the allosteric effector or ligand) to a site that is different from the active site (the allosteric site).

4.6 Sequential model

The sequential model of allosteric regulation holds that subunits are not connected in such a way that a conformational change in one induces a similar change in the others. Thus, all enzyme subunits do not necessitate the same conformation. Moreover, the sequential model dictates that molecules of a substrate bind via an induced fit protocol. While such an induced fit converts a subunit from the tensed state to relaxed state, it does not propagate the conformational change to adjacent subunits. Instead, substrate-binding at one subunit only slightly alters the structure of other subunits so that their binding sites are more receptive to substrate. To summarize:

- Subunits need not exist in the same conformation
- Molecules of substrate bind via induced-fit protocol
- Conformational changes are not propagated to all subunits

4.7 Morpheein model

The morpheein model of allosteric regulation is a dissociative concerted model. A morpheein is a homo-oligomeric structure that can exist as an ensemble of physiologically significant and

functionally different alternate quaternary assemblies. Transitions between alternate morpheein assemblies involve oligomer dissociation, conformational change in the dissociated state, and reassembly to a different oligomer. The required oligomer disassembly step differentiates the morpheein model for allosteric regulation from the classic MWC and KNF models. Porphobilinogen synthase (PBGS) is the prototype morpheein.

Regulatory enzymes are characterized by increased or decreased activity in response to chemical signals. Metabolic pathways are regulated by controlling the activity of one or more enzymatic steps along that path. Regulatory control allows cells to meet changing demands for energy and biomolecules. Enzymatic activity is regulated in four general ways:

- Allosteric control. Allosteric enzymes have distinct binding sites for effector molecules which control their rates of reaction.
- Control proteins participate in cellular regulatory processes. For example, calmodulin is a Ca^{2+} binding protein which binds with high affinity and modulates the activity of many Ca^{2+}-regulated enzymes. In this way, Ca^{2+} acts as "second messenger" to allow crosstalk and feedback control in metabolic pathways.
- Enzyme activity controlled by reversible covalent modification is carried out with a variety of chemical groups. A common example is the transfer of phosphate groups, catalyzed by enzymes known as kinases. Glucose **metabolism** is modulated by phosphate transfer.
- Proteolytic activation. The above example is reversible in nature. Irreversible hydrolytic cleavage of peptide fragments from inactive forms of enzymes known as zymogens converts them into fully active forms. Proteolytic enzymes are controlled by this mechanism.

4.8 Chemical modification

The chemical modification of enzymes has played and will continue to play an important role in probing the mechanism of enzyme activity. This technique can be utilized for identification of those individual amino acid residues responsible for the catalytic properties of the entire protein. In chemical modification experiments, changes in enzymatic specificity have been

noted, but often not predicted. However, in recent years, rational approaches for the alteration of enzymatic properties have become feasible by means of site-specific mutagenesis and chemical methodology. In the first method, one amino acid can be replaced by a new one; in the second method, not only can new amino acid residues be introduced but also new catalytic entities (such as flavins) can be affixed to the protein molecule. Both methodologies are in their infancy, yet they represent a potentially powerful approach toward the design and synthesis of enzymes possessing new specificities.

Creative Enzymes has developed a series of services for all types of enzyme modifications. One of our highlighted services is chemical modifications of enzymes. The proper approaches and efficient operations guarantee extraordinary stability and catalytic efficiency. Creative Enzymes delivers excellent custom service for enzyme modifications using chemical approaches.

- Site-specific or residue-specific modifications
- Permanent modifications leading to consistency and stability in the long term
- Professional site analysis and chemical modifications
- Downstream sequencing and testing

4.8.1 Advantage of chemical modification

For decades, researchers have tried to develop more practical methods, and chemical modification is an important probe that has been used widely to study the enzyme structures. In chemical modification, a reagent is placed in contact with the enzyme and binds covalently to amino acid side-chains in the enzyme, which will produce changes in some measurable property (or properties) of the enzyme. This covalent derivatization is then correlated to the enzyme property under consideration, so that a function can be suggested for the modified residue. This technique can be utilized for identification of those individual amino acid residues responsible for the catalytic properties of the entire protein. Therefore, the method is also considerably important to stabilize the enzyme, through modifications to the key amino acids that determine the structure or function of the enzyme. In other cases, the surface of the enzyme could also be covalently linked to oligomers and polymers that may improve the stability, biodistribusion, and compatibility with the end-use environment. The chemical modification of enzymes has played and will continue playing an important role in probing enzymatic mechanisms and stabilizing enzymes.

4.9 Feedback inhibition

Feedback inhibition**,** in enzymology, suppression of the activity of an enzyme, participating in a sequence of reactions by which a substance is synthesized, by a product of that sequence. When the product accumulates in a cell beyond an optimal amount, its production is decreased by inhibition of an enzyme involved in its synthesis. After the product has been utilized or broken down and its concentration thus decreased, the inhibition is relaxed, and the formation of the product resumes. Such enzymes, whose ability to catalyze a reaction depends upon molecules other than their substrates (the ones upon which they act to form a product), are said to be under allosteric control. Feedback inhibition is a mechanism by which the concentration of certain cell constituents is limited. Feedback inhibition, in enzymology, suppression of the activity of an enzyme, participating in a sequence of reactions by which a substance is synthesized, by a product of that sequence. When the product accumulates in a cell beyond an optimal amount, its production is decreased by inhibition of an enzyme involved.

The intracellular concentrations of enzymes and other proteins are maintained at genetically predetermined "setpoints." Deviation of a concentration from its set point triggers events that can act at any point in a pathway to inhibit or promote synthesis of a protein, inactivate or activate an enzyme, promote or inhibit incorporation of a substance into a cell, etc. Feedback inhibition plays an important role in most regulatory processes. In feedback inhibition, the level of a variable is monitored, and a change in the positive direction triggers a response that counteracts the change. A simple example of feedback inhibition is a thermostat connected to a heater. A sensor detects the temperature in the room, and when the temperature reaches a predetermined set point, the thermostat signals the furnace to shut off. When the temperature drops below the set point, the inhibition is released, and the furnace is turned back on.

Feedback inhibition can be part of a more complex regulatory mechanism. The same thermostat can control both the heater and the air conditioner, for example, so that any deviation up or down triggers a change in the opposite direction. When the temperature reaches above the set point the air conditioner kicks in, and when it drops the furnace kicks in.

4.10 Control of a protein's concentration

The top figure shows a plot frame for concentration of a specific protein versus time. The dashed line indicates the setpoint level, that is, the concentration that has been genetically predetermined. The middle figure shows what we might observe if we were able to measure extremely minute variations in the concentration of protein x. The bottom figure illustrates the effect of this kind of regulation, namely to maintain the variable at a constant level.

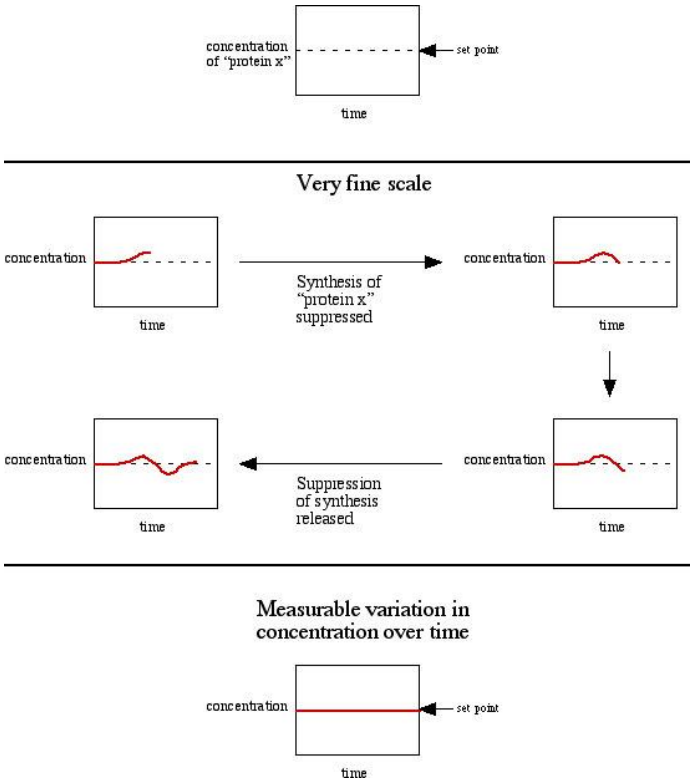

Fig. 6

4.10.1 Control of blood pressure

The regulation of blood pressure is highly complex, involving multiple mechanisms that act in both the short term and the long term. Here is a simple scheme in which two processes act together to exert control over blood pressure. Vasodilation and vaso constriction refer respectively to the expansion or narrowing of the diameter of the arterioles.

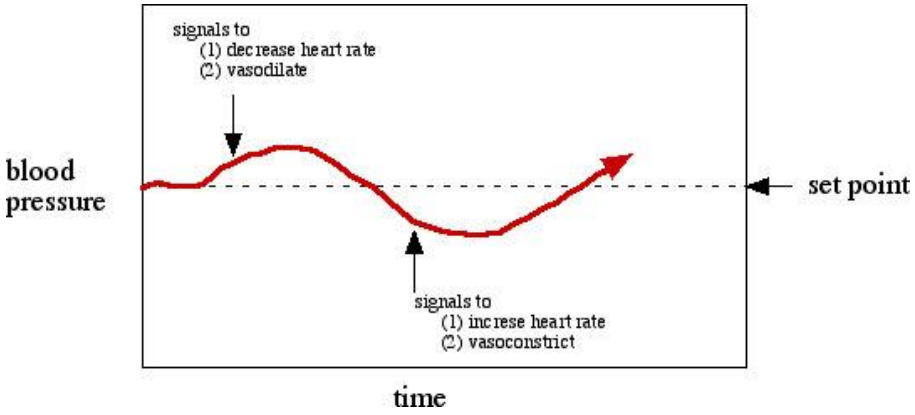

Fig. 7

In this example a change in blood pressure may result in initiation rather than inhibition of a process such as increased sympathetic nervous system activity which in turn causes vasoconstriction. Thus there is feedback control, but not every process involved in this regulation is necessarily feedback inhibition. With blood pressure we actually are able to measure changes, sometimes very dramatic changes in fact. The physiological condition of the organism determines the setpoint for blood pressure. Genes are involved, of course, but genes are not the sole determinants of the setpoint.

4.10.2 Positive feedback

Contrast feedback inhibition with *positive* feedback. In the latter, a change in a variable triggers mechanisms that move the variable *in the same direction*. If the thermostat was miswired so as to trigger the furnace when the temperature increased, then when the furnace was first turned on the thermostat would keep it on until the room became unbearably hot. Global catastrophes have resulted from positive feedback. For example, global warming once was completely out of hand here on Earth; on the other hand it has been postulated that during one era the crust of the planet completely froze, also due to a kind of positive feedback. The planet Venus is believed to be extremely hot due to a runaway greenhouse effect ("runaway" is a euphemism for positive feedback). Another example of positive feedback is malignant hypertension. In this condition, an increase in blood pressure triggers mechanisms that cause pressure to rise further. Malignant hypertension quickly leads to coma and death.

Some positive feedback mechanisms are incorporated into our physiology. For example, as the urinary bladder fills mechanoreceptors are stimulated, leading to contraction of the smooth

muscle of the bladder. As the bladder becomes progressively distended, the contractions increase and the feeling become more urgent. Runaway positive feedback doesn't occur, though. Contractions stop and start up again periodically. The smooth muscle of the uterus becomes more and more active at the end stages of pregnancy. Again, positive feedback is responsible. In both examples, relief is eventually necessary - urinate, or your bladder will rupture. I won't offend my female readers by suggesting that I have any idea what childbirth is like, but I imagine that when it is over it is a much greater relief than relieving one's bladder.

4.11 Cooperativity

Cooperativity**,** in enzymology, a phenomenon in which the shape of one subunit of an enzyme consisting of several subunits is altered by the substrate (the substance upon which an enzyme acts to form a product) or some other molecule so as to change the shape of a neighbouring subunit. The result is that the binding of a second substrate molecule to the second subunit of the enzyme differs in strength or velocity from that of the first, the third from the second, and so on. If the change in shape of the first subunit makes easier the binding of substrate to the second subunit, the effect is called positive cooperativity. In negative cooperativity, the binding of a molecule to the first subunit makes more difficult the binding of substrate to the second.

Allosteric control**,** in enzymology, inhibition or activation of an enzyme by a small regulatory molecule that interacts at a site (allosteric site) other than the active site (at which catalytic activity occurs). The interaction changes the shape of the enzyme so as to affect the formation at the active site of the usual complex between the enzyme and its substrate (the compound upon which it acts to form a product). As a result, the ability of the enzyme to catalyze a reaction is modified. This is the basis of the so-called induced-fit theory, which states that the binding of a substrate or some other molecule to an enzyme causes a change in the shape of the enzyme so as to enhance or inhibit its activity.

The regulatory molecule may be a product of a synthetic pathway and inhibit an enzyme in that pathway, thereby preventing the further formation of itself. Other molecules act as activators; *i.e.*, they interact with an enzyme so as to enhance the binding of the substrate to the enzyme, thus enhancing catalytic activity. The enzyme adenyl cyclase, itself activated by the hormone adrenaline (epinephrine), which is released when a mammal requires energy, catalyzes a reaction that results in the formation of the compound cyclic adenosine

monophosphate (cyclic AMP). Cyclic AMP, in turn, activates enzymes that metabolize carbohydrates for energy production. A combination of allosteric activation and inhibition thus provides a way by which the cell can rapidly regulate needed substances.

Cooperativity is a phenomenon displayed by systems involving identical or near-identical elements, which act dependently of each other, relative to a hypothetical standard non-interacting system in which the individual elements are acting independently. One manifestation of this is enzymes or receptors that have multiple binding sites where the affinity of the binding sites for a ligand is *apparently* increased, positive cooperativity, or decreased, negative cooperativity, upon the binding of a ligand to a binding site as in given figure. For example, when an oxygen atom binds to one of hemoglobin's four binding sites, the affinity to oxygen of the three remaining available binding sites increases; i.e. oxygen is more likely to bind to a hemoglobin bound to one oxygen than to an unbound hemoglobin. This is referred to as cooperative binding.

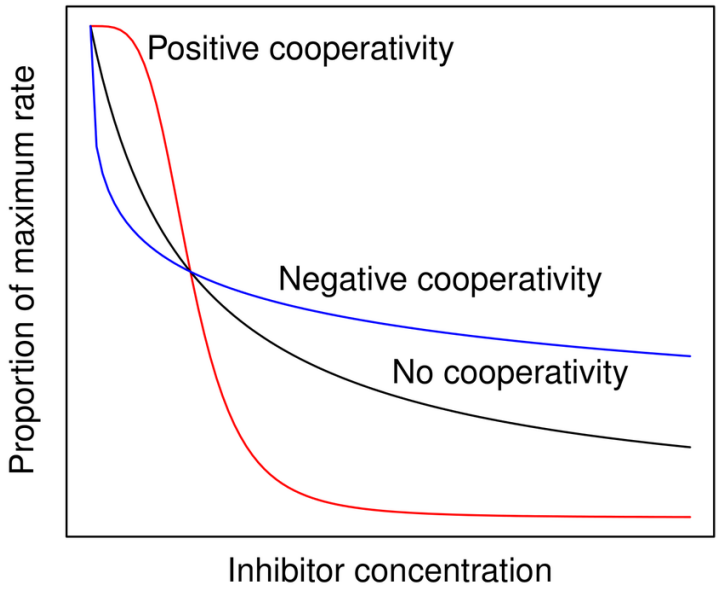

Fig. 8 Positive and negative cooperativity in inhibitor binding an enzyme.

We also see cooperativity in large chain molecules made of many identical (or nearly identical) subunits (such as DNA, proteins, and phospholipids), when such molecules undergo phase transitions such as melting, unfolding or unwinding. This is referred to as subunit cooperativity. However, the definition of cooperativity based on apparent increase or decrease in affinity to successive ligand binding steps is problematic, as the concept of "energy" must

always be defined relative to a standard state. When we say that the affinity is increased upon binding of one ligand, it is empirically unclear what we mean since a non-cooperative binding curve is required to rigorously define binding energy and hence also affinity. A much more general and useful definition of positive cooperativity is: A process involving multiple identical incremental steps, in which intermediate states are statistically *underrepresented* relative to a hypothetical standard system (null hypothesis) where the steps occur independently of each other.

Likewise, a definition of negative cooperativity would be a process involving multiple identical incremental steps, in which the intermediate states are over represented relative to a hypothetical standard state in which individual steps occur independently. These latter definitions for positive and negative cooperativity easily encompass all processes which we call "cooperative", including conformational transitions in large molecules (such as proteins) and even psychological phenomena of large numbers of people (which can act independently of each other, or in a co-operative fashion).

4.12 Allosteric enzymes

Allosteric enzymes are enzymes that change their conformational ensemble upon binding of an effector (allosteric modulator) which results in an apparent change in binding affinity at a different ligand binding site. This "action at a distance" through binding of one ligand affecting the binding of another at a distinctly different site, is the essence of the allosteric concept. Allostery plays a crucial role in many fundamental biological processes, including but not limited to cell signaling and the regulation of metabolism. Allosteric enzymes need not be oligomers as previously thought, and in fact many systems have demonstrated allostery within single enzymes. In biochemistry, allosteric regulation (or allosteric control) is the regulation of a protein by binding an effector molecule at a site other than the enzyme's active site. The site to which the effector binds is termed the *allosteric site*. Allosteric sites allow effectors to bind to the protein, often resulting in a conformational change involving protein dynamics. Effectors that enhance the protein's activity are referred to as *allosteric activators*, whereas those that decrease the protein's activity are called *allosteric inhibitors*.

Allosteric regulations are a natural example of control loops, such as feedback from downstream products or feedforward from upstream substrates. Long-range allostery is especially important in cell signaling. Allosteric regulation is also particularly important in

the cell's ability to adjust enzyme activity. The term *allostery* comes from the Greek *allos*, "other," and *stereos*, "solid (object)." This is in reference to the fact that the regulatory site of an allosteric protein is physically distinct from its active site. The protein catalyst (enzyme) may be part of a multi-subunit complex, and/or may transiently or permanently associate with a Cofactor (e.g. adenosine triphosphate). Catalysis of biochemical reactions is vital due to the very low reaction rates of the uncatalysed reactions. A key driver of protein evolution is the optimization of such catalytic activities via protein dynamics.

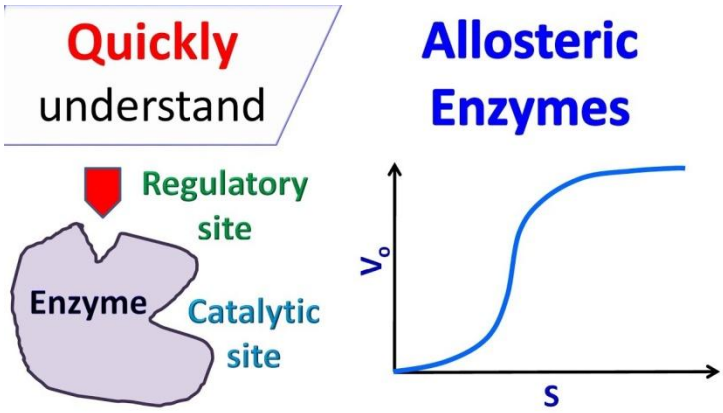

Fig. 9

Whereas enzymes without coupled domains/subunits display normal Michaelis-Menten kinetics, most allosteric enzymes have multiple coupled domains/subunits and show cooperative binding. Generally speaking, such cooperativity results in allosteric enzymes displaying a sigmoidal dependence on the concentration of their substrates in positively cooperative systems. This allows most allosteric enzymes to greatly vary catalytic output in response to small changes in effector concentration.

Effector molecules, which may be the substrate itself (homotropic effectors) or some other small molecule (heterotropic effector), may cause the enzyme to become more active or less active by redistributing the ensemble between the higher affinity and lower affinity states. The binding sites for heterotropic effectors, called allosteric sites, are usually separate from the active site yet thermodynamically coupled. Allosteric Database (ASD, http://mdl.shsmu.edu.cn/ASD) provides a central resource for the display, search and analysis of the structure, function and related annotation for allosteric molecules, including allosteric enzymes and their modulators. Each enzyme is annotated with detailed

description of allostery, biological process and related diseases, and each modulator with binding affinity, physicochemical properties and therapeutic area.

4.13 Kinetic Properties

Hemoglobin, though not an enzyme, is the canonical example of an allosteric protein molecule - and one of the earliest to have its crystal structure solved (by Max Perutz). More recently, the *E. coli* enzyme aspartate carbamoyltransferase (ATCase) has become another good example of allosteric regulation. The kinetic properties of allosteric enzymes are often explained in terms of a conformational change between a low-activity, low-affinity "tense" or T state and a high-activity, high-affinity "relaxed" or R state. These structurally distinct enzyme forms have been shown to exist in several known allosteric enzymes.

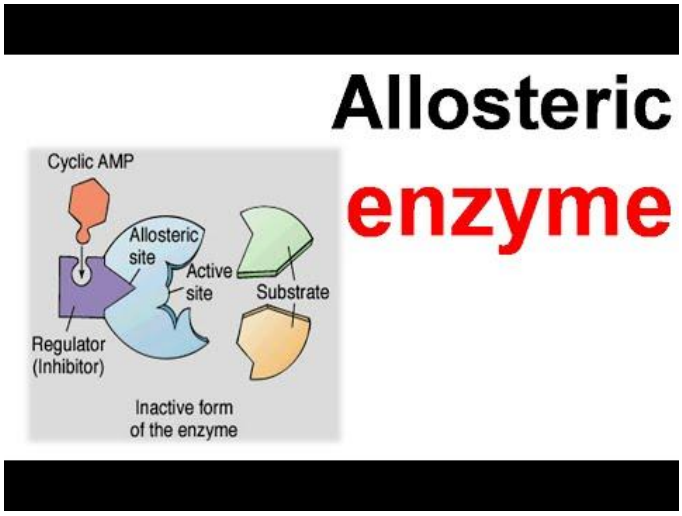

Fig. 10

However the molecular basis for conversion between the two states is not well understood. Two main models have been proposed to describe this mechanism: the "concerted model" of Monod, Wyman, and Changeux, and the "sequential model" of Koshland, Nemethy, and Filmer. In the concerted model, the protein is thought to have two “all-or-none” global states. This model is supported by positive cooperativity where binding of one ligand increases the ability of the enzyme to bind to more ligands. The model is not supported by negative cooperativity where losing one ligand makes it easier for the enzyme to lose more.

In the sequential model there are many different global conformational/energy states. Binding of one ligand changes the enzyme so it can bind more ligands more easily, i.e. every time it binds a ligand it wants to bind another one. Neither model fully explains allosteric binding, however. The recent combined use of physical techniques (for example, x-ray crystallography and solution small angle x-ray scattering or SAXS) and genetic techniques (site-directed mutagenesis or SDM) may improve our understanding of allostery.

4.14 Marker enzymes

Marker enzymes are enzymes, which are not ubiquitous but confined to a specific type of organelle, a subcomponent, or a cell. Since marker enzymes are not to be found just anywhere else, their detection can indicate the presence of the source whereas their absence means the lack of that source. Marker enzymes are one of the cell biomarkers used to characterize a cell type. They are also used in the isolation of the target cellular component. For example, succinate dehydrogenase is a marker enzyme for mitochondrion whereas the acid phosphatase for lysozome.

Enzymes are highly specialized complex proteins that aid chemical changes in every part of the body. For example, they help break down food so your body can use it effectively. They also help your blood clot. And they're present in every organ and cell in your body. Enzymes are necessary for your body to function properly. Enzyme markers are blood tests that analyze specific enzyme activity in the body. Some inherited diseases or conditions can cause these enzymes to stop working or be less efficient. Monitoring the rise or fall of enzyme levels can aid in the diagnosis of a variety of conditions. Your doctor can order a blood test for enzyme markers, or a routine blood test to help uncover abnormalities. In some cases, you may need to take a test multiple times over the course of several days to measure changes over time.

4.14.1 How are enzyme marker tests performed?

The test is a routine blood test that takes place in a laboratory. No fasting or special preparation is necessary. But tell your doctor before the test about all prescription and OTC medications and supplements you take. A blood test involves the following steps:

- A healthcare provider will use an antiseptic to clean a small area of your arm, usually the inside of your elbow or the back of your hand.

- They'll then wrap an elastic band around your upper arm to create pressure and make it easier to access a vein.
- They will insert a needle into your vein and blood will flow into a small vial. You'll likely feel the stick of the needle or a stinging sensation.
- After filling the vial, the healthcare provider will remove the elastic band and the needle.
- They will place a bandage over the puncture site and send the blood sample to a lab for analysis.
- The procedure should take only a few minutes.

4.14.2 What are the risks associated with enzyme marker tests?

Your arm may be sore at the puncture site, and you might have some mild bruising or brief throbbing. Most people have no serious or lasting side effects from a blood test. Rare complications include:

- bleeding
- lightheadedness
- fainting
- infection, which is a small risk whenever the skin is broken

4.1.5 Enzyme region

Enzyme**,** a substance that acts as a catalyst in living organisms, regulating the rate at which chemical reactions proceed without itself being altered in the process. The biological processes that occur within all living organisms are chemical reactions, and most are regulated by enzymes. Without enzymes, many of these reactions would not take place at a perceptible rate. Enzymes catalyze all aspects of cell metabolism. This includes the digestion of food, in which large nutrient molecules (such as proteins, carbohydrates, and fats) are broken down into smaller molecules; the conservation and transformation of chemical energy; and the construction of cellular macromolecules from smaller precursors. Many inherited human diseases, such as albinism and phenylketonuria, result from a deficiency of a particular enzyme.

4.1.6 Chemical nature

All enzymes were once thought to be proteins, but since the 1980s the catalytic ability of certain nucleic acids, called ribozymes (or catalytic RNAs), has been demonstrated, refuting this axiom. Because so little is yet known about the enzymatic functioning of RNA, this discussion will focus primarily on protein enzymes. A large protein enzyme molecule is composed of one or more amino acid chains called polypeptide chains. The amino acid sequence determines the characteristic folding patterns of the protein's structure, which is essential to enzyme specificity. If the enzyme is subjected to changes, such as fluctuations in temperature or pH, the protein structure may lose its integrity (denature) and its enzymatic ability. Denaturation is sometimes, but not always, reversible. Bound to some enzymes is an additional chemical component called a cofactor, which is a direct participant in the catalytic event and thus is required for enzymatic activity. A cofactor may be either a coenzyme—an organic molecule, such as a vitamin—or an inorganic metal ion; some enzymes require both. A cofactor may be either tightly or loosely bound to the enzyme. If tightly connected, the cofactor is referred to as a prosthetic group.

An enzyme will interact with only one type of substance or group of substances, called the substrate, to catalyze a certain kind of reaction. Because of this specificity, enzymes often have been named by adding the suffix "-ase" to the substrate's name (as in urease, which catalyzes the breakdown of urea). Not all enzymes have been named in this manner, however, and to ease the confusion surrounding enzyme nomenclature, a classification system has been developed based on the type of reaction the enzyme catalyzes. There are six principal categories and their reactions:

- Oxidoreductases, which are involved in electron transfer.
- Transferases, which transfer a chemical group from one substance to another.
- Hydrolases, which cleave the substrate by uptake of a water molecule (hydrolysis).
- Lyases, which form double bonds by adding or removing a chemical group.
- Isomerases, which transfer a group within a molecule to form an isomer.
- Ligases, or synthetases, which couple the formation of various chemical bonds to the breakdown of a pyrophosphate bond in adenosine triphosphate or a similar nucleotide.

4.1.6.1 Factors affecting enzyme activity

Because enzymes are not consumed in the reactions they catalyze and can be used over and over again, only a very small quantity of an enzyme is needed to catalyze a reaction. A typical enzyme molecule can convert 1,000 substrate molecules per second. The rate of an enzymatic reaction increases with increased substrate concentration, reaching maximum velocity when all active sites of the enzyme molecules are engaged. The enzyme is then said to be saturated, the rate of the reaction being determined by the speed at which the active sites can convert substrate to product. Enzyme activity can be inhibited in various ways. Competitive inhibition occurs when molecules very similar to the substrate molecules bind to the active site and prevent binding of the actual substrate. Penicillin, for example, is a competitive inhibitor that blocks the active site of an enzyme that many bacteria use to construct their cell walls.

Noncompetitive inhibition occurs when an inhibitor binds to the enzyme at a location other than the active site. In some cases of noncompetitive inhibition, the inhibitor is thought to bind to the enzyme in such a way as to physically block the normal active site. In other instances, the binding of the inhibitor is believed to change the shape of the enzyme molecule, thereby deforming its active site and preventing it from reacting with its substrate. This latter type of noncompetitive inhibition is called allosteric inhibition; the place where the inhibitor binds to the enzyme is called the allosteric site. Frequently, an end-product of a metabolic pathway serves as an allosteric inhibitor on an earlier enzyme of the pathway. This inhibition of an enzyme by a product of its pathway is a form of negative feedback.

Allosteric control can involve stimulation of enzyme action as well as inhibition. An activator molecule can be bound to an allosteric site and induce a reaction at the active site by changing its shape to fit a substrate that could not induce the change by itself. Common activators include hormones and the products of earlier enzymatic reactions. Allosteric stimulation and inhibition allow production of energy and materials by the cell when they are needed and inhibit production when the supply is adequate.

4.16.2 Stererio specificity of enzymes

A number of enzymes have the ability to discriminate between enantiomeric substrates or products; such enzymes are referred to as stereospecific/stereoselective enzymes. The substrate specificity of these enzymes are further sub-categorized according to the handedness

of the substrates they catalyse. For instance, the L-haloacid dehalogenase from *Pseudomonas putida* S3 which catalyses the stereospecific hydrolysis of only L-isomer of 2-haloacids. Such enzymes are unique and display chiral preferences in specificity i.e. stereospecificity in their catalysis. The ability of certain microorganisms to produce stereospecific enzyme stems from an evolutionary adaption towards the utilisation of chiral of substrates in their surrounding environment, which are essential for growth. Most compounds in nature are, in fact, chiral. Kinetically, enzyme stereospecificity is expressed as enantiomeric ratio (E), the ratio of specificity constants (K_{cat} /K_m) of the enzyme for the fast (reactive) and slow (non-reactive) enantiomers (Equation 1). Depending on the enzyme, the fast and slow enantiomers can be D- or L-form of the chiral substrate.

$$E = \frac{\left(K_{cat} / K_m\right)_{\text{fast enantiomer}}}{\left(K_{cat} / K_m\right)_{\text{slow enantiomer}}}$$

Where K_{cat} the maximum number amount of substrate the enzyme is can convert to product per catalytic site and per unit time. The K_m is the Michaelis-Menten constant and it is the substrate concentration when the reaction velocity is half the maximum velocity of the reaction. These constants are mathematically essential to illustrate the chirality of a particular enzyme. An enzyme that is not chiral would typically display an E value = 1, whilst stereospecific enzymes would have E values higher than 1.

Stereospecificity is one of the key properties of enzymes as biocatalysts. Stereospecific enzymes especially hydrolases, are useful in synthesis of pure enantiomers required for pharmaceuticals, agrochemicals and chiral intermediates in chemical industries. Although the molecular details of stereospecificity are not completely understood, many studies carried out by different research groups succeeded in enhancing and even in some cases reversing the stereospecificity of various enzymes using protein engineering. In this review, we focus on current understanding on enzymes stereospecificity, and success in protein engineering to improve stereospecificity.

4.16.3 Molecular basis for stereospecificity

Determining the molecular basis for stereospecificity of an enzyme requires the identification of the reacting orientations for both fastand slow-reacting substrate enantiomers. The differences between the two orientations along with the accompanying interactions essentially form the molecular basis for the stereospecificity of enzymes. Identifying the reacting

orientation for the fast-reacting enantiomer is straightforward; because the reacting atoms of the substrate are oriented in a way that they will best interact with the active site residues, whereas the non-reacting substrate moiety is positioned in the most fitted complementary pocket nearby. Hence, the enantiomeric excess of the correctly positioned or fast-reacting substrate would in turn, be higher than that of the slow-reacting counterpart. In such cases, the fast reacting substrate enantiomer that an enzyme preferentially catalyses would register a percentage that approaches 100. Conversely, due to possibilities of compromises, orienting the slow-reacting substrate enantiomer to is often less straightforward. In some instances, certain features in the fast-reacting enantiomer will clash with the slow-reacting enantiomer within the tight active site pocket, as the two enantiomers are mirror images of each other. Therefore the difficulty in identifying the molecular basis of enzyme stereospecificity emanated from orienting the slow-reacting enantiomer.

Literature has so far, proposed two approaches that are generally used to orient the slow-reacting enantiomer to interact with the catalytic residues of an enzyme. The first approach involves the fitting of the slow-reacting enantiomer by exchanging two substituents of the fast-reacting enantiomer. The process occurs by swapping the positions of any two of the four substituents that are attached to a chiral atom on the fast-reacting enantiomer to form the slow-reacting orientation, a substrate showing an absolute configuration opposite to that of the fast-reacting substrate. For clarity, absolute configuration defines the spatial arrangement of chiral molecule (group) and its stereochemistry. While the swapping of substituents preserves the location of the stereocenter, it also produces two mismatches between the exchanged substituents and the binding site. It is noteworthy to highlight here that preservation of the stereocenter is important for reactions that involve bond breaking and forming such as dehalogenation.

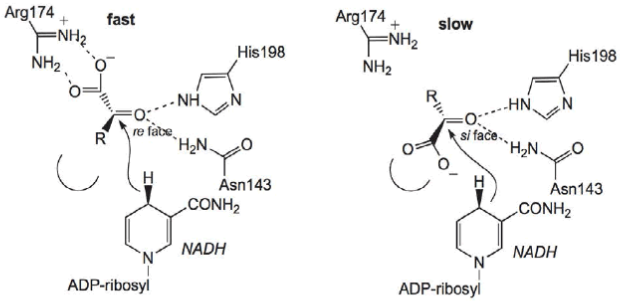

Figure 1: Structural representations of the two possible 2-ketocarboxylic orientations in the active site of L-HicDH from Lactobacillus confuses [11]. The two orientations differ by the position of R- group and carboxylate substituent. In both cases, the 2-carbonyl oxygen is positioned by H-bonds from His198 and Asn143. In the fast-reacting orientation, the carboxylate forms hydrogen bonds to Arg174 and the hydrophobic R-group sits in a hydrophobic pocket, represented by a semicircle. Whereas in the slow-reacting orientation the R-group is positioned near Arg174 and the carboxylate in the hydrophobic pocket, resulting in the two mismatches.

Fig.11

The second approach to orient the slow-reacting enantiomer is *via* an umbrella-like inversion. This approach involves changing the position of a single substituent (usually hydrogen) in the fast-reacting enantiomer to generate the slow-reacting enantiomer orientation. Umbrella-like inversion essentially occur by an inversion through the stereocenter hence, inverting the location of all the four substituents to generate the opposite substrate enantiomer. This is followed by a displacement that reverses the position of the substituents except the hydrogen, relative to their previous positions as in given figure. Therefore, the hydrogen substituent in the final orientation points to a new direction, and the stereocenter is slightly displaced from its original position. Unlike the substituents exchange approach, umbrella-like inversion generates orientation with lower energy, as the position alteration creates only one mismatch between the substituents and the binding site, rather than two mismatches in the orientation generated by the substituents exchange approach.

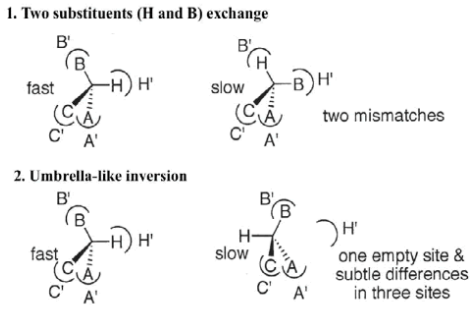

Figure 2: Schematic of the two ways by which the slow enantiomer can fit the optimum active site for the fast enantiomer [14]. All the interactions between the four binding sites (A', B', C' and H') and the chiral substituents (A, B, C and H) match in the fast enantiomer for both approaches. For the slow enantiomer (generated by exchanging two substituents, H and B), all the substituents point to the interacting site while only two interactions match. Umbrella-like inversion creates slow enantiomer with one empty binding site, one mismatch and subtle displacement of the stereocenter.

Fig.12

Re-orientation of a substrate via umbrella-like inversion often occurs in catalytic reactions that are executed adjacent to the stereocenter, and seldom occur in reactions that involve formation and breaking of bond at the stereocenter. X-ray structures of enantiomeric substrate configurations bound to various enzymes suggest umbrella-like inversion exist more commonly. For example, comparing the crystal structure of transition state of the fast- and slow-reacting enantiomers of menthol in the active site of *Candida rugosa* lipase, Cygler and colleagues showed the slow reacting enantiomer is oriented in an umbrella-like orientation.

4.16.4 Prediction of stereospecificity

By nature, the active sites of many enzymes are chiral; hence they specifically prefer specific enantiomer of chiral substrates and inhibitors. Therefore, substrate stereopreference needs to be considered when choosing a biocatalyst for reaction that involves specific substrate enantiomer. Based on X-ray crystal structures of enzyme-substrate complex and observed stereospecificity, generalisation was attempted in order to summarize the earlier results and to guide prediction of new substrate behaviours. These generalizations are either based on the substrate properties, in which case are referred to as ‘empirical rules, or based on the active site properties and are called ‘box models’.

The empirical rule model specifically focuses on the features of the chiral substituents such as shape and size. This is because the strongest destabilising intermolecular interaction involves steric clash between atoms, which is greatly influenced by the size and shape of the substituents. Other rules also consider polar and nonpolar feature of the substituents in predicting the stereospecificity. The simplest empirical rule only specifies the relative size of the chiral substituents (e.g. large, small or medium); hence it is known as size rule [18]. This rule predicts the stereospecificity of reducing ketones based on relative size of the two substituents adjacent to the carbonyl group as in given figure. The rule suggested that increasing the difference in size of the two substituents could enhance the stereospecificity.

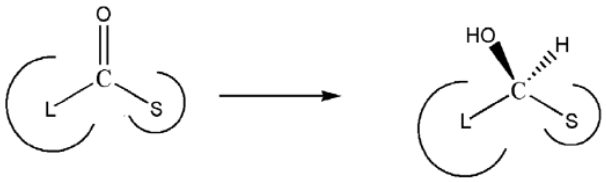

Figure 3: Size rule for predicting the stereospecificity for the reduction of carbonyl compounds. L represents large substituent e.g. pheny1. S represents small substituent e.g. methyl.

Fig. 13

4.17 Summary

The cells of our body are capable of making many different enzymes. In living systems hundreds of different enzyme catalysed reactions occur simultaneously. Regulation of enzyme activity is important to coordinate the different metabolic processes. It is also important to maintained cellular homeostasis i.e. to maintain the internal environment of the organism constant. These reactions must be regulated for the proper functioning of a living system. Regulatory enzymes exhibit increased or decreased catalytic activity in response to certain signals. An enzyme's catalytic activity can be directly controlled through structural alterations that influence the enzyme's substrate-binding affinity.

Enzyme activity may be turned "up" or "down" by activator and inhibitor molecules that bind specifically to the enzyme active site. The binding of an activator or inhibitor's is reversible. It means doesn't permanently attach to the enzyme. There to types of Inhibitors mostly affects enzyme activity: An inhibitor has binds to the active site of the enzymes and blocks the binding of the substrate is called competitive inhibition. As a result, competitive inhibition increase only the Km, leaving the Vmax the same. For example Inhibition of the enzyme succinate dehydrogenase by malonate and many other pharmaceutical drugs. The inhibitor doesn't block the substrate binding to the active site. Instead, it attaches at another site and blocks the enzyme activity. This inhibition is said to be "noncompetitive Inhibition. As a results Noncompetitive Inhibitors decreased Vmax but Do Not Affect Km For example , the action of pepstatin on enzyme renin.

Terminal questions

Q. 1 How activity of an enzyme is regulated by irreversible changes in covalent structure? Explain with example.

Answer:----------

Q. 2 Discuss the detail effect of substrate concentration on enzyme catalyzed reaction.

Answer:----------

Q. 3 What is positive co-operativity? Explain with suitable example.

Answer:----------

Q. **4** Define the terms:

(i) Apoenzyme (ii) Holoenzyme

(iii) Allosteric site (iv) Cofactor

Answer:----------

Q. 5 Study of pre-steady kinetics determines the mechanism of enzyme catalysis. Explain.

Answer:----------

Further readings

1. Biochemistry- Lehninger A.L.
2. Biochemistry –J.H.Weil.
3. Biochemistry fourth edition-David Hames and Nigel Hooper.
4. Textbook of Biochemistry for Undergraduates - Rafi, M.D.

5. Biochemistry and molecular biology- Wilson Walker.

Block- III

Industrial enzymes and purification

Unit-5
Industrial enzymes

Unit-6
Isolation and purification of enzymes

Introduction

This is the third block on Industrial enzymes and purification**.** It consists of following two units as given below:

Unit: 5 Industrial enzymes are enzymes that are commercially used in a variety of industries such as pharmaceuticals, chemical production, biofuels, food & beverage,

and consumer products. Due to advancements in recent years, biocatalysis through isolated enzymes is considered more economical than use of whole cells. Enzymes may be used as a unit operation within a process to generate a desired product, or may be the product of interest. Industrial biological catalysis through enzymes has experienced rapid growth in recent years due to their ability to operate at mild conditions, and exceptional chiral and positional specificity, things that traditional chemical processes lack. Isolated enzymes are typically used in hydrolytic and isomerization reactions. Whole cells are typically used when a reaction requires a co-factor. Although co-factors may be generated in vitro, it is typically more cost-effective to use metabolically active cells.

Despite their excellent catalytic capabilities, enzymes and their properties must be improved prior to industrial implementation in many cases. Some aspects of enzymes that must be improved prior to implementation are stability, activity, inhibition by reaction products, and selectivity towards non-natural substrates. This may be accomplished through immobilization of enzymes on a solid material, such as a porous support. Immobilization of enzymes greatly simplifies the recovery process, enhances process control, and reduces operational costs. Many immobilization techniques exist, such as adsorption, covalent binding, affinity, and entrapment. Ideal immobilization processes should not use highly toxic reagents in the immobilization technique to ensure stability of the enzymes. After immobilization is complete, the enzymes are introduced into a reaction vessel for biocatalysis.

Unit: 6 A varieties of enzyme purification services are available at creative Enzymes. We provide purification and quality analysis in small trial scales or large industrial scales from natural resources or production mixtures, for clinical, therapeutic, research, and chemical industries. Our services also cover the preliminary steps, as well as post-purification recovery and analysis. Although initial characterizations of an enzyme in a mixture or sample matrix is practical, such as activity measurement and preliminary quantification, more fundamental knowledge relies on more advanced studies of the

enzyme, which can only performed with pure enzyme samples. Pure enzymes also mean easier assays with less interference.

Some analysis methods, such as crystallography, are sensitive to sample purity and give desired results only with the highest samples purity. In large scale production for industrial applications, enzyme purification is directly related to product quality, in addition to regulatory requirements. Therefore, enzyme purification must be thoroughly considered and cautiously operated for both research and production purposes. However, the task is not straightforward.

Unit-5: Industrial enzymes

Structure

Objectives

5.1 Introduction

5.2 Industrial enzymes

5.3 Enzymes in detergent

5.4 Enzymes in food

5.5 Enzymes can serve as a natural alternative to chemical processing for food

5.6 Leather manufacturing

5.7 Enzymes in dairy

5.8 Enzymes in medicines

5.9 Application of Enzymes in Disease Diagnosis

5.9.1 Medicinal and clinical use

5.9.2 Industrial purpose

5.9.3 Enzymes as food and in food industry

5.9.4 Beverage industry

5.9.5 Ice Cream:-

5.9.6 Research:-

5.10 Enzymes used in chemical industry

5.10.1 Enzymatic Catalysis:

5.11 Industrial uses of enzymes

5.1 Introduction

Many factors could change the efficiency, the yield, and stability of activity during purification, and the effects of these factors vary largely from one enzyme to another. At creative Enzymes, we depend on the knowledgeable scientists and their years of experiences to design and perform the most suitable purification process for each enzyme. We understand that the customers may have different preferences on the purity, yield, and stability in different cases, and we will further customize the purification process to satisfy these needs. Almost all samples need to be prepared before the actual purification. For the enzymes from cell sources, they need to be fractionated into components before purification. The first step usually involves homogenization of cells, which disrupt the cell wall to release the enzyme into the homogenate, along with other components.

Depending on the cell type, the homogenization could be easy as in the case of mammalian tissue without rigid cell wall, or it may need harsher conditions such as abrasion, freezing, and high pressure due to the rigid cell wall of the plant tissue. Sometimes, additional hydrolytic enzymes or detergents are added for better extraction.

The mixture is then fractionated by centrifugation, yielding a dense pellet of heavy material at the bottom of the centrifuge tube and a lighter supernatant. The supernatant is again centrifuged at a greater force to yield yet another pellet and supernatant. The procedure, called differential centrifugation, yields several fractions of decreasing density, each still containing hundreds of different proteins, which are subsequently assayed for the activity being purified. Usually, one fraction will be enriched for such activity, and it then serves as the source of material to which more discriminating purification techniques are applied. The choice of temperature, pH, buffering salt, buffer strength, ionic strength, osmolarity, additives (EDTA, SDS, non-ionic detergents etc.), and homogenization technique are important of the success of purification.

Objectives

This is the third block on industrial enzymes and purification**.** It consists of following two units. Under first unit (industrial enzymes) we have following objectives. These are as under:

- To know different industrial enzymes
- To know various uses of enzymes.
- To know about application of enzymes in disease diagnosis
- To know about purification of enzymes
- To know about techniques of purification of enzymes
- To know enzyme engineering & enzyme immobilization

5.2 Industrial enzymes

Industrial enzymes are enzymes that are commercially used in a variety of industries such as pharmaceuticals, chemical production, biofuels, food & beverage, and consumer products. Due to advancements in recent years, biocatalysis through isolated enzymes is considered more economical than use of whole cells. Enzymes may be used as a unit operation within a process to generate a desired product, or may be the product of interest. Industrial biological catalysis through enzymes has experienced rapid growth in recent years due to their ability to operate at mild conditions, and exceptional chiral and positional specificity, things that traditional chemical processes lack. Isolated enzymes are typically used in hydrolytic and isomerization reactions. Whole cells are typically used when a reaction requires a co-factor. Although co-factors

may be generated in vitro, it is typically more cost-effective to use metabolically active cells.

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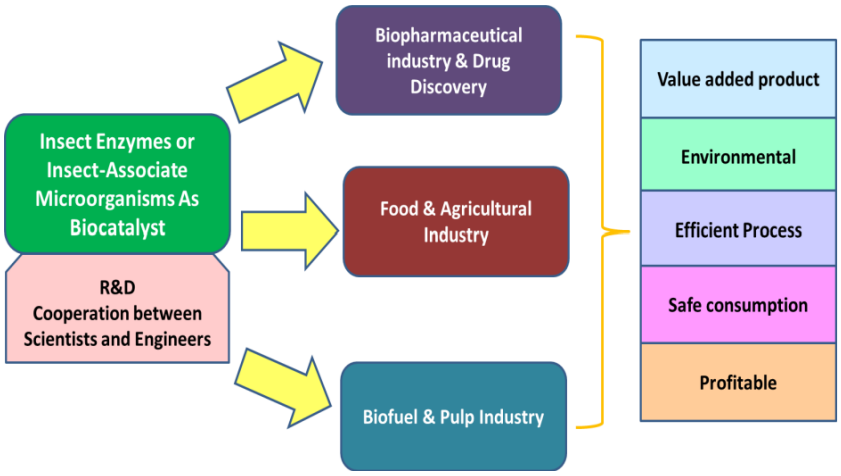

Fig. 1

Enzyme adsorption onto carriers functions based on chemical and physical phenomena such as van der Waals forces, ionic interactions, and hydrogen bonding. These forces are weak, and as a result, do not affect the structure of the enzyme. A wide variety of enzyme carriers may be used. Selection of a carrier is dependent upon the surface area, particle size, pore structure, and type of functional group. Many binding chemistries may be used to adhere an enzyme to a surface to varying degrees of success. The most successful covalent binding techniques include binding via glutaraldehyde to amino groups and N-hydroxysuccinide esters. These immobilization techniques occur at

ambient temperatures in mild conditions, which have limited potential to modify the structure and function of the enzyme.

Immobilization using affinity relies on the specificity of an enzyme to couple an affinity ligand to an enzyme to form a covalently bound enzyme-ligand complex. The complex is introduced into a support matrix for which the ligand has high binding affinity, and the enzyme is immobilized through ligand-support interactions. Immobilization using entrapment relies on trapping enzymes within gels or fibers, using non-covalent interactions. Characteristics that define a successful entrapping material include high surface area, uniform pore distribution, tunable pore size, and high adsorption capacity. Enzymes typically constitute a significant operational cost for industrial processes and in many cases, must be recovered and reused to ensure economic feasibility of a process.

Although some biocatalytic processes operate using organic solvents, the majority of processes occur in aqueous environments, improving the ease of separation. Most biocatalytic processes occur in batch, differentiating them from conventional chemical processes. As a result, typical bioprocesses employ a separation technique after bioconversion. In this case, product accumulation may cause inhibition of enzyme activity. Ongoing research is performed to develop *in situ* separation techniques, where product is removed from the batch during the conversion process. Enzyme separation may be accomplished through solid-liquid extraction techniques such as centrifugation or filtration, and the product-containing solution is fed downstream for product separation.

Enzymes are proteins that catalyze chemical reactions. As such, enzymes have been widely used to facilitate industrial processes and the production of products, and these enzymes are referenced as industrial enzymes. Although it has dated back to the ancient times when enzymes were used in baking, brewing, cheese making, etc., they were used either as spontaneously growing microorganisms or as added preparations such as calves' rumen or papaya fruit. Only during the past few decades, the development in recombinant DNA technology and advanced bioprocesses made it possible to produce enzymes as purified, well-characterized preparations on a large scale, allowing the wide application of enzymes in various industrial products and processes, such as

chemical, detergent, textile, food, animal-feed, leather, and pulp and paper industries. The latest developments in protein engineering and site-directed evolution have enabled us to tailor-make enzymes with new activities and/or for new process conditions.

This leads to a highly diversified industry that is currently growing both in terms of size and complexity. According to a report by Business Communications Co., the global market for industrial enzymes should increase from $2.2 billion in 2006 at a compound annual growth rate of 4% and will reach $2.7 billion by 2012. The technical enzyme applications include detergent, textile, pulp and paper, leather, and miscellaneous chemicals. New and emerging applications are helping drive demand for industrial enzymes, and the industry is responding with a continuous stream of innovative products.

Enzymes as a Unit Operation		
Enzyme	**Industry**	**Application**
Palatase	Food	Enhance cheese flavor
Lipozyme	Food	Interesterification of vegetable oil
Lipase	Pharmaceutical	Synthesis of chiral compounds
Lipopan	Food	Emulsifier
Cellulase	Biofuel	Class of enzymes that degrade cellulose to glucose monomers
Amylase	Food/biofuel	Class of enzymes that degrade starch to glucose monomers
Xylose isomerase	Food	High-fructose corn syrup production

Resinase	Paper	Pitch control in paper processing
Penicillin amidase	Pharmaceutical	Synthetic antibiotic production
Amidase	Chemical	Class of enzymes used for non-proteinogenic enantiomerically pure amino acid production

5.3 Enzymes in detergent

Detergent enzymes are biological enzymes that are used with detergents. They catalyze the reaction between stains and the water solution, thus aiding stain removal and improving efficiency. Laundry detergent enzymes are the largest application of industrial enzymes. Otto Röhm introduced the use of enzymes in detergent by using trypsin extracted from the tissues of slaughtered animals. Röhm's formula, though more successful than German household cleaning methods, was considered unstable when used with alkali and bleach. In 1959 yields were improved by microbial synthesis of proteases. Laundry enzymes must be able to function normally in a wide array of conditions: water temperatures ranging from 0 to 60 °C; alkaline and acidic environments; solutions with high ionic strength; and the presence of surfactants or oxidizing agents.

The five classes of enzymes found in laundry detergent include proteases, amylases, lipases, cellulases, and mannanases. They break down proteins (e.g. in blood and egg stains), starch, fats, cellulose (e.g. in vegetable puree), and mannans (e.g. in bean gum stains) respectively. For stain removal, conventional household washing machines use heated water, as this increases the solubility of stains. However, heating the water to the required temperature uses a considerable amount of energy; energy usage can be reduced by using detergent enzymes which perform well in cold water, allowing low-temperature washes and removing the need for heated water.

Clothes made of delicate materials such as wool and silk can be damaged in high-temperature washes, and jeans and denim can fade due to their dark dyes. Low-

temperature washes with detergent enzymes can prevent this damage, meaning that consumers can buy clothes from a wider range of materials without worrying about damaging them during washing.

5.4 Enzymes in food

Enzymes are proteins produced by all living organisms. They are biological catalysts which are responsible for all chemical reactions in nature. When your body wants to transform food such as starch in bread or pasta into energy enzymes are used to convert the starch to simple sugars which can be used by your cells. Enzymes are efficient and specific performing typically only one defined reaction over and over again. The fact that they come from nature means that they act at moderate pH and temperature conditions which make them sustainable alternatives to chemical processing in the food industry.

5.5 Enzymes can serve as a natural alternative to chemical processing for food

Enzymes have been used in food production for thousands of years. Our early ancestors discovered that cows stomach could turn milk into cheese. Today, we use enzymes in the manufacture of everything from bread, wine, beer, juice and dairy processing and much more besides. Enzymes are a natural way to keep bread softer for longer, they enable us to use local grains like cassava to make beer and make dairy products suitable for those with lactose intolerance.

Fig. 2

Lactose, the sugar found in dairy products, can cause problems like bloating and other gastrointestinal discomforts in people with lactose intolerance. Lactose intolerance

affects a significant amount of people worldwide, especially in places where dairy farming is not common. The incidence of lactose intolerance can be as high as 75% of the population in these areas.

Enzymes can help lactose intolerant individuals enjoy dairy products with minimal side effects. Lactose is a sugar made of two smaller sugars: galactose and glucose. Lactase is an enzyme that cleaves lactose into these two smaller sugars, neither of which cause the negative side effects of lactose in those with lactose intolerance. This is why you see the ingredient 'lactase' in lactose-free milks, for example.

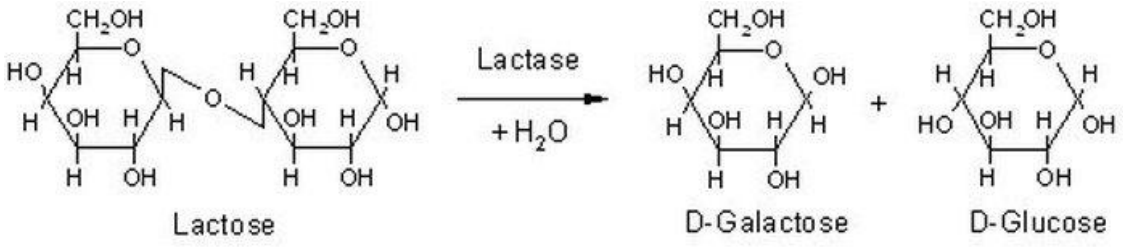

Fig. 3

The breakdown of the lactose sugar molecule gives glucose and galactose. These sugars have a greater relative sweetness than lactose meaning that lactose free or low-lactose products that have been made with the lactase enzyme are sweeter in taste than those not treated with lactase. In the food industry this can allow dairy products like yoghurt to be made with a reduced amount of added sugar but with the same taste profile.

5.6 Leather manufacturing

Leather industry uses proteolytic and lipolytic enzymes in leather processing. The use of these enzymes is associated with the structure of animal skin as a raw material. Enzymes are used to remove unwanted parts. Alkaline proteases are added in the soaking phase. This improves water uptake by the dry skins, removal and degradation of protein, dirt, and fats and reduces the processing time. In some cases, pancreatic trypsin is also used in this phase. Proteases are used in dehairing and dewooling of leather, and improve its quality (cleaner and stronger surface, softer leather, less spots). Lipases are used in this phase or in bating phase to specifically remove grease. The use of lipases is a fairly new development in leather industry.

The leather industry was historically considered noxious due to the leather-making process. The traditional procedure involved soaking animal hides in a mixture of urine and lime to remove unwanted hairs, flesh and fat, then kneading them in dog or pigeon feces with bare feet. The subsequent discharge and refuse disposal was severely hazardous to both human health and the environment because of the high amounts of concentrated sulfide and chromium in the effluence.

This method was eventually discarded by the industry in the early 20th century following Röhm's discovery, replaced by a more eco-friendly process involving detergent enzymes. Consequently, hazardous sodium sulfide (used to remove animal hair from hides) usage is lessened by 60%, while water usage for soaking and hair cutting is lowered by 25%. Additionally, toxic pollution and emissions have been reduced by 30%. These enzymes have never completely substituted the industrial chemicals. Nevertheless, the working conditions, wastewater quality, and processing times have been greatly improved.

Increased legislation has led to a limit on the laundry detergent industry's use of environmentally-unfriendly synthetic surfactants and phosphate salts. In a bid to produce more environmentally-friendly products, several detergent manufacturers have increased their use of enzymes in the production process in combination with lower concentrations of the sufactants and phosphates. These biologically active enzymes include bacteria, yeast, and mushrooms, which produce less chemical pollution and decompose certain toxicants. In contrast to the benefits of low-temperature washing, a study of the effects of detergent enzymes on untreated knit and woolen fabrics showed damage proportional to both soaking time and the enzyme concentration.

Consumers' responses to detergent enzymes have varied. It is reported that some Philippine consumers who are used to laundering by hand slightly suffered from powder detergents, which mainly consisted of laundry enzyme formulations. As a result, it was thought that laundry enzymes have the potential to increase the likelihood of getting occupational type 1 allergic responses. However, a large-scale skin prick test (SPT) containing 15,765 volunteers with 8 different types of detergent enzymes found that the allergy reaction is extremely rare among the public, with only 0.23% showing a reaction. The issue in Filipino consumers is believed to be the rushed hand-

laundering method. After various tests with several volunteers worldwide, it is found that exposure to laundry enzymes leads to neither skin allergy (Type I sensitization) nor skin erosion.

The global leather industry produced about 18 billion ft^2 leather in 2003, with an estimated value of about US$40 billion. Developing countries now produce over 60% of the world's leather needs. Conversion of raw hide into leather requires several mechanical and chemical operations involving use of many chemicals in an aqueous medium, including acids, alkalis, chromium salts, tannins, solvents, auxiliaries, surfactants, acids, metallorganic dyes, natural or synthetic tanning agents, sulfonated oils, and salts. The quantity of effluent generated is about 30 L/kg hide or skin processed. The total quantity of effluent discharged by Indian tanneries is about 50,000 m^3/day and contains high concentrations of organic pollutants.

Chromium is an important heavy metal used in leather, electroplating, and metallurgical industries. More than 170,000 tons of Cr wastes are discharged into the environment worldwide each year. In India in 1999 700,000 tons of wet salted hides and skins were processed in about 3000 tanneries, which discharged a total of 30 billion liters of waste water with a concentration of suspended solids between 3000–5000 mg/L and chromium as Cr^6 between 100–200 mg/L.

Reduction and recycle of the various streams at source appear to be a good approach to dramatically decrease the present quality and quantity of effluent generated by this industry. Disposal of the sludge after biochemical treatment also has not been satisfactorily solved. Use of enzymes and microorganisms for dehairing and stabilizing could reduce the use of toxic metals and chemicals. However, the leather industry needs major breakthroughs to achieve nontoxic discharge.

5.7 Enzymes in dairy

In the field of biotechnology, there are many industrial applications that result in biotech products that we use every day at home. Some of these are food science applications that utilize enzymes to produce or make improvements in the quality of different foods. In the dairy industry, some enzymes are required for the production of cheeses, yogurt, and

other dairy products, while others are used in a more specialized fashion to improve texture or flavor. Five of the more common types of enzymes and their role in the dairy industry are described below.

5.7.1 Rennet

Milk contains proteins, specifically caseins, that maintain its liquid form. Proteases are enzymes that are added to milk during cheese production, to hydrolyze caseins, specifically kappa casein, which stabilizes micelle formation preventing coagulation. Rennet and rennin are general terms for any enzyme used to coagulate milk. Technically rennet is also the term for the lining of a calf's fourth stomach. The most common enzyme isolated from rennet is chymosin. Chymosin can also be obtained from several other animals, microbial or vegetable sources, but indigenous microbial chymosin (from fungi or bacteria) is ineffective for making cheddar and other hard cheeses.

Limited supplies of calf rennet have prompted genetic engineering of microbial chymosin by cloning calf prochymosin genes into bacteria. Bioengineered chymosin may be involved in the production of up to 70% of cheese products. While the use of bioengineered enzymes spares the lives of calves, it presents ethics issues for those opposed to eating foods prepared with GEMs.

5.7.2 Lactalbumin and Lactoglobulin

Milk contains a number of different types of proteins, in addition to the caseins. Cow milk also contains whey proteins such as lactalbumin and lactoglobulin. The denaturing of these whey proteins, using proteases, results in a creamier yogurt product. Destruction of whey proteins is also essential for cheese production. During the production of soft cheeses, whey is separated from the milk after curdling and may be sold as a nutrient supplement for bodybuilding, weight loss, and lowing blood pressure, among other things. There have even been reports of dietary whey for cancer therapies, and having a role in the induction of insulin production for those with type 2 diabetes. Proteases are used to produce hydrolyzed whey protein, which is whey protein broken down into shorter polypeptide sequences. Hydrolyzed whey is less likely to cause allergic reactions and is used to prepare supplements for infant formulas and medical uses.

5.7.3 Lactase

Lactase is a glycoside hydrolase enzyme that cuts lactose into its constituent sugars, galactose, and glucose. Without sufficient production of lactase enzyme in the small intestine, humans become lactose intolerant, resulting in discomfort (cramps, gas, and diarrhea) in the digestive tract upon ingestion of milk products. Lactase is used commercially to prepare lactose-free products, particularly milk, for such individuals. It is also used in the preparation of ice cream, to make a creamier and sweeter tasting product. Lactase is usually prepared from *Kluyveromyces* sp. of yeast and *Aspergillus* sp. of fungi.

5.7.4 Catalase

The enzyme Catalase has found limited use in one particular area of cheese production. Hydrogen peroxide is a potent oxidizer and toxic to cells. It is used instead of pasteurization, when making certain cheeses such as Swiss, in order to preserve natural milk enzymes that are beneficial to the end product and flavor development of the cheese. These enzymes would be destroyed by the high heat of pasteurization. However, residues of hydrogen peroxide in the milk will inhibit the bacterial cultures that are required for the actual cheese production, so all traces of it must be removed. Catalase enzymes are typically obtained from bovine livers or microbial sources and are added to convert the hydrogen peroxide to water and molecular oxygen.

5.7.5 Lipases

Lipases are used to break down milk fats and give characteristic flavors to cheeses. Stronger flavored cheeses, for example, the Italian cheese, Romano, are prepared using lipases. The flavor comes from the free fatty acids produced when milk fats are hydrolyzed. Animal lipases are obtained from kid, calf, and lamb, while microbial lipase is derived by fermentation with the fungal species *Mucor meihei*. Although microbial lipases are available for cheese-making, they are less specific in what fats they hydrolyze, while the animal enzymes are more partial to short and medium-length fats. Hydrolysis of the shorter fats is preferred because it results in the desirable taste of many kinds of cheese. Hydrolysis of the longer chain fatty acids can result in either soapiness or no flavor at all.

5.8 Enzymes in medicines

Even before the inception of biotechnology, enzymes and proteins were used as drugs. Enzymes bind with their targets very specifically and with high affinity. They convert

multiple target molecules to the desired products by catalytic action, increasing the rate of chemical interconversions and metabolic processes. This property differentiates them from all other types of drugs. Biochemical diversity and the ease with which the enzyme concentration may be increased by environmental and genetic manipulation give the advantage of using enzymes of microbial origin. Enzymes that are used therapeutically have the great advantage of being economically viable and reliable. Due to high yields, they are easy for product modification and optimization. Digestive and metabolic enzymes can be used either alone or in permutation with other therapies for the treatment of a number of diseases such as leukemia, skin ulcers, cardiovascular diseases, Parkinson's disease, inflammation, digestive disorders, pancreatic disorders, etc. They are also in use in the diagnosis, biochemical investigation, and monitoring of many alarming diseases.

5.9 Application of Enzymes in Disease Diagnosis

Enzyme metabolism is a basic biological process, which is essential for the survival of all species. Their special function is to catalyze chemical reactions. Enzymes have a wide range of applications in improving the reaction rate close to equilibrium. Enzymes play a key role in the metabolic activities of all organisms, whether human, animal, plant or microbial, enzyme has a wide range of applications in microbial biotechnology and its diagnosis process. The abnormality of enzyme metabolism system leads to many metabolic diseases. Studies have shown that many diseases are related to many components of enzyme metabolism system, and have been widely used in clinical examination as a specific marker of disease.

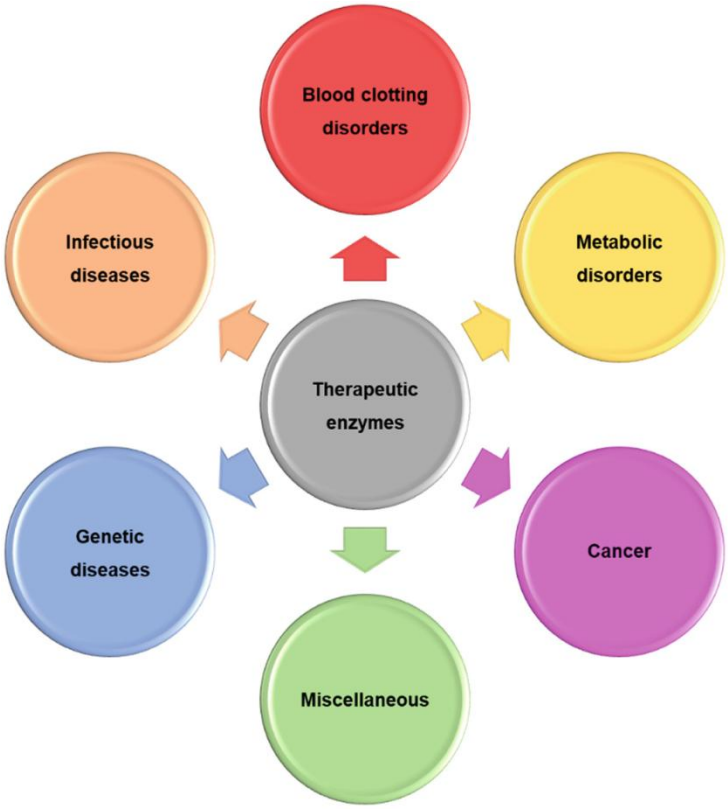

Fig. 4

Enzymes used as drugs have two important characteristics, which are different from traditional drugs. Firstly, unlike drugs they bind and act on their targets with great affinity. Secondly, they are highly specific and act as catalyst to convert multiple target molecules to the desired products. These two features make enzymes specific and potent drugs that can accomplish therapeutic biochemistry in the body that small molecule cannot. The catalytic activity of enzymes is exploited in industrial manufacturing of drugs. Enzymes are also used as digestive aid where they are used to supplement digestive enzymes like amylase, lipase, and protease. Almost all enzyme therapies developed till date deal with the genetic disorders. Also the enzyme replacement therapy is used for relatively rare, inborn error of metabolism. Several enzymes are also used to prevent and treat common diseases like heart attack and stroke. The enzyme collagenase has been reported for healing burn wound in children and enzyme chondroitinase ABC in the treatment of spinal cord injury.

It is often thought that enzyme transformations (or biocatalysis), is a new and emerging technology in the manufacture of small molecule active pharmaceutical ingredients (APIs). In fact a relatively large number of pharmaceuticals already on the market contain intermediates produced by biocatalysis. An explosion in the number and quantities of enzymes available to the synthetic organic chemist has made biocatalysis an increasingly attractive and viable manufacturing option. As always, cost of goods and process productivity are key drivers of adoption. In addition, sustainability and the adoption of greener and safer technologies are also clear factors influencing manufacturing route selection today and going forward. Biocatalysis is a green technology, and life cycle analysis shows that the use of recombinant technologies plays a major part in maximising the sustainability benefit of a biocatalysed process compared to a traditional chemical process. Uses of enzymes can be classified as:-

5.9.1 Medicinal and clinical use

- Enzymes can be used for Aiding Digestion. Example: Amylases, Proteases and Lipase.
- They can also be used as Deworming agents. Example: Papain.
- Enzymes act as anti-clotting agents like Fibrinolytic and Thrombolytic. Examples: Urokinase and Streptokinase.
- Enzymes can be used as surface disinfectants. Example: Trypsin.
- They can also be used in the diagnosis purpose. Example: Glucose oxidase along with peroxidase to detect the level of glucose (colorimetric estimation).

5.9.2 Industrial purpose

- Enzymes can be used in the textile industry. Example: Amylase as softening agent for starched clothes.
- They can also be used for Leather purpose. Example: Proteolytic purpose.
- Enzymes have the importance in the paper manufacturing. Examples: Endoxylanases for bleaching of Wood pulp.
- They can be used in the manufacturing of organic compounds. Example: Bacterial enzymes for the manufacturing of acetone, butanol, lactic acid etc.

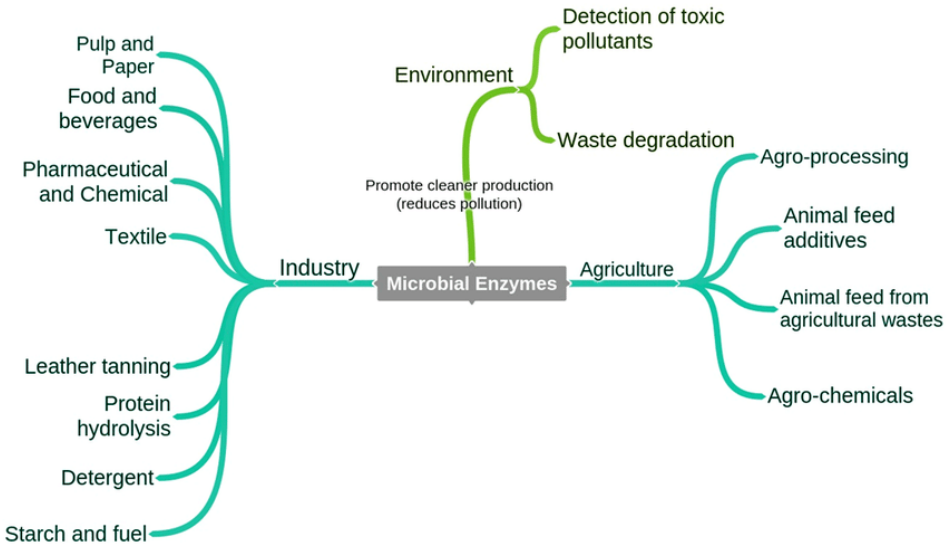

Fig. 5

5.9.3 Enzymes as food and in food industry

- Enzymes can be used in the meat packing industry. Example: Papain which is proteolytic in action, therefore hydrolyses peptide bonds thus for tenderizing meat and beef .
- Enzymes have their role in Manufacturing of cheese. Example: Rennin (chymosin) Found in stomach, converts milk protein casein to curd like calcium paracaseinate.

5.9.4 Beverage industry

- Papain is used to stabilize chill proof bear. Yeast enzymes are also used in beverage industry.

5.9.5 Ice Cream:-

- Lactose is used to prevent the formation of lactose crystals in ice-cream preparations.

5.9.6 Research:-

- Several enzymes are used for detection of biochemical reactions.

5.10 Enzymes used in chemical industry

Enzymes are biological molecules that catalyze (increase the rates of) chemical reactions.

- Since enzymes are selective for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell.
- Synthetic molecules called artificial enzymes also display enzyme-like catalysis.
- Enzymes are used in the chemical industry and other industrial applications when extremely specific catalysts are required.
- **Chemical reactions**: Processes that lead to the transformation of one set of chemical substances to another.
- **Rennin**: a proteolytic enzyme, obtained the gastric juice of the abomasum of calves, used to coagulate milk and make cheese
- **Enzymes**: Biological molecules that catalyze (i.e., increase the rates of) chemical reactions.
- **Synthetic**: The combination of two or more parts, whether by design or by natural processes. It may imply being prepared or made artificially, in contrast to naturally.

Enzymes are biological molecules that catalyze (increase the rates of) chemical reactions. In enzymatic reactions, the molecules at the beginning of the process, called substrates, are converted into different molecules, called products. Almost all chemical reactions in a biological cell need enzymes in order to occur at rates sufficient for life. Since enzymes are selective for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell.

5.10.1 Enzymatic Catalysis:

Like all catalysts, enzymes work by lowering the activation energy for a reaction, thus dramatically increasing the rate of the reaction. As a result, products are formed faster and reactions reach their equilibrium state more rapidly. Most enzyme reaction rates are millions of times faster than those of comparable un-catalyzed reactions. As with all catalysts, enzymes are not consumed by the reactions they catalyze, nor do they alter the equilibrium of these reactions. However, enzymes do differ from most other

catalysts in that they are highly specific for their substrates. A few RNA molecules called ribozymes also catalyze reactions, with an important example being some parts of the ribosome. Synthetic molecules, called artificial enzymes, also display enzyme-like catalysis.

Enzyme activity can be affected by other molecules. Inhibitors can decrease enzyme activity; activators can increase activity. Many drugs and poisons are enzyme inhibitors. Activity is also affected by temperature, pressure, chemical environment (e.g., pH), and substrate concentration. Some enzymes are used commercially; for example, in the synthesis of antibiotics. In addition, some household products use enzymes to speed up biochemical reactions (e.g., enzymes in biological washing powders break down protein or fat stains on clothes; enzymes in meat tenderizers break down proteins into smaller molecules, making the meat easier to chew).

Enzymes are used in the chemical industry and other industrial applications when extremely specific catalysts are required. However, enzymes in general are limited in the number of reactions they have evolved to catalyze, and by their lack of stability in organic solvents and at high temperatures. As a consequence, protein engineering is an active area of research and involves attempts to create new enzymes with novel properties, either through rational design or in vitro evolution. These efforts have begun to be successful, and a few enzymes have now been designed “from scratch” to catalyze reactions that do not occur in nature.

In food processing, the enzymes used include amylases from fungi and plants. These enzymes are used in the production of sugars from starch, such as in making high-fructose corn syrup. In baking, they catalyze the breakdown of starch in the flour to sugar. Yeast fermentation of sugar produces the carbon dioxide that raises the dough. Proteases are used by biscuit manufacturers to lower the protein level of flour. Trypsin is used to predigest baby foods. For the processing of fruit juices, cellulases and pectinases are used to clarify fruit juices. Papain is used to tenderize meat for cooking. In the dairy industry, rennin, derived from the stomachs of young ruminant animals (like calves and lambs) is used to manufacture of cheese, used to hydrolyze protein. Lipases are implemented during the production of Roquefort cheese to enhance the

ripening of the blue-mold cheese. Lactases are used to break down lactose to glucose and galactose.

In the brewing industry, enzymes from barley are released during the mashing stage of beer production. They degrade starch and proteins to produce simple sugar, amino acids, and peptides that are used by yeast for fermentation. Industrially-produced barley enzymes are widely used in the brewing process to substitute for the natural enzymes found in barley. Amylase, glucanases, and proteases are used to split polysaccharides and proteins in the malt. Betaglucanases and arabinoxylanases are used to improve the wort and beer filtration characteristics. Amyloglucosidase and pullulanases are used for low-calorie beer and adjustment of fermentability. Proteases are used to remove cloudiness produced during storage of beers.

In the starch industry, amylases, amyloglucosideases, and glucoamylases convert starch into glucose and various syrups. Glucose isomerase converts glucose into fructose in production of high-fructose syrups from starchy materials. In the paper industry, amylases, xylanases, cellulases, and ligninases are used to degrade starch to lower viscosity, aiding sizing and coating paper. In the biofuel industry, cellulases used to break down cellulose into sugars that can be fermented. In the production of biological detergents, proteases, produced in an extracellular form from bacteria, are used in pre-soak conditions and direct liquid applications, helping with the removal of protein stains from clothes. In molecular biology, restriction enzymes, DNA ligase, and polymerases are used to manipulate DNA in genetic engineering, important in pharmacology, agriculture and medicine, and are essential for restriction digestion and the polymerase chain reaction. Molecular biology is also important in forensic science.

5.11 Industrial uses of enzymes

Enzymes are used in the food, agricultural, cosmetic, and pharmaceutical industries to control and speed up reactions in order to quickly and accurately **obtain a valuable final product**. Enzymes are crucial to making cheese, brewing beer, baking bread, extracting fruit juice, tanning leather, and much more. The **industrial uses of enzymes** are also increasing since they are being used in the production of biofuels and

biopolymers. The enzymes can be harvested from microbial sources or can be made synthetically. Yeast and E. coli are commonly engineered to overexpress an enzyme of interest. This type of enzyme engineering is a **powerful way to obtain large amounts of enzyme for biocatalysis** in order to replace traditional chemical processes.

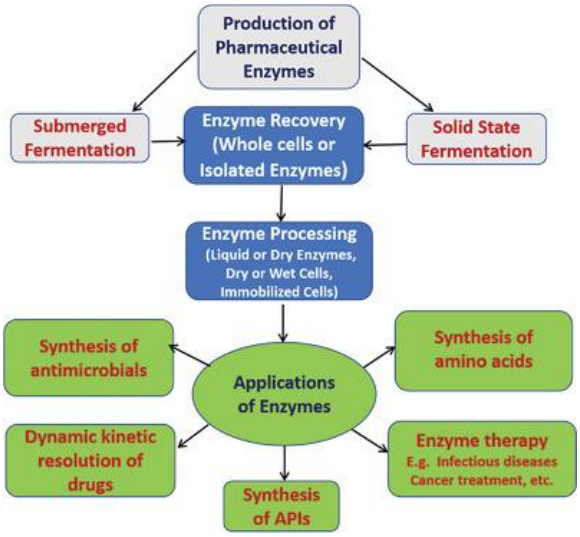

Fig. 6

Examples Breweries wouldn't be able to brew our beer without enzymes and the yeast that contain them. One of the first steps of the brewing process involves sprouting grain and breaking that starch into maltose and glucose sugar molecules via amylase enzymes. Yeast then consume these simple sugars and produce alcohol and carbon dioxide via glycolysis and alcoholic fermentation. These processes together require a whopping 12 enzymes! Using the whole yeast organism is much more efficient that trying to recreate this process with synthetic enzymes. The alcoholic fermentation process takes two pyruvate molecules from glycolysis and converts them to ethanol via pyruvate dehydrogenase and alcohol dehydrogenase. The production of cheese follows a similar process, but instead requires bacteria to perform glycolysis to convert the sugars in milk to the lactic acid that gives cheese and yogurt its exceptional flavor.

Enzymes are transforming the non-food industrial sectors to improve processes and decrease energy usage. For example acrylamide is made from acrylonitrile using nitrile

hydratase. The organism *Rhodococcus rhodochrous* J1 was directed to overexpress the enzyme nitrile hydratase. This enzyme efficiently converts acrylonitrile into acrylamide under mild conditions and offers an improvement over more traditional techniques. The conventional method of producing glycolic acid involved reacing formaldehyde with carbon monoxide over an acid catalyst at high temperature and pressure. Enzymes have offered a more mild alternative. E. Coli can be made to overexpress nitrilase, which, when combined with other enzymes such as lactoaldehyde reductase and lactoaldehyde dehydrogenase in a chain reaction provides an easier method for glycolic acid production.

5.12 Enzyme engineering

If whole organisms can't be used for an industrial process then it might require a particular enzyme structure and orientation**.** This is difficult to accomplish with traditional harvesting or chemical synthesis methods. Many times a specific enantiomer is required to improve the efficiency of a reaction, and it can be difficult to find a high proportion of a specific molecule in nature. However, with new directed evolution technologies it has become possible to develop designer enzymes by forcing mutations in the enzyme production processes of bacteria or yeast**.** These mutations sometimes produce an organism that is particularly useful for producing enzymes in industry**.** This process can improve organism and enzyme stability**,** substrate specificity, and enantioselectivity. Most industrial processes demand that an enzyme be highly specific to the substrate, and there is always room for improvement to the process.

5.13 Enzyme immobilization

Enzymes or 'biocatalysts' are remarkable discovery in the field of bioprocess technology. Biocatalysis has been widely accepted in diverse sectors owing to their ease of production, substrate specificity and green chemistry. However, for large extent commercialization of these bio-derived catalysts, their reusability factor becomes mandatory, failing which they would no longer be economic. Maintenance of their structural stability during any biochemical reaction is highly challenging. Consequently, immobilized enzymes with functional efficiency and enhanced

reproducibility are used as alternatives in spite of their expensiveness. Immobilized biocatalysts can either be enzymes or whole cells. Enzyme immobilization is confinement of enzyme to a phase (matrix/support) different from the one for substrates and products. Inert polymers and inorganic materials are usually used as carrier matrices. Apart from being affordable, an ideal matrix must encompass characteristics like inertness, physical strength, stability, regenerability, ability to increase enzyme specificity/activity and reduce product inhibition, nonspecific adsorption and microbial contamination. Immobilization generates continuous economic operations, automation, high investment/capacity ratio and recovery of product with greater purity.

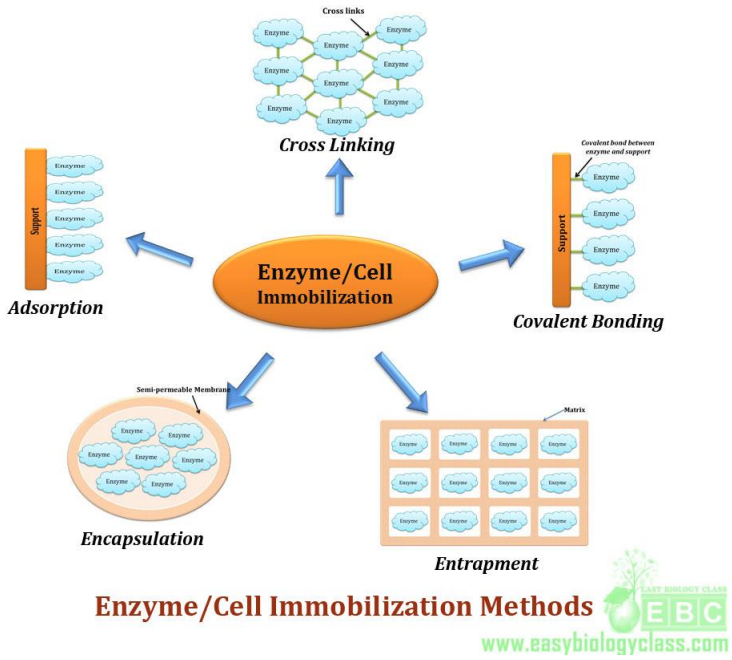

Fig. 7

Several methods are used for immobilization and various factors influence the performance of immobilized enzymes. Adsorption/carrier-binding method uses water-insoluble carriers such as polysaccharide derivatives, synthetic polymers and glass. In cross-linking/covalent method, bi/multifunctional reagents such as glutaraldehyde, bisdiazobenzidine and hexamethylene diisocyanate are used. Polymers like collagen, cellulose and κ-carrageenan are employed by entrapment method, while the membrane confinement method includes formulation of liposomes and microcapsules

Enzyme immobilization technology is one of the key modern industrial biotechnologies. Since the commercial use of first immobilized enzymes in the 1960s, enzyme immobilization technologies and theories as well as immobilization materials and chemistry have gained rapid development. Nowadays, the design of the immobilized enzymes, which suits different specific applications, has abandoned the traditional trial-and-error approach and gradually transited to the rational design, which is characterized by the fact that the enzyme immobilization technology is now used not only to realize the reuse of the costly enzymes a better control of the process, but also to improve the enzyme catalytic functions such as activity, stability, and selectivity. To some extent, the enzyme immobilization technology is becoming a complimentary technology to the genetic engineering.

Currently, it is becoming increasingly appreciated that the availability of a robust immobilized enzyme in the early stage of process development will definitively enable early insight into process development and save costs not only in process development but also in production. However, the lack of guidelines for selection of the method of immobilization and the performance to be expected of an immobilized enzyme for a specific application seriously hampers application of a rational approach to the design of such robust immobilized enzymes. In this context, the article systematically delineates the basic principles governing the individual approaches in the design of robust enzymes, which can be classified into the following four approaches:

- Rational versus trial–error,
- Diversity versus versatility,
- Complimentary versus alternative, and
- Modification versus immobilization approach. Moreover, the article attempts to provide a rational basis for future development of immobilized enzymes.

5.14 Factors influencing performance of immobilized enzymes

Factors	**Implications of immobilization**
Hydrophobic partition	Enhancement of reaction rate of hydrophobic substrate

Microenvironment of carrier	Hydrophobic nature stabilizes enzyme
Multipoint attachment of carrier	Enhancement of enzyme thermal stability
Spacer or arm of various types of immobilized enzymes	Prevents enzyme deactivation
Diffusion constraints	Enzyme activity decreases and stability increases
Presence of substrates or inhibitors	Higher activity retention
Physical post-treatments	Improvement of enzyme performance
Different binding mode	Activity and stability can be affected
Physical structure of the carrier such as pore size	Activity retention was often pore-size dependent
Physical nature of the carrier	Carriers with large pore size mitigate diffusion limitation, leading to higher activity retention

5.15 Different techniques used for immobilization

5.15.1 Adsorption

Enzyme adsorption results from hydrophobic interactions and salt linkages where either the support is bathed in enzyme for physical adsorption or the enzyme is dried on electrode surfaces. Adsorbed enzymes are shielded from aggregation, proteolysis and interaction with hydrophobic interfaces. Researchers have used eco-friendly supports like coconut fibers having good water-holding capacity and high cation exchange property; microcrystalline cellulose with irreversible binding capacity; kaolin with high enzyme retainability by chemical acetylation; and micro/mesoporous materials having thiol functionalized, large surface area ideally suited for reduction and oxidation reactions. Silanized molecular sieves have also been successfully used as supports for enzyme adsorption owing to the presence of silanols on pore walls that facilitate enzyme immobilization by hydrogen bonding. Various chemical modifications of the currently used supports would definitely help in better immobilization. Water activity profiles of lipase adsorbed using polypropylene-based hydrophobic granules/Accurel EP-100 has been reported. It would be important to note that Accurel with smaller particle sizes increases reaction rates and enantiomeric ratios during biocatalyzation.

For better process control and economic production, *Yarrowia lipolytica* lipase was immobilized on octyl-agarose and octadecyl-sepabeads supports by physical adsorption that resulted in higher yields and greater (tenfold) stability than that of free lipase. This was accounted by the hydrophobicity of octadecyl-sepabeads that enhances affinity between the enzyme and support. *Candida rugosa* lipase adsorbed on biodegradable poly (3-hydroxybutyrate-co-hydroxyvalerate) showed 94 % residual activity after 4 h at 50 °C and reusability till 12 cycles. These supports were preferred because they are less tough and crystalline than polyhydroxybutyrate. 1, 4-Butenediol diglycidyl ether-activated byssus threads have been suitable basement for urease that increased pH stability and retained 50 % enzyme activity under dried conditions. Eco-friendly supports of biological origin not only prevent cropping up of ethical issues, but also cut down the production costs. Of late, biocompatible mesoporous silica nanoparticles (MSNs) supports have been used for biocatalysis in energy applications owing to their long-term durability and efficiency.

5.15.2 Covalent binding

Covalent association of enzymes to supports occurs owing to their side chain amino acids like arginine, aspartic acid, histidine and degree of reactivity based on different functional groups like imidazole, indolyl, phenolic hydroxyl, etc. Peptide-modified surfaces when used for enzyme linkage results in higher specific activity and stability with controlled protein orientation. Cyanogen bromide (CNBr)-agarose and CNBr-activated-Sepharose containing carbohydrate moiety and glutaraldehyde as a spacer arm have imparted thermal stability to covalently bound enzymes. Highly stable and hyperactive biocatalysts have been reported by covalent binding of enzymes to silica gel carriers modified by silanization with elimination of unreacted aldehyde groups and to SBA-15 supports containing cage-like pores lined by Si–F moieties. Increase in half-life and thermal stability of enzymes has been achieved by covalent coupling with different supports like mesoporous silica, chitosan, etc. Cross-linking of enzymes to electrospun nanofibers has shown greater residual activity due to increased surface area and porosity. Use of such nanodiametric supports have brought a turning point in the field of biocatalyst immobilization. Covalent binding of alcohol dehydrogenase on attapulgite nanofibers (hydrated magnesium silicate) has been opted owing to its

thermal endurance and variable nano sizes. Biocatalytic membranes have been useful in unraveling effective covalent interactions with silicon-coated enzymes. Cross-linked enzyme aggregates produced by precipitation of enzyme from aqueous solution by addition of organic solvents or ionic polymers have been reported. Different orientations of immobilized enzyme on magnetic nanoclusters obtained by covalent binding have found their applications in pharmaceutical industries owing to their enhanced longevity, operational stability and reusability. Maintaining the structural and functional property of enzymes during immobilization is one of the major roles played by a cross-linking agent. One such agent is glutaraldehyde, popularly used as bifunctional cross-linker, because they are soluble in aqueous solvents and can form stable inter- and intra-subunit covalent bonds.

5.15.3 Affinity immobilization

Affinity immobilization exploits specificity of enzyme to its support under different physiological conditions. It is achieved by two ways: either the matrix is precoupled to an affinity ligand for target enzyme or the enzyme is conjugated to an entity that develops affinity toward the matrix. Affinity adsorbents have also been used for simultaneous purification of enzymes. Complex affinity supports like alkali stable chitosan-coated porous silica beads and agarose-linked multilayered concanavalin A harbor higher amounts of enzymes which lead to increased stability and efficiency. Bioaffinity layering is an improvisation of this technique that exponentially increases enzyme-binding capacity and reusability due to the presence of non-covalent forces such as coulombic, hydrogen bonding, van der Waals forces, etc.

5.15.4 Entrapment

Entrapment is caging of enzymes by covalent or non-covalent bonds within gels or fibers (Singh 2009). Efficient encapsulation has been achieved with alginate–gelatin–calcium hybrid carriers that prevented enzyme leakage and provided increased mechanical stability. Entrapment by nanostructured supports like electrospun nanofibers and pristine materials have revolutionalized the world of enzyme immobilization with their wide-ranging applications in the field of fine chemistry, biomedicine biosensors and biofuels. Prevention of friability and leaching and augmentation of entrapment efficiency and enzyme activity by *Candida rugosa* lipase entrapped in chitosan have been reported. This support has also been reported to be

non-toxic, biocompatible and amenable to chemical modification and highly affinitive to protein due to its hydrophilic nature. Entrapment by mesoporous silica is attributed to its high surface area, uniform pore distribution, tunable pore size and high adsorption capacity. Simultaneous entrapment of lipase and magnetite nanoparticles with biomimetic silica enhanced its activity in varying silane additives. Sol–gel matrices with supramolecular calixarene polymers have been used for entrapment of *C. rugosa* lipase keeping in view their selective binding and carrying capacities. Lipases entrapped κ-carrageenan has been reported to be highly thermostable and organic solvent tolerant.

5.16 Materials used for fabrication of immobilization supports

5.16.1 Natural polymers as supports

5.16.1.1 Alginate

Alginate derived from cell walls of brown algae are calcium, magnesium and sodium salts of alginic acid and have been extensively used for immobilization as xanthan–alginate beads, alginate–polyacrylamide gels and calcium alginate beads with enhanced enzyme activity and reusability. Cross-linking of alginate with divalent ions (like Ca^{2+}) and glutaraldehyde improves the stability of enzymes.

5.16.1.2 Chitosan and chitin

Natural polymers like chitin and chitosan have been used as supports for immobilization. The protein or carbohydrate moieties of enzymes are used for binding them to chitosan. Chitosan has been used in combination with alginate where chitosan-coated enzymes had less leaching effect compared to alginate owing to the physical and ionic interactions between the enzyme and support. Similarly, a wet composite of chitosan and clay proved to be more reliable for enzyme trapping, because it has hydroxyl and amino groups, which easily link with enzymes, together with good hydrophilicity and high porosity. Chitosan in the form of beads can entrap twice as much of the enzymes. According to Chern and Chao, the chitin-binding domain of chitinase A1 from *Bacillus circulans* has a high affinity to chitin; so, this property has been exploited to retain D-hydantoinase.

5.16.1.3 Collagen

Being a natural polymer, collagen has been used for immobilization of tannase employing glutaraldehyde as cross-linking agent. Fe^{3+}-collagen fibers proved to be excellent supporting matrix for catalase immobilization by retaining significant activity even after 26 reuses.

5.16.1.4 Carrageenan

Carrageenan, a linear sulfated polysaccharide, has been consistently used for immobilizing a variety of enzymes, like lipase for improving stability. This support is pseudoplastic in nature, which helps it to thin under shear stress and recover its viscosity once the stress is removed. Jegannathan et al. could achieve an encapsulation efficiency of 42.6 % by the co-extrusion method using the same support for biodiesel production. Carrageenan has been reported as a cheap and durable support with better entrapment for lactic acid and α-galactosidase enzyme.

5.16.1.5 Gelatin

Gelatin is a hydrocolloid material, high in amino acids, and can absorb up to ten times its weight in water. Its indefinite shelf life has attracted attention for enzyme immobilization. Gelatin has been utilized in mixed carrier system with polyacrylamide where cross-linking with chromium (III) acetate proved better than chromium (III) sulfate and potassium chromium (III) sulfate. Calcium alginate with gelatin forms a good template for calcium phosphate deposition for enzyme immobilization, and gelatin in combination with polyester films promoted 75 % loading efficiency, compared to previous studies which had 50 % loading efficiency.

5.16.1.6 Cellulose

This most abundant natural polymer has been widely used to immobilize fungi laccase, penicillin G acylase, glucoamylase, α-amylase, tyrosinase, lipase and β-galactosidase. Diethylaminoethyl (DEAE)-modified cellulosic supports have longer storage capacity. Cellulose-coated magnetite nanoparticles have been used for starch degradation where the attachment of α-amylase to cellulose dialdehyde-coated magnetite nanoparticles

resulted in the formation of a novel starch degrading system. Immobilization with ionic liquid-cellulose film activated by glutaraldehyde gave better formability and flexibility.

5.16.1.7 Starch

Made of linear amylase and branched amylopectin units, starch has been used as enzyme immobilizer. Calcium alginate–starch hybrid supports were applied for surface immobilization and entrapment of bitter gourd peroxidase. Entrapped enzyme was more stable in the presence of denaturants like urea due to internal carbohydrate moieties, while surface-immobilized enzyme had superior activity. Radiation grafting of substances like acrylamide and dimethylaminoethyl methacrylate onto starch are among the widely used industrial techniques for a high product yield.

5.16.1.8 Pectin

This structural heteropolysaccharide along with 0.2–0.7 % glycerol acts as plasticizer to reduce brittleness of support and has been used to immobilize papain and for development of new materials for skin injury treatment. Pectin–chitin and pectin–calcium alginate support have enhanced thermal and denaturant resistance and catalytic properties of entrapped enzymes due to the formation of high stable polyelectrolyte complexes between the enzyme and the pectin-coated support.

5.16.1.9 Sepharose

CNBr-activated Sepharose-4B has been used to immobilize amylase and glucoamylase owing to its porosity and easy adsorption of macromolecules. Further matrix modifications like alkyl substituted Sepharose with multipoint attachment between hydrophobic clusters of the enzyme and alkyl residues of the support play a major role in retaining the catalytic properties at extremes of pH, high salt concentrations and elevated temperatures. Another example of modified Sepharose matrix is concanavalin A (Con A)–Sepharose 4B where biospecific interaction between the glycosyl chains of the enzyme and Con A plays a pivotal role in fabrication of various biosensors.

5.16.2.0 Synthetic polymers as supports

Ion exchange resins/polymers are insoluble supports with porous surface for enzyme trapping. Amberlite and DEAE cellulose, renewable matrices with large surface area,

have been used for immobilization of α-amylase. During white radish peroxidase immobilization, glutaraldehyde and polyethylene glycol act as an additive and protective layer around the active center of the enzyme to prevent the attack of free radicals. Some synthetic polymers used as enzyme supports are stated as follows: polyvinyl chloride that prevents enzyme, cyclodextrin glucosyltransferase from thermal inactivation; polyurethane microparticles derived from polyvinyl alcohol and hexamethyl diisocyanate in the ratio of 1:3 with high enzyme loading and efficiency; UV-curable methacrylated/fumaric acid-modified epoxy that is proposed to be useful for industrial applications; polyaniline in two different forms, viz. emeraldine salt and emeraldine base powder used for covalent binding of α- amylase; glutaraldehyde-activated nylon for immobilizing lipase and UV-activated polyethylene glycol having high porosity employed for wastewater treatment.

5.1.7 Inorganic materials as supports

5.17.1 Zeolites

Zeolites or 'molecular sieves' are microporous crystalline solids with well-defined structures and shape-selective properties and are widely used in molecular adsorption. Microporous zeolites were found to be a better support for α-chymotrypsin immobilization than microporous dealuminized ones because of the presence of more hydroxyl groups that form strong hydrogen bonds with the enzyme. Likewise, Na Y zeolite was used to immobilize lysozyme because it had higher activity compared to other supports as reported. The heterogeneous surface of zeolites with multiple adsorption sites are considered to be suitable for modulating the enzyme and support interactions.

5.17.2 Ceramics

Immobilization of *Candida antarctica* lipase on ceramic membrane showed that this inert support could be exploited for carrying out hydrolytic and synthetic reactions by limiting feedback inhibition. Ceramic foams containing both macro (77 nm) and micropores (45 μm) were found to be efficient in lowering diffusion rate and increasing the specific surface area. Another example of ceramics is toyonite whose variable pore structure can be modified using different organic coatings.

5.17.3 Celite

Celite is highly porous diatomaceous, bioaffinity material and has been used for immobilization of lipase, polyphenol oxidases and β-galactosidase, because it is an inexpensive support having low polarity and large adhesion area. It provides resistance against high pH or temperature, urea, detergents and organic solvents. Celite acts as an additive in sol–gel matrix for ω-transaminases immobilization. It has been preferred due to its chemical inertness and interconnected pore structure.

5.17.4 Silica

Enzymes like lignin peroxidase and horseradish peroxidase (HRP) immobilized on activated silica have been effectively used for the removal of chlorolignins from eucalyptus kraft effluent. α-Amylase immobilized on silica nanoparticles improves cleaning performance of detergents. They have been used because of their nano-sized structures with high surface area, ordered arrangement and high stability to chemical and mechanical forces. Surface modifications of silica by amination of hydroxyl and reactive siloxane groups and addition of methyl or polyvinyl alcohol groups strengthen enzyme and support bonds.

5.17.5 Glass

Glass is a highly viscous liquid and has been employed in immobilizing α-amylase; phthaloyl chloride containing amino group functionalized glass beads was found to be robust and renewable for the process. Another enzyme nitrite reductase was immobilized on controlled pore glass beads, which served as a biosensing device for continuous monitoring. Urease immobilized on glass pH-electrodes has provided a stable biosensor for monitoring as low as 52 μg/ml urea in blood samples.

5.17.6 Activated carbon

Both natural and hydrochloric acid-modified activated carbon has provided valuable support for enzyme adsorption. Lately, mesoporous-activated carbon particles containing large contact sites for enzyme immobilization have been used for immobilizing acid protease and acidic lipases where catalytic efficiency has been significantly maintained after 21 cycles of reuse. It was also found that activated carbon

with a high surface area and a significant fraction of its pore volume in the 300–1,000 Å range was suitable for enzyme immobilization.

5.17.7 Charcoal

Chemical modification of charcoal by adsorbing papain with sulfhydryl groups increased the number of active sites and has been utilized for recovery of mercury from aqueous solution and efficiently employed for industrial wastewater treatment. Charcoal supports have been also used in food industries for immobilizing amyloglucosidase for starch hydrolysis without any cross-linking agent and has 90 % catalytic activity. As reported earlier by Kibarer and Akovali, charcoal is an excellent adsorbent with high adsorptive capacity and minimum fine particulate matter release.

5.18 Applications and scope

Biocatalysts are the key players in various industrial processes. Constant efforts are being made to improve the enzyme's activity, efficiency, reproducibility and stability during industrial processes. Production of regioselective and enantioselective compounds for biomedical application has been possible by immobilized enzymes. Glucose biosensors have been developed using electrospun PVA and surface-modified carbon nanotubes. Hydrogen peroxide biosensors have been devised using γ-aluminum trioxide nanoparticles/chitosan film-modified electrode. Agarose–guar has been successfully utilized for designing phenol biosensors. Currently, keen efforts are being taken for increasing the stability of biosensors. Immobilization of biosensing enzymes into nanocavities showed significant results.

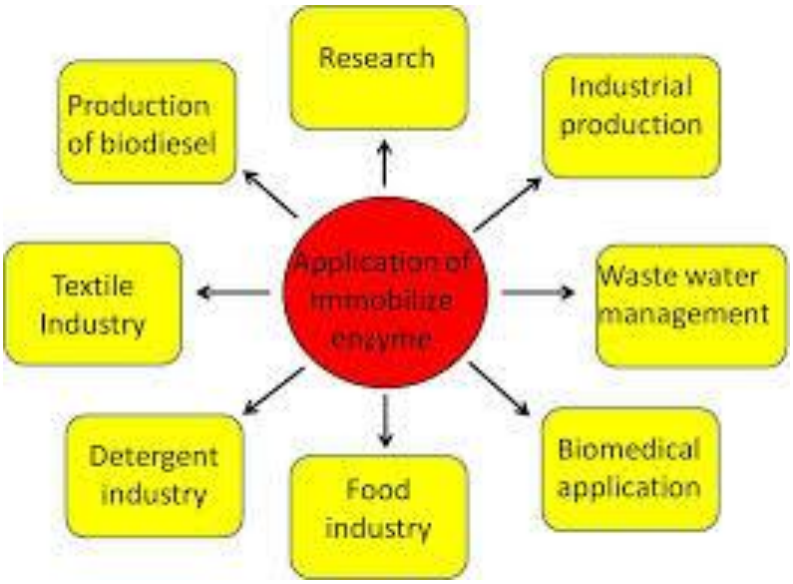

Fig. 8

Biosynthesis of polyester has been facilitated by immobilized *C. antarctica* lipase B, a greener alternative to petroleum-based conventional catalysts. With the advent of nanotechnology, silica nanoparticles with immobilized laccase have been applied for elimination of micropollutants from wastewater. Increasing environmental concerns have led to the use of immobilized biocatalysts for biodiesel production. The different factors influencing enzyme immobilization and the possible modifications for their enhancement in activity have been chalked out in given figure.

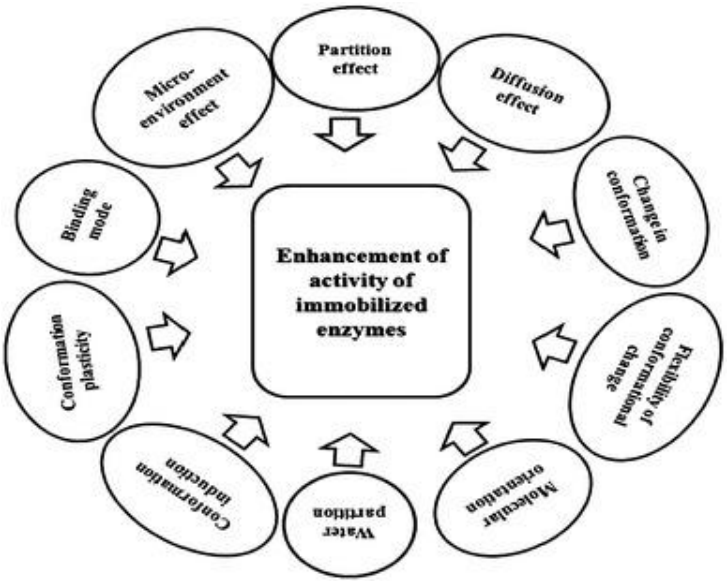

Fig. 9 Determinants of enzyme immobilization and activity

5.19 Summary

Under this unit we have discussed the use of enzymes or microorganisms in food preparations is an age-old process. With the advancement of technology, novel enzymes with wide range of applications and specificity have been developed and new application areas are still being explored. Microorganisms such as bacteria, yeast and fungi and their enzymes are widely used in several food preparations for improving the taste and texture and they offer huge economic benefits to industries. Microbial enzymes are the preferred source to plants or animals due to several advantages such as easy, cost-effective and consistent production. The present review discusses the recent advancement in enzyme technology for food industries

Enzymes as industrial biocatalysts offer numerous advantages over traditional chemical processes with respect to sustainability and process efficiency. Enzyme catalysis has been scaled up for commercial processes in the pharmaceutical, food and beverage industries, although further enhancements in stability and biocatalyst functionality are required for optimal biocatalytic processes in the energy sector for biofuel production and in natural gas conversion.

The technical barriers associated with the implementation of immobilized enzymes suggest that a multidisciplinary approach is necessary for the development of immobilized biocatalysts applicable in such industrial-scale processes. Specifically, the overlap of technical expertise in enzyme immobilization, protein and process engineering will define the next generation of immobilized biocatalysts and the successful scale-up of their induced processes. This review discusses how biocatalysis has been successfully deployed, how enzyme immobilization can improve industrial processes, as well as focuses on the analysis tools critical for the multi-scale implementation of enzyme immobilization for increased product yield at maximum market profitability and minimum logistical burden on the environment and user.

Terminal questions

Q.1. Describe commercially prepared enzymes and explain the microbial production of proteases.

Answer:----------

Q.2. Explain the isolation and purification of commercially important enzymes.

Answer:----------

Q.3. Explain traditional and modern biotechnology giving a few examples.

Answer:----------

Q.4. Describe the industrial process for manufacture of citric acid with a neat flowsheet.

Answer:----------

Q.5. Explain the application of enzymes in disease diagnosis.

Answer:----------

Q.6. Explain the enzyme immobilization.

Answer:----------

Q. 7 What are the factors influencing performance of immobilized enzyme**?**

Answer:----------

Further readings

1. Biochemistry- Lehninger A.L.
2. Biochemistry –J.H.Weil.
3. Biochemistry fourth edition-David Hames and Nigel Hooper.
4. Textbook of Biochemistry for Undergraduates - Rafi, M.D.
5. Biochemistry and molecular biology- Wilson Walker.

Unit-6: Isolation and purification of enzymes

Structure

6.1 Introduction

6.2 Properties of enzymes

6.3 Isolation of enzymes

6.3.1 Test Systems

6.4 Purification of enzymes

6.5 Large-scale production of enzymes

6.6 Enzyme structure and substrate binding

6.7 Mechanism of enzyme action

6.8 Gel-filtration chromatography

6.9 Selection of Operating Conditions

6.9.1 Matrix Choice

6.9.2 Types of gel filtration chromatography Group Separations

6.9.3 High-resolution fractionation of biomolecules

6.10 Principle of gel filtration chromatography

6.11 Applications of gel-filtration chromatography

6.12 Advantages of gel filtration chromatography

6.13 Limitations of Gel Filtration Chromatography

6.14 Ion exchange chromatography

6.14.1 Cationic exchangers

6.14.2 Anionic exchangers

6.14.3 Principle of ion exchange chromatography

6.14.4 Procedure of ion exchange chromatography

6.14.5 Applications of ion exchange chromatography

6.15 Advantages of ion exchange chromatography

6.16 Limitations of ion exchange chromatography

6.17 Affinity chromatography

6.17.1 Principle of affinity chromatography

6.18 Centrifugation

6.19 Electrophoretic techniques

6.1 Introduction

Most of the biological reactions are enzyme mediated. There are several standard protocols, techniques and methods available to purify and characterize such biocatalysts present either in plant, animal or microbial systems. Most of the regulatory enzymes catalyzing several such reactions are intracellular in nature. But in the lower organisms such as microbes, the enzyme is also extracellular in nature and in these cases the level of contaminants is not that high as compared to the intracellular enzyme, the product (enzyme) is generally in diluted form. Thus, in developing a downstream process for isolating and purifying an enzyme, one of the main objectives remains to bring down the cost. This requires strategic selection of the purification processes. Though there are plenty of protocols and methods available for this, the selection of process largely depends upon the nature of the targeted enzyme. If the enzyme is intracellular, contamination level will be high and if it is extracellular, dilution problem predominates. There is no standard protocol available, which could be universally accepted either for intracellular or extracellular enzyme purification.

6.2 Properties of enzymes

Enzymes are the complex protein molecules, often called biocatalysts, which are produced by living cells. They are highly specific both in the reactions that they

catalyze and in their choice of reactants, which are known as substrates. An enzyme typically catalyzes a single chemical reaction or a set of closely related reaction. Side reactions resulting in the wasteful formation of by-products are rare in enzyme-catalyzed reactions, in comparison to uncatalyzed ones. Enzymes can also be defined as soluble, colloidal and organic catalysts that are produced by living cells, but are capable of acting independently of the cells. Enzymes are currently being used in diverse areas in the food, feed, paper, leather, agriculture and textiles industries, resulting in major cost reductions. Simultaneously, rapid scientific progress is now encouraging the chemistry and pharmacological industries to embrace enzyme technology, a trend supported by concerns regarding energy, raw materials, health and the environment. One of the most common advantages of enzymes is their ability to function continuously even after their removal or separation from the cells. This means that even after the separation of cells from *in vivo* environments, they continue to work efficiently under *in vitro* conditions; we can conclude that these biocatalysts remain in an active state even after their isolation.

Principally, enzymes are non-toxic, biodegradable and can be produced in ample amounts by micro-organisms for industrial applications. In this chapter, the isolation, production, purification, utilization and application of enzymes (in soluble and immobilized or insoluble form) are discussed in detail. Procedures such as recombinant DNA technology and protein engineering are frequently used to produce more efficient and beneficial enzymes. The industrial production and utilization of enzymes is an important part of industry. Interdisciplinary collaboration between areas such as chemistry, process engineering, microbiology and biochemistry is required to develop the best possible enzyme technology, and eventually to achieve increased production and maintain the enzyme's physico-chemical properties under *in vitro* environments.

6.3 Isolation of enzymes

In living organisms, enzymes are components of mixtures that contain numerous organic and inorganic substances. These substances may interact with the enzymes, coenzymes, substances, or products of the enzymatic reaction. Study of the properties of an enzyme requires that it be purified. At present, several hundred enzymes are

available in purified form. It should be remembered that the characteristics of purified enzymes may not be the same as those they possess in their natural environment. Such variable factors as the stabilizing effect of mitochondria, diffusion rates, and inhibitory feedback mechanisms may alter the behavior of enzymes *in vivo*. Competition of other substances (e. g., nonspecific proteins) for substrates and inhibitors may also change enzyme activity *in vivo*. Nevertheless, the investigation of enzyme activity *in vivo* and the possibility of increasing or inhibiting this activity for experimental or therapeutic purposes is greatly facilitated by studies *in vitro* of the properties of purified enzymes. Investigation of the properties of purified enzymes requires the availability of:

(a) A test system;

(b) Extraction methods; and

(c) Fractionation methods.

6.3.1 Test Systems

The first requirement for the purification of an enzyme is the development of a quantitative test of its activity. The progress of purification is followed by the appropriate test until maximum activity per weight has been reached. It is recommended by the International Commission on Enzymes that enzyme assays should, whenever possible, be based on the measurement of the initial rates of activity; in this way, complications due to reversibility of reactions or to formation of inhibitory products are avoided. The concentration of the substrate should be large enough to saturate the enzyme, so that the reaction rate approximates a zero-order reaction. Under these test conditions, one unit of enzyme is defined as the amount that will catalyze the transformation of 1 μ mole of substrate per minute. Use of this absolute unit permits a comparison of different enzymes. Specific activity is expressed as units of enzyme per milligram of protein. When the enzyme has a catalytic center, the concentration of which can be determined, the catalytic power can be expressed in terms of catalytic center activity that is the number of molecules of substrate acted upon per minute per catalytic center.

Enzyme reactions may be measured by a variety of methods. Many substrates or products absorb in the visible or ultraviolet range, and their formation or disappearance can "be followed by spectrophotometric methods. Instruments are available that record

optical densities continuously at any desired wavelength. These instruments are equipped with thermostatically controlled cuvette holders for maintaining constant temperature during the assay. Enzyme reactions that involve the production or consumption of gases or the production of acids can be followed by manometric methods.

In case acid is formed, the reaction is carried out in the presence of a salt such as bicarbonate, which, on interaction with acid, releases gas. Oxidases, decarboxylases, hydrogenases, or carbonic anbydrases can be studied by this method. Another method requires measurement of the time necessary for the decoloration (reduction) of a certain amount of dye by the enzyme. This method is used extensively in the study of dehydrogenases. Enzyme reactions that involve pH changes can be followed by continuous titration. The pH is kept approximately constant by the addition of alkali and the reaction velocity is determined from the amount of alkali added per minute, as measured by an automatic apparatus like the Radiometer-pH Stat. Polarimetric measurements can also be used to follow enzyme reactions when an optically active substrate is converted to an inactive product. Sometimes it is necessary to follow the formation of products by chromatography. Many enzyme reactions can be followed by withdrawing samples from the reactor at intervals and measuring the product by chemical methods.

6.4 Purification of enzymes

Enzyme purification is of great importance in to acquire knowledge about structural and functional properties and to foretell its applications. The ultimate degree of purity of a particular enzyme depends upon its end use. The objective behind deciding the strategy for purification is to obtain the greatest possible yield of the desired enzyme with the highest catalytic activity and the greatest possible purity. Most of the purification methods used in laboratory research can be easily scaled to industrial processes. These methods are as under:

- Filtration
- Centrifugation

- Ultrafiltration
- Diafiltration
- Precipitation
- Chromatography, such as ion-exchange chromatography and gel filtration chromatography.

Enzyme purification is often a complex process, and a number of methods are usually applied in sequence to attain sufficiently high purity levels. The recommended strategy is to use less expensive and simple methods at early stages, when the volume handled is large, and let the more sophisticated and expensive techniques appear when the volume remaining is already small. The basic requirements for an efficient scheme aimed at enzyme purification are:

- High final degree of purity
- High overall recovery of enzyme activity
- Reproducibility.

Recall that extraction methods release the wanted enzyme into the medium, as well as a number of other cell components, for example, other enzymes and proteins, nucleic acids, and polysaccharides, which tend to increase the viscosity of the solution owing to their polymeric structure. After homogenization of the original raw material, the first step in purification is separation of the remaining cell debris, via either differential sedimentation or precipitation of the supernatant, or resorting to centrifugation or filtration for a faster and clearer extract. Digestion with nuclease will reduce the viscosity of the solution, and the extra enzyme can be easily removed at later stages of purification. Hence, soluble proteins (including enzymes) will be separated from organelle-sequestered proteins.

After the aforementioned removal of nucleic acids and cell debris, the supernatant containing the enzyme will be subjected to removal of further unwanted contaminants, namely small organic and inorganic molecules, as well as other proteins, using salting out (e.g., ammonium sulfate) or solvent (e.g., acetone/ethanol) to promote precipitation,

followed by dialysis against its corresponding buffer to remove enzyme-bound salts. Aqueous separation of enzymes can also be achieved, using counter-solvents such as butanol or octanol. Ultrafiltration is another technique used to separate enzymes, provided the molecular weight cutoff of the membrane is compatible with that of the target enzyme.

This technique, which leads to an aqueous concentrate of enzyme, is faster and easier to implement than the two-step processes of precipitation and dialysis. Chromatographic techniques will then follow, namely ion exchange or gel filtration, which give rise to purer fractions of enzyme, along with a significant increase in its specific activity. Such purification procedures can also be used to estimate the molecular mass of the enzyme, using a mixture of proteins of known molecular mass as reference standards. Affinity chromatography may be used as the final step of purification, when an inhibitor is used as ligand in the stationary phase.

The purpose of purification is to isolate specific enzymes from a crude extract of cells containing many other unwanted components in order to obtain the maximum specific activity with the best possible recovery of the initial activity. There are several procedures that are widely used for enzyme purification. The key to success for the enzyme purification process is the selection of the most appropriate treatment stage. The degree of purification of the enzymes depends on the purpose for which the enzyme will be used. There are several methods for the purification and isolation of enzymes produced on a large scale in industry.

- Molecular weight-based separation processes: dialysis and ultrafiltration; gradient centrifugation of density; chromatography of exclusion molecular.
- Separation processes based on solubility differences: isoelectric precipitation; solubilization and/or precipitation of the proteins by salting (salting-in and salting-out); solvent fractionation.
- Separation processes based on electrical charge of molecule.
- Separation of proteins by selective adsorption.

- Separation based on specificity of binders: chromatography affinity.

The purification stage will not be uniform for all enzymatic products. The processes described above may be made separately or together, allowing the separation of complex mixtures of enzymes. These stages should be rigorously controlled to obtain high-quality enzymatic products.

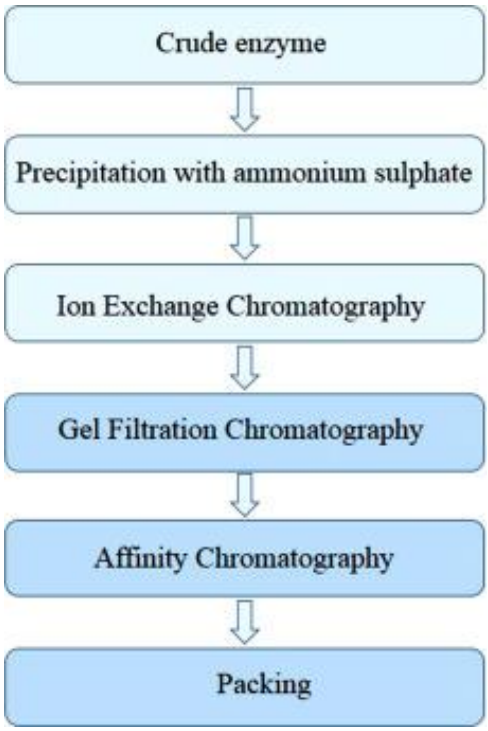

Fig. 1 Steps involved in the purification of enzymes

6.5 Large-scale production of enzymes

Among various enzymes produced at large scale are proteases (subtilisin, rennet), hydrolases (pectinase, lipase, lactase), isomerases (glucose isomerase), and oxidases (glucose oxidase). These enzymes are produced using overproducing strains of certain organisms. Separation and purification of an enzyme from an organism require disruption of cells, removal of cell debris and nucleic acids, precipitation of proteins, ultrafiltration of the desired enzyme, chromatographic separations (optional), crystallization, and drying. The process scheme varies depending on whether the enzyme is intracellular or extracellular. In some cases, it may be more advantageous to use inactive (dead or resting) cells with the desired enzyme activity in immobilized

form. This approach eliminates costly enzyme separation and purification steps and is therefore economically more feasible.

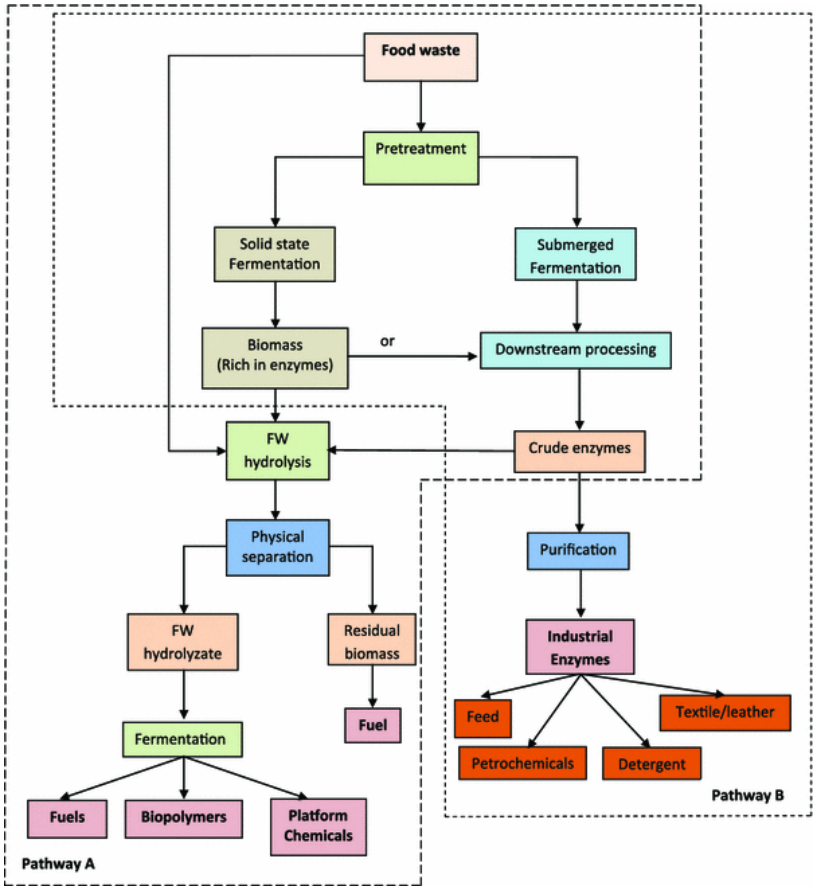

Fig. 2 Enzyme production from food wastes using a biorefinery concept

The first step in the large-scale production of enzymes is to cultivate the organisms producing the desired enzyme. Enzyme production can be regulated and fermentation conditions can be optimized for overproduction of the enzyme. Proteases are produced by using overproducing strains of *Bacillus*, *Aspergillus*, *Rhizopus*, and *Mucor*; pectinases are produced by *Aspergillus niger*; lactases are produced by yeast and *Aspergillus*; lipases are produced by certain strains of yeasts and fungi; glucose isomerase is produced by *Flavobacterium arborescens* or *Bacillus coagulans*. After the cultivation step, cells are separated from the media usually by filtration or sometimes by centrifugation. Depending on the intracellular or extracellular nature of the enzyme,

either the cells or the fermentation broth is further processed to separate and purify the enzyme. The recovery of intracellular enzymes is more complicated and involves the disruption of cells and removal of cell debris and nucleic acids. The given figure depicts a schematic of an enzyme plant producing intracellular enzymes.

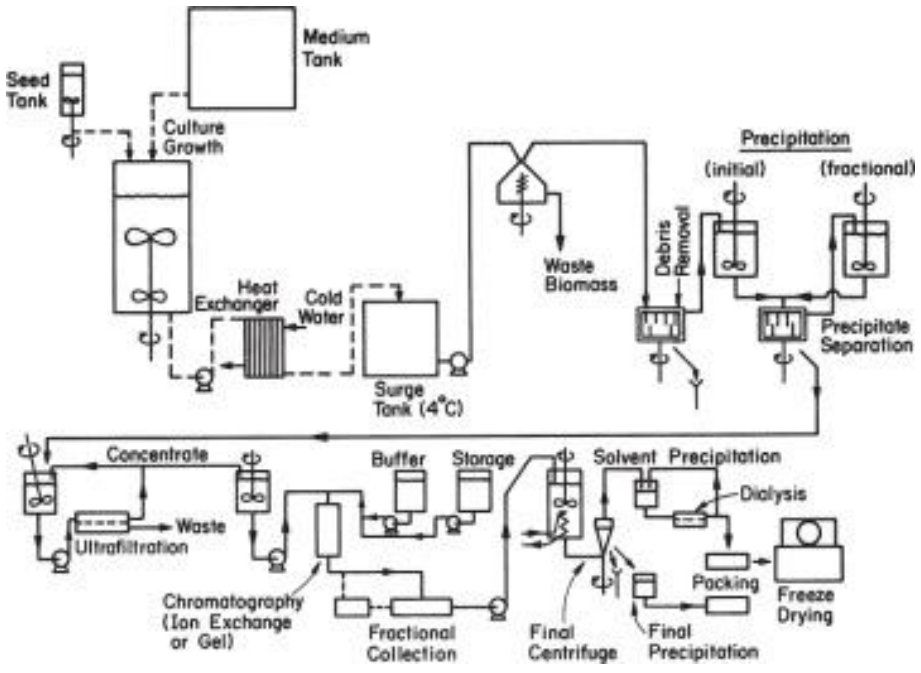

Fig. 3 A flowsheet for the production of an extracellular enzyme.

In some cases, an enzyme may be both intracellular and extracellular, which requires processing of both broth and cells. Intracellular enzymes may be released by increasing the permeability of the cell membrane. Certain salts, such as $CaCl_2$, and other chemicals, such as dimethylsulfoxide (DMSO), and pH shift may be used for this purpose. If enzyme release is not complete, then cell disruption may be essential.

6.6 Enzyme structure and substrate binding

Amino acid-based enzymes are globular proteins that range in size from less than 100 to more than 2 000 amino acid residues. These amino acids can be arranged as one or more polypeptide chains that are folded and bent to form a specific three-dimensional structure, incorporating a small area known as the active site in given figure, where the

substrate actually binds. The active site may well involve only a small number (less than 10) of the constituent amino acids.

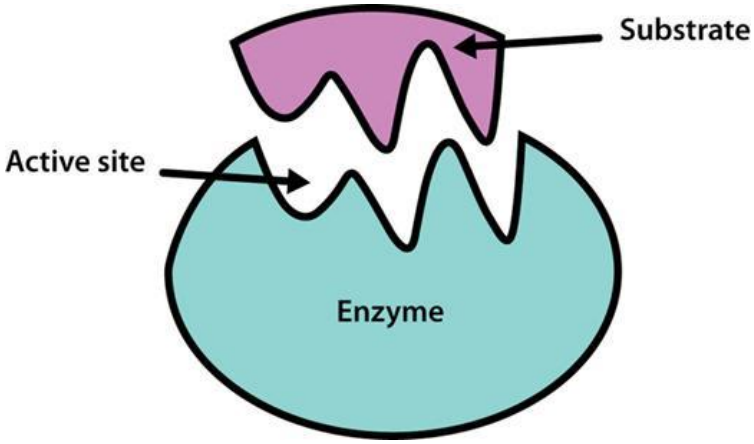

Fig. 4 Representation of substrate binding to the active site of an enzyme molecule.

It is the shape and charge properties of the active site that enable it to bind to a single type of substrate molecule, so that the enzyme is able to demonstrate considerable specificity in its catalytic activity. The hypothesis that enzyme specificity results from the complementary nature of the substrate and its active site was first proposed by the German chemist Emil Fischer in 1894, and became known as Fischer's ‘lock and key hypothesis’, whereby only a key of the correct size and shape (the substrate) fits into the keyhole (the active site) of the lock (the enzyme). It is astounding that this theory was proposed at a time when it was not even established that enzymes were proteins. As more was learned about enzyme structure through techniques such as X-ray crystallography, it became clear that enzymes are not rigid structures, but are in fact quite flexible in shape. In the light of this finding, in 1958 Daniel Koshland extended Fischer's ideas and presented the ‘induced-fit model’ of substrate and enzyme binding, in which the enzyme molecule changes its shape slightly to accommodate the binding of the substrate. The analogy that is commonly used is the ‘hand-in-glove model’, where the hand and glove are broadly complementary in shape, but the glove is moulded around the hand as it is inserted in order to provide a perfect match.

Since it is the active site alone that binds to the substrate, it is logical to ask what is the role of the rest of the protein molecule. The simple answer is that it acts to stabilize the active site and provide an appropriate environment for interaction of the site with the substrate molecule. Therefore the active site cannot be separated out from the rest of the protein without loss of catalytic activity, although laboratory-based directed (or forced) evolution studies have shown that it is sometimes possible to generate smaller enzymes that do retain activity. It should be noted that although a large number of enzymes consist solely of protein, many also contain a non-protein component, known as a cofactor, that is necessary for the enzyme's catalytic activity. A cofactor may be another organic molecule, in which case it is called a coenzyme, or it may be an inorganic molecule, typically a metal ion such as iron, manganese, cobalt, copper or zinc. A coenzyme that binds tightly and permanently to the protein is generally referred to as the prosthetic group of the enzyme. When an enzyme requires a cofactor for its activity, the inactive protein component is generally referred to as an apoenzyme, and the apoenzyme plus the cofactor (i.e. the active enzyme) is called a holoenzyme in given figure.

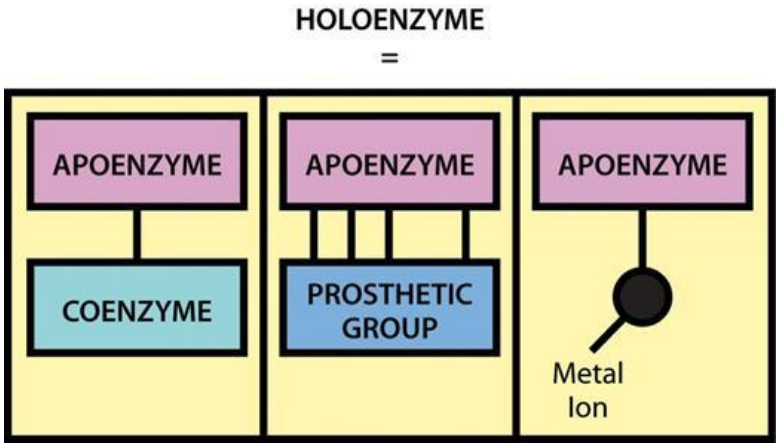

Fig. 5 The components of a holoenzyme

6.7 Mechanism of enzyme action

Enzyme kinetics

Enzyme kinetics is the study of factors that determine the speed of enzyme-catalysed reactions. It utilizes some mathematical equations that can be confusing to students when they first encounter them. However, the theory of kinetics is both logical and simple, and it is essential to develop an understanding of this subject in order to be able

to appreciate the role of enzymes both in metabolism and in biotechnology. Assays of enzyme activity can be performed in either a discontinuous or continuous fashion. Discontinuous methods involve mixing the substrate and enzyme together and measuring the product formed after a set period of time, so these methods are generally easy and quick to perform. In general we would use such discontinuous assays when we know little about the system (and are making preliminary investigations), or alternatively when we know a great deal about the system and are certain that the time interval we are choosing is appropriate.

In continuous enzyme assays we would generally study the rate of an enzyme-catalysed reaction by mixing the enzyme with the substrate and continuously measuring the appearance of product over time. Of course we could equally well measure the rate of the reaction by measuring the disappearance of substrate over time. Apart from the actual direction (one increasing and one decreasing), the two values would be identical. In enzyme kinetics experiments, for convenience we very often use an artificial substrate called a chromogen that yields a brightly coloured product, making the reaction easy to follow using a colorimeter or a spectrophotometer. However, we could in fact use any available analytical equipment that has the capacity to measure the concentration of either the product or the substrate.

In almost all cases we would also add a buffer solution to the mixture. As we shall see, enzyme activity is strongly influenced by pH, so it is important to set the pH at a specific value and keep it constant throughout the experiment. Our first enzyme kinetics experiment may therefore involve mixing a substrate solution (chromogen) with a buffer solution and adding the enzyme. This mixture would then be placed in a spectrophotometer and the appearance of the coloured product would be measured. This would enable us to follow a rapid reaction which, after a few seconds or minutes, might start to slow down, as shown in given figure.

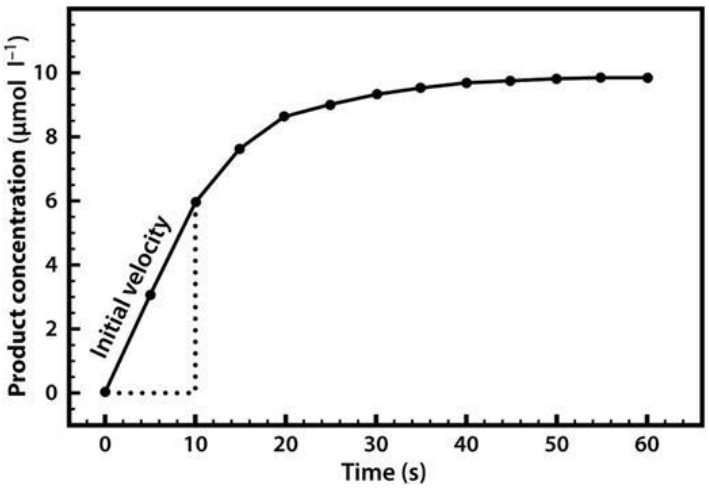

Fig. 6 Formation of product in an enzyme-catalysed reaction, plotted against time

A common reason for this slowing down of the speed (rate) of the reaction is that the substrate within the mixture is being used up and thus becoming limiting. Alternatively, it may be that the enzyme is unstable and is denaturing over the course of the experiment, or it could be that the pH of the mixture is changing, as many reactions either consume or release protons. For these reasons, when we are asked to specify the rate of a reaction we do so early on, as soon as the enzyme has been added, and when none of the above-mentioned limitations apply. We refer to this initial rapid rate as the initial velocity (v_0). Measurement of the reaction rate at this early stage is also quite straightforward, as the rate is effectively linear, so we can simply draw a straight line and measure the gradient (by dividing the concentration change by the time interval) in order to evaluate the reaction rate over this period.

We may now perform a range of similar enzyme assays to evaluate how the initial velocity changes when the substrate or enzyme concentration is altered, or when the pH is changed. These studies will help us to characterize the properties of the enzyme under study.

The relationship between enzyme concentration and the rate of the reaction is usually a simple one. If we repeat the experiment just described, but add 10% more enzyme, the reaction will be 10% faster, and if we double the enzyme concentration the reaction will proceed twice as fast. Thus there is a simple linear relationship between the reaction rate and the amount of enzyme available to catalyse the reaction as shown figure.

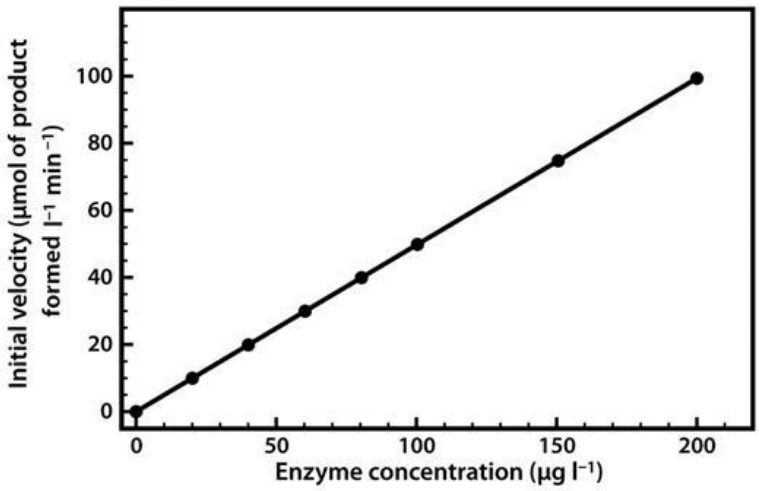

Fig. 7 Relationship between enzyme concentration and the rate of an enzyme-catalysed reaction.

This relationship applies both to enzymes *in vivo* and to those used in biotechnological applications, where regulation of the amount of enzyme present may control reaction rates. When we perform a series of enzyme assays using the same enzyme concentration, but with a range of different substrate concentrations, a slightly more complex relationship emerges, as shown in given figure. Initially, when the substrate concentration is increased, the rate of reaction increases considerably. However, as the substrate concentration is increased further the effects on the reaction rate start to decline, until a stage is reached where increasing the substrate concentration has little further effect on the reaction rate. At this point the enzyme is considered to be coming close to saturation with substrate, and demonstrating its maximal velocity (V_{max}). Note that this maximal velocity is in fact a theoretical limit that will not be truly achieved in any experiment, although we might come very close to it.

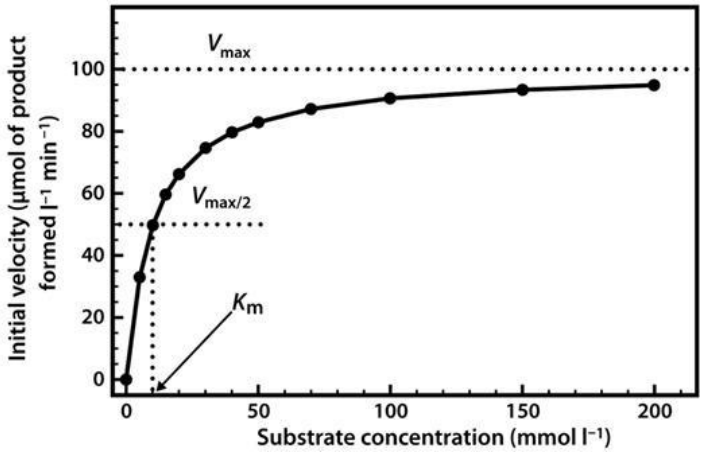

Fig. 8

6.8 Gel-filtration chromatography

Gel-filtration chromatography is a form of partition chromatography used to separate molecules of different molecular sizes. This technique has also frequently been referred to by various other names, including gel-permeation, gel-exclusion, size- exclusion, and molecular- sieve chromatography. The basic principle of gel filtration is quite straightforward. Molecules are partitioned between a mobile phase and a stationary phase (comprising a porous matrix of defined porosity) as a function of their relative sizes. A column constructed of such a matrix, typically in bead form, will have two measurable liquid volumes, the external volume, consisting of the liquid between the beads, and the internal volume, consisting of the liquid within the beads. The external volume is usually referred to as the void volume (V_0), while the sum of the external and internal volumes is the total volume (V_t). Following sample application, molecules larger than the pores of the stationary phase matrix will be excluded from the internal volume within the beads and will, therefore, migrate quite rapidly through the column, emerging at V_0, while molecules both smaller than the matrix pores, as well as those intermediate in size, will equilibrate with both the external and internal liquid volumes, causing them to migrate much more slowly and emerge at a volume (V_e) greater than V_0. Molecules are, therefore, eluted in order of decreasing molecular size. The elution volume, V_e, of a particular molecule depends on the fraction of the stationary phase available to it for diffusion.

6.9 Selection of Operating Conditions

Various factors should be considered when designing a gel-filtration system. These include: (1) matrix choice, (2) sample size and concentration, (3) column parameters, (4) choice of eluent, (5) effect of flow rate, and (6) column cleaning and storage.

6.9.1 Matrix Choice

Under separation conditions, matrices should be inert with respect to the molecules being separated in order to avoid partial adsorption of the molecules to the matrix, not only retarding their migration through the column, but also resulting in "tailed" peaks (*see*, for example. The stability of the matrix to organic solvents, pH, and temperature is also an important consideration and these variables should be compatible with the properties of the molecules being separated. When choosing a suitable matrix, one with a molecular mass fractionation range should be selected which will allow the molecule of interest to elute after V_0 and before V_t. The most suitable fractionation range, however, will not only be dictated by the molecular mass of the target molecule, but also by the composition of the sample being applied to the column. Therefore, the best separation of molecules within a sample having similar molecular masses is achieved using a matrix with a narrow fractionation range.

Gel-filtration matrices may be made from a wide variety of materials including dextrans, agarose, polyacrylamide, polyvinyl ethyl carbitol, polyvinylpyrrolidone, cellulose, silica-based materials, or from mixtures such as dextran-polyacrylamide or dextran–agarose. Each of these materials has advantages and disadvantages. Dextran may be greatly improved as a support material by means of enhanced cross-linking and its "swelling" may be controlled by the surrounding ionic milieu. With agarose, this "swelling" is nearly independent of ionic strength and pH but high binding capacities may be obtained with synthesis of highly porous agarose particles. Polyacrylamide-based matrices have a swelling behavior similar to dextrans while cellulose has a hydrophilic surface (which is good for reducing nonspecific hydrophobic interactions). Although cellulose is relatively inexpensive, it requires extra cross-linking for stability. Acrylate-copolymer supports are stable across a wide range of pH values. Commonly used gel-filtration matrices consist of porous beads composed of cross-linked

polyacrylamide, agarose, dextran, or combinations of these, and are supplied either in suspended form or as dried powders.

Polystyrene-divinylbenzene matrices have hydrophobic surfaces which make them unsuitable for the separation of proteins. Silica matrices have good rigidity (making them suitable for high-pressure fast flow-rate systems) but have hydrophobic surfaces and are unstable at pH > 8. This has led to the development of coated polystyrene-divinyl benzene and silica bead matrices, which have hydrophilic surfaces.

There has been a drive to further reduce particle size in order to achieve faster speed and greater chromatographic resolution. Weaker polymeric resins may compress under pressure and flow, a drawback that limits the extent to which the particle size can be reduced for chromatographic applications. Porous hybrid organic/inorganic particles have been employed as the supports for size-exclusion chromatography. More recently, bridged ethyl hybrid (BEH) particles, which have their surface modified with diol groups, have been developed to minimize nonspecific ionic interactions with proteins. In addition, the high mechanical strength of BEH particles enables a reduction in particle size to 1.7 μm, providing significant improvements in chromatographic efficiency.

6.9.2 Types of gel filtration chromatography

Group Separations

- The components of a sample are separated into two major groups according to the size range.
- A group separation can be used to remove high or low molecular weight contaminants (such as phenol red from culture fluids) or to desalt and exchange buffers.

6.9.3 High-resolution fractionation of biomolecules

- The components of a sample are separated according to differences in their molecular size.

- High-resolution fractionation can be used to isolate one or more components, to separate monomers from aggregates, to determine the molecular weight or to perform a molecular weight distribution analysis.

6.10 Principle of gel filtration chromatography

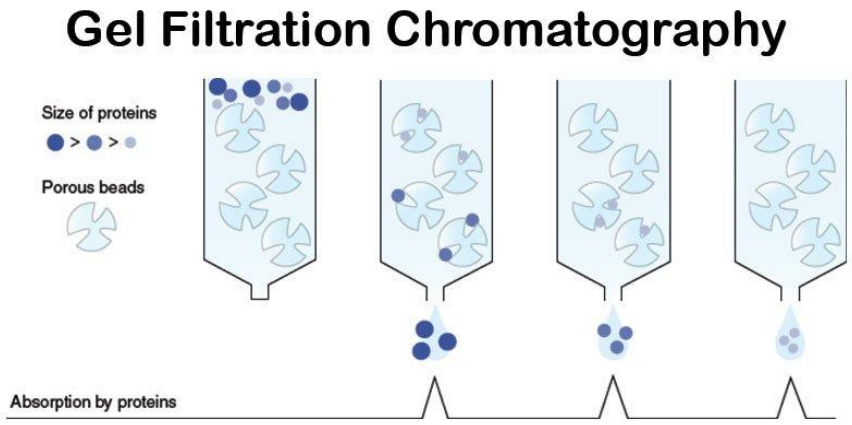

Fig. 9

To perform a separation, the gel filtration medium is packed into a column to form a packed bed. The medium is a porous matrix in the form of spherical particles that have been chosen for their chemical and physical stability, and inertness (lack of reactivity and adsorptive properties). The packed bed is equilibrated with a buffer which fills the pores of the matrix and the space in between the particles. The liquid inside the pores is sometimes referred to as the stationary phase and this liquid is in equilibrium with the liquid outside the particles, referred to as the mobile phase.

- The stationary phase used is a porous polymer matrix whose pores are completely filled with the solvent to be used as the mobile phase.
- The molecules in the sample are pumped through specialized columns containing such microporous packing material (gel).
- The basis of the separation is that molecules above a certain size are totally excluded from the pores, while smaller molecules access the interior of the pores partly or wholly.

- The flow of the mobile phase hence will cause larger molecules to pass through the column unhindered, without penetrating the gel matrix, whereas smaller molecules will be retarded according to their penetration of the gel.

6.11 Applications of gel-filtration chromatography

One of the principal advantages of gel-filtration chromatography is that separation can be performed under conditions specifically designed to maintain the stability and activity of the molecule of interest without compromising resolution. Absence of a molecule-matrix binding step also prevents unnecessary damage to fragile molecules, ensuring that gel-filtration separations generally give high recoveries of activity. This technique, however, is not without its disadvantages. When separating proteins by gel-filtration chromatography, for example, proteolysis becomes an increasing problem, since the target protein frequently becomes the abundant substrate for proteases also present in the mixture, consequently reducing recovery of activity. Because of the large size of gel-filtration columns, large volumes of eluent are usually required for their operation, often creating excessive running costs. Gel filtration also has an inherent low resolution compared to other chromatographic techniques because none of the molecules are retained by the column and non ideal flow occurs around the beads. In addition, this technique has a low sample-handling capacity dictated by the need to optimize resolution.

6.12 Advantages of gel filtration chromatography

- Gel filtration is a robust technique that is well suited to handling biomolecules that are sensitive to changes in pH, the concentration of metal ions or co-factors and harsh environmental conditions.
- A significant advantage of gel filtration is that conditions can be varied to suit the type of sample or the requirements for further purification, analysis or storage without altering the separation.
- Separations can be performed in the presence of essential ions or cofactors, detergents, urea, guanidine hydrochloride, at high or low ionic strength, at 37 °C or in the cold room according to the requirements of the experiment.

- Unlike ion exchange or affinity chromatography, molecules do not bind to the chromatography medium so buffer composition does not directly affect resolution (the degree of separation between peaks).
- Short analysis time.
- Well defined separation.
- Narrow bands and good sensitivity.
- There is no sample loss.
- The small amount of mobile phase required.
- The flow rate can be set.

6.13 Limitations of Gel Filtration Chromatography

- The limited number of peaks that can be resolved within the short time scale of the run.
- Filtrations must be performed before using the instrument to prevent dust and other particulates from ruining the columns and interfering with the detectors.
- The molecular masses of most of the chains will be too close for the separation to show anything more than broad peaks.

6.14 Ion exchange chromatography

Ion-exchange chromatography (IEC) is part of ion chromatography which is an important analytical technique for the separation and determination of ionic compounds, together with ion-partition/interaction and ion-exclusion chromatography. Ion chromatography separation is based on ionic (or electrostatic) interactions between ionic and polar analytes, ions present in the eluent and ionic functional groups fixed to the chromatographic support. Two distinct mechanisms as follows; ion exchange due to competitive ionic binding (attraction) and ion exclusion due to repulsion between similarly charged analyte ions and the ions fixed on the chromatographic support, play a role in the separation in ion chromatography. Ion exchange has been the predominant form of ion chromatography to date. This chromatography is one of the most important adsorption techniques used in the separation of peptides, proteins, nucleic acids and related biopolymers which are charged molecules in different molecular sizes and

molecular nature. The separation is based on the formation of ionic bonds between the charged groups of biomolecules and an ion-exchange gel/support carrying the opposite charge. Biomolecules display different degrees of interaction with charged chromatography media due to their varying charge properties.

- Chromatography is the separation of a mixture of compounds into its individual components based on their relative interactions with an inert matrix.
- Ion exchange chromatography (or ion chromatography) is a process that allows the separation of ions and polar molecules based on their affinity to ion exchangers.
- The principle of separation is thus by reversible exchange of ions between the target ions present in the sample solution to the ions present on ion exchangers.

In this process two types of exchangers i.e., cationic and anionic exchangers can be used.

6.14.1 Cationic exchangers possess negatively charged group, and these will attract positively charged cations. These exchangers are also called “Acidic ion exchange” materials, because their negative charges result from the ionization of acidic group.

6.14.2 Anionic exchangers have positively charged groups that will attract negatively charged anions. These are also called “Basic ion exchange” materials.

- Ion exchange chromatography is most often performed in the form of column chromatography. However, there are also thin-layer chromatographic methods that work basically based on the principle of ion exchange.

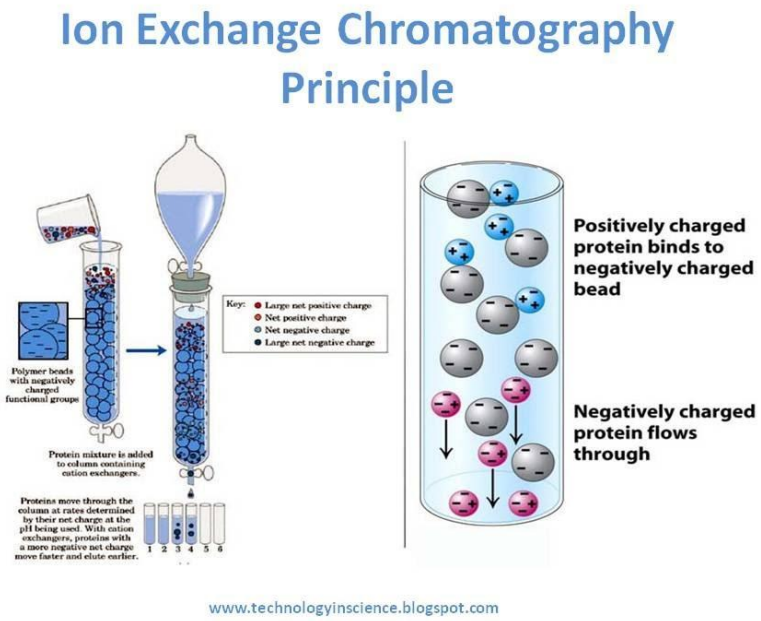

Fig. 10

6.14.3 Principle of ion exchange chromatography

This form of chromatography relies on the attraction between oppositely charged stationary phase, known as an ion exchanger, and analyte.

- The ion exchangers basically contain charged groups covalently linked to the surface of an insoluble matrix.
- The charged groups of the matrix can be positively or negatively charged.
- When suspended in an aqueous solution, the charged groups of the matrix will be surrounded by ions of the opposite charge.
- In this “ion cloud”, ions can be reversibly exchanged without changing the nature and the properties of the matrix.

6.14.4 Procedure of ion exchange chromatography

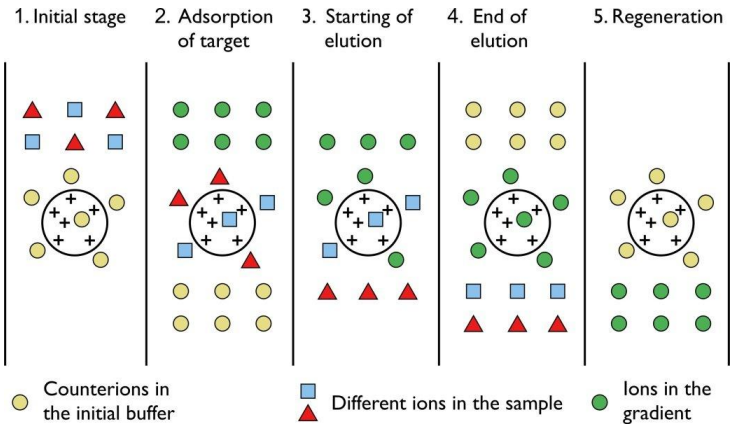

Fig. 11

Ion exchange separations are carried out mainly in columns packed with an ion-exchanger.

- These ionic exchangers are commercially available. They are made up of styrene and divinyl benzene. Example. DEAE-cellulose is an anionic exchanger, CM-cellulose is a cationic exchanger.
- The choice of the exchanger depends upon the charge of particle to be separated. To separate anions “Anionic exchanger” is used, to separate cations “Cationic exchanger” is used.
- First the column is filled with ion exchanger then the sample is applied followed by the buffer. The tris-buffer, pyridine buffer, acetate buffer, citrate and phosphate buffers are widely used.
- The particles which have high affinity for ion exchanger will come down the column along with buffers.
- In next step using corresponding buffer separates the tightly bound particles. Then these particles are analyzed spectroscopically.

6.14.5 Applications of ion exchange chromatography

- An important use of ion-exchange chromatography is in the routine analysis of amino acid mixtures.
- The 20 principal amino acids from blood serum or from the hydrolysis of proteins are separated and used in clinical diagnosis.
- This is most effective method for water purification. Complete deionization of water (or) a non-electrolyte solution is performed by exchanging solute cations

for hydrogen ions and solute anions for hydroxyl ions. This is usually achieved by method is used for softening of drinking water.

- In the analysis of products of hydrolysis of nucleic acids. In this way, information is gained about the structure of these molecules and how it relates to their biological function as carriers of hereditary information.
- Chelating resins are used to collect trace metals from seawater.
- To analyze lunar rocks and rare trace elements on Earth.

6.15 Advantages of ion exchange chromatography

- It is one of the most efficient methods for the separation of charged particles.
- It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids.
- Ion exchange is used for both analytical and preparative purposes in the laboratory, the analytical uses being the more common.
- Inorganic ions also can be separated by ion-exchange chromatography.

6.16 Limitations of ion exchange chromatography

- Only charged molecules can be separated.
- Buffer requirement.

6.17 Affinity chromatography

Affinity chromatography is primarily used to capture a specific protein or a class of proteins within complex mixtures. The principle of affinity chromatography is based on the ability of a biologically active molecule to bind specifically and reversibly to a complementary molecule, which is often bound to a solid support. These ligand molecules may include antibodies, metals, lectins, biotin, aptamers, and other molecules. The binding sites of the immobilized substances must be sterically accessible after their coupling to the solid support and should not be deformed by immobilization. In the case of specific proteins, an affinant is attached to the active surface of the column packing material or column surface. The sample is injected onto the column and the protein(s) of interest is captured by the affinant. Proteins that do not

possess a complementary binding site for the bound ligand will either pass directly through the column or be eluted by a low-stringency washing step.

The bound proteins are recovered by washing the column with a competitive substrate or a solution that disrupts the interaction between the protein and the affinant (e.g., denaturants). Although an antibody is directed to a specific protein, many other affinity methods have been developed to capture a class of proteins. These methods include immobilized metal affinity columns containing nickel to capture histidine-containing peptides, gallium to isolate phosphopeptides, or titanium dioxides for phosphopeptides. In addition, affinity methods have been developed to select peptides containing specific types of residues such as cysteine, tryptophan, or methionine. There are a variety of different lectins that have been used to selectively separate glycoproteins based on the composition of the carbohydrate side chain. A method was also developed that utilizes phosphoprotein isotope affinity tags, combining stable isotope and biotin labeling for proteome-wide affinity separation and quantitation of phosphoproteins.

Affinity chromatography is a method of separating a biomolecule from a mixture, based on a highly specific macromolecular binding interaction between the biomolecule and another substance. The specific type of binding interaction depends on the biomolecule ofinterest; antigen and antibody, enzyme and substrate, receptor and ligand,or protein a nd nucleic acid binding interactions are frequently exploited for isolation of various biomolecules. Affinity chromatography is useful for its high selectivity and resolution of separation, compared to other chromatographic methods.

6.17.1 Principle of affinity chromatography

Affinity chromatography takes advantage of specific binding interactions between the analyte of interest (normally dissolved in the mobile phase), and a binding partner or ligand (immobilized on the stationary phase). In a typical affinity chromatography experiment, the ligand is attached to a solid, insoluble matrix--usually a polymer such as agarose or polyacrylamide--chemically modified to introduce reactive functional groups with which the ligand can react, forming stable covalent bonds. The stationary

phase is first loaded into a column to which the mobile phase is introduced. Molecules that bind to the ligand will remain associated with the stationary phase. A wash buffer is then applied to remove non-target biomolecules by disrupting their weaker interactions with the stationary phase, while the biomolecules of interest will remain bound. Target biomolecules may then be removed by applying a so-called elution buffer, which disrupts interactions between the bound target biomolecules and the ligand. The target molecule is thus recovered in the eluting solution.

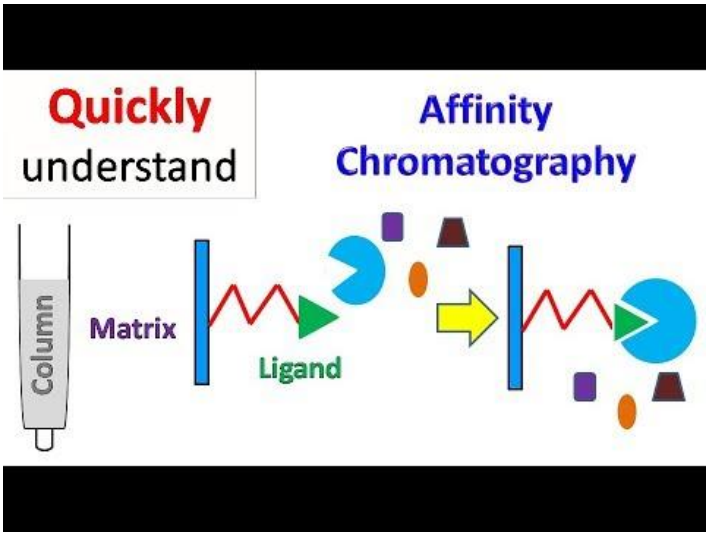

Fig. 12

Affinity chromatography does not require the molecular weight, charge, hydrophobicity, or other physical properties of the analyte of interest to be known, although knowledge of its binding properties is useful in the design of a separation protocol. Types of binding interactions commonly exploited in affinity chromatography procedures are summarized in the table below.

Typical biological interactions used in affinity chromatography

Sr. No	Types of Ligand	Target Molecule	Sr. No	Types of Ligand	Target Molecule
1.	Substrate analogue	Enzymes	**6.**	Avidin	Biotin/Biotin-conjugated molecule
2.	Antibody	Antigen	**7.**	Calmodulin	Calmodulin binding partner

3.	Lectin	Polysaccharide	**8.**	Glutathione	GST fusion protein
4.	Nucleic acid	Complementary base sequence	**9.**	Proteins A and G	Immunoglobulins
5.	Hormone	Receptor	**10.**	Metal ions	Poly-histidine fusion protein

6.18 Centrifugation

Centrifugation has been used to separate colloids from aqueous solution on the basis of particle size and density. The samples are prefiltered to remove particulate material (by definition through a 0.45-μm filter paper) and then placed in centrifuge tubes. Samples usually undergo centrifugation at 25,000 rpm for a minimum of 45 min. This is repeated until the conductance and surface tension correspond to that of pure water. Again it takes protracted time scales to process samples, making the method unsuitable for marine and estuarine solutions where large sample volumes are required. However, it is suggested that centrifugation is far more effective than diafiltration and ion exchange at removing LMW molecules, and that centrifugation tubes are readily decontaminated and sterilized. The main disadvantage of centrifugation is therefore the limited sample capacity of the centrifuge.

It is a technique that helps to separate mixtures by applying centrifugal force. A centrifuge is a device, generally driven by an electric motor, that puts an object, e.g., a rotor, in a rotational movement around a fixed axis. A centrifuge works by

using the principle of sedimentation: Under the influence of gravitational force (g-force), substances separate according to their density. Different types of separation are known, including isopycnic, ultrafiltration, density gradient, phase separation, and pelleting.

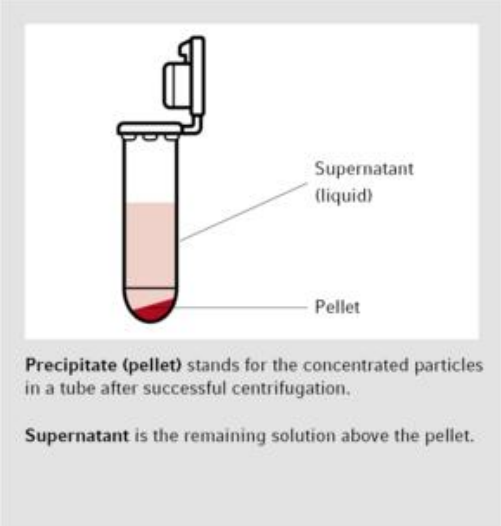

Fig. 13

Pelleting is the most common application for centrifuges. Here, particles are concentrated as a pellet at the bottom of the centrifuge tube and separated from the remaining solution, called supernatant. During phase separation, chemicals are converted from a matrix or an aqueous medium to a solvent (for additional chemical or molecular biological analysis). In ultrafiltration, macromolecules are purified, separated, and concentrated by using a membrane. Isopycnic centrifugation is carried out using a "self-generating" density gradient established through equilibrium sedimentation. This method concentrates the analysis matches with those of the surrounding solution. Protocols for centrifugation typically specify the relative centrifugal force (rcf) and the degree of acceleration in multiples of g (g-force). Working with the rotational speed, such as revolutions per minute (rpm), is rather imprecise.

6.18.1 Basic Principle of Centrifuge

The centrifuge works on the principle of gravity and generation of the centrifugal force to sediment different fractions. The rate of sedimentation depends on the applied centrifugal field (G) being directed radially outwards G depends on-

- Angular velocity (ω in radians/sec)
- Radial distance (r in cm) of the particle from the axis of rotation
- $G = \omega^2 r$

6.18.2 Rate of sedimentation

It depends on factors other than centrifugal force like the mass of the particle, density and volume, the density of the medium, shape of the particle, and friction.

6.18.3 Sedimentation time

It depends on the following factors and they are-

- Size of particle
- The density difference between particle and medium
- Radial distance from the axis of rotation to the liquid meniscus (rt)
- Radial distance from the axis of rotation to the bottom of the tube(rb)
- Revolutions Per Minute (RPM)

6.18.4 Differential Centrifugation in bio separation

The force of gravity will cause sedimentation of particles from a heterogeneous mixture; larger and denser particles sedimentfaster than the smaller and less dense particles. This phenomenon is useful for separating heterogeneous solutions into independent components, and for the isolation and enrichment of target molecules, cells, and cell organelles. **Differential centrifugation** accelerates the **separation process** by introducing centripetal forces many times greater than gravity. The precipitated particles form a pellet at the bottom of the tube during centrifugation. The rate of sedimentation is dependent on the size and density of the particles, so centrifugation can be used to isolate target particles simply by controlling centrifugal force or the rate of centrifugation. The rate of centrifugation is reported as angular velocity by revolutions per minute (rpm) or as acceleration(g). RPM is dependent on the radius of the rotor in the centrifuge.

Fig. 14 Differential Centrifugation in bio separation

6.18.5 Differential centrifugation vs density gradient centrifugation

There are two main methods of separating particles by centrifugation**:** differential centrifugation and density gradient centrifugation. Differential centrifugation capitalizes upon the differential rates of sedimentation of particles of varying density**.** The larger, denser particles have a higher rate of sedimentation**.** Density gradient centrifugation produces a cleaner separation of particles than differential centrifugation by employing a density matrix for the particles to move through. The differential centrifugation process involves multiple centrifugation steps of incrementally increased centrifugal force. The largest and densest particles with the greatest rate of sedimentation will comprise the pellet during the initial low-force spin while the smaller, less dense particles remain in the supernatant**.** The pellet and supernatant can then be separated**,** and **the** supernatant can be placed back into the centrifuge at a higher centrifugal force to pull out the next group of particles with a lower rate of sedimentation.

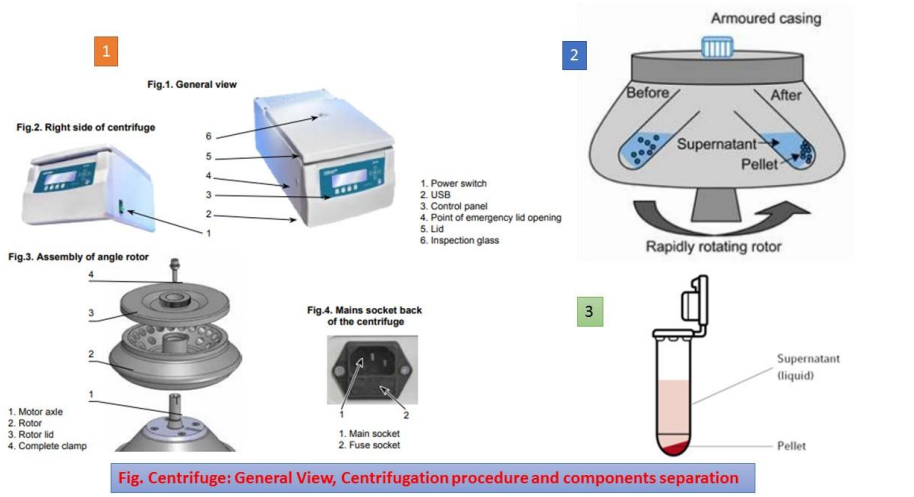

Fig. 15 Centrifuge: introduction, principle, types, handling procedure and uses

This process is repeated as many times as necessary to isolate each desired group of particles. As an example, cells that are lysed in a detergent-free buffer and all membrane-bound proteins will remain associated with their specific membranes. After centrifugation the soluble cytosolic proteins will be located in the supernatant while the membrane associated proteins will be in the heavier pellet. To further enrich proteins from a specific cellular organelle, differential centrifugation can be used. Nuclei will form a pellet when centrifuged at 600 g for 10 minutes. The supernatant can be further centrifuged at 15,000 g for 10 minutes to bring mitochondria and lysosomes into the pellet, while the supernatant from this step can be centrifuged again at 100,000 g for 10 minutes to collect a microsomal pellet. The heterogeneity of biological particles makes isolates from differential centrifugation prone to contamination and poor recovery. This issue can be fixed by washing the pellet and repeating the centrifugation protocol, and by further filtering the sample.

Density gradient centrifugation employs a tube packed with a material that forms a gradient of increasing density and viscosity. Various types of media are used for density gradient separation including polyhydric alcohols, polysaccharides, inorganic salts, and silica. The type of matrix used is chosen based on the target molecule. The density gradient matrix allows for more stringent separation of particles with less

contamination**.** The particles move through the matrix at different sedimentation rates and settle out into clean bands.

6.18.6 Uses of Centrifuge

In the clinical laboratory, a centrifuge is applicable for the following purposes and they are-

- Remove cellular elements from blood to provide cell-free plasma or serum for analysis.
- Remove chemically precipitated protein from an analytical specimen.
- Separate protein-bound from free ligand in immunochemical and another assay.
- Separation of the subcellular organelle, DNA, RNA.
- Extract solutes in biological fluids from aqueous to organic solvents.
- Separate lipid components.

6.19 Electrophoretic techniques

The term electrophoresis describes the migration of a charged particle under the influence of an electric field. Many important biological molecules, such as amino acids, peptides, proteins, nucleotides and nucleic acids, possess ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (−). Under the influence of an electric field these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.

The equipment required for electrophoresis consists basically of two items, a power pack and an electrophoresis unit. Electrophoresis units are available for running either vertical or horizontal gel systems. Vertical slab gel units are commercially available and routinely used to separate proteins in acrylamide gels. The gel is formed between two glass plates that are clamped together but held apart by plastic spacers. The most commonly used units are the so-called minigel apparatus (Fig. 10.1). Gel dimensions are typically 8.5 cm wide × 5 cm high, with a thickness of 0.5−1 mm. A plastic comb is placed in the gel solution and is removed after polymerisation to provide loading wells for up to 10 samples. When the apparatus is assembled, the lower electrophoresis tank

buffer surrounds the gel plates and affords some cooling of the gel plates. A typical horizontal gel system is shown in given figure.

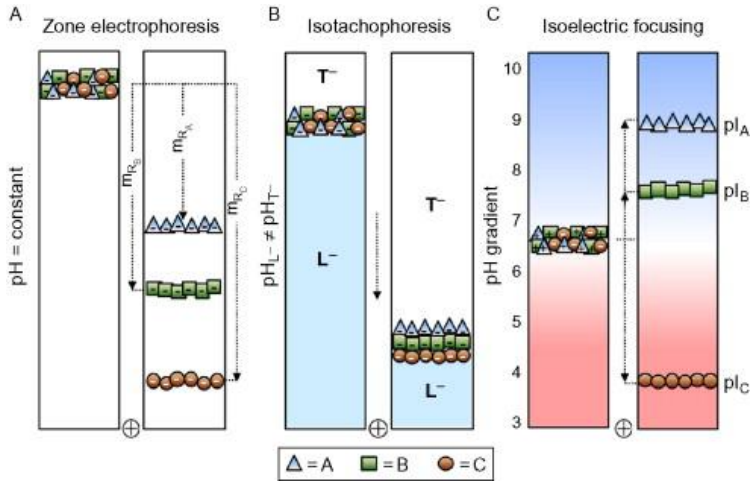

Fig. 16 Electrophoretic technique

6.19.1 Principle of electrophoresis

Electrophoresis is defined as the migration of charged particles, under the influence of an electric field at a definite pH. In a mixture of proteins, each protein with its electrical charge will move differently in an electric field. This electrophoretic mobility depends on the pH of the medium, strength of the field, net charge of the molecule and size/shape of the molecule. Electrophoresis is used for the analysis of large molecules (proteins and nucleic acids) and simpler charged molecules (peptide, simpler ions).

6.19.2 Types of electrophoresis

The following are different types or electrophoresis.

- Paper electrophoresis
- Cellulose acetate electrophoresis
- Capillary electrophoresis
- Gel electrophoresis

Agarose gel electrophoresis, Polyacrylamide Gel Electrophoresis (SDS PAGE, Native PAGE and two- dimensional electrophoresis).

6.19.3 Polyacrylamide gel electrophoresis

Polyacrylamide gel is prepared from acrylamide and bis-acrylamide in a suitable buffer. Polymerization of Acrylamide and bisacrylamide is achieved by a free radical reaction promoted by N,N,N',N'tetramethylethylenediamine (TEMED). This free radical process is initiated by Ammonium per sulfate (APS) used in gel. Acrylamide and bisacrylamide monomers are weak neurotoxin whereas, the polymerised polyacrylamide is non-toxic. While handling acrylamide solutions, care should be exercised and spectacles, gloves and mask should be worn.

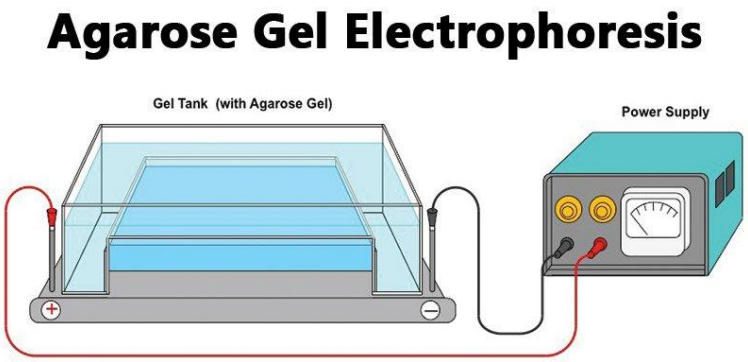

Fig. 17 Polyacrylamide gel electrophoresis

6.19.4 Sodium dodecyl Sulphate (SDS) polyacrylamide gel electrophoresis

Sodium dodecyl Sulphate Polyacrylamide Gel Electrophoresis(SDS-PAGE) is an electrophoretic technique very commonly used in Biochemistry, Molecular biology and forensic science. This technique was first described by Laemmli in the year 1970 and till now dominates in scientific research.

6.19.5 Factors influencing Electrophoresis: Movement of proteins depends on various aspects. Within the gel the molecules must pass through as they are moving from one pole to another. The smaller molecules can weave in and out of the matrix of the gel with more ease, compared with larger molecules. As a general rule, the molecules move rapid if it has more net charge, has a shape of ball and shorter diameter

6.19.6 The buffer pH

It will influence the direction and rapid of the protein migration. Movement of proteins depends on various aspects; one of them is the charges on the proteins. Proteins are sequence of amino acids that can be ionized depend on their acid or basic character. The protein's net electric charge is the sum of the electric charges found on the surface of the molecule as a function of the environment.

The rate of migration will depend on the strength of their net surface charges: The protein that carries more +ve charges will move towards the cathode at a faster rate. On the contrary, the protein that carries more -ve charges will move towards the anode at a faster rate. In this regard, proteins can be separated based on their electric charges. Depending on the pH of the buffer, proteins in a sample will carry different charges. At the pI (isoelectric point) of a specific protein, the protein molecule carries no net charge and does not migrate in an electric field. At pH above the pI, the protein has a net negative charge and migrates towards the anode. At pH below the pI the protein obtains a net positive charge on its surface and migrates towards the cathode.

6.19.7 The buffer ionic strength

It influences the proportion of the current carried by the proteins
At low ionic strength the proteins will carry a relatively large proportion of the current and so will have a relatively fast migration. At high ionic strength, most of the current will be carried by the buffer ions and so the proteins will migrate relatively slowly. An analogy might be useful in visualizing this effect of ionic strength. Imagine a bank where there are two counters – one for deposits the anode) and one for withdrawals (= the cathode), with electrons being the money. The ions may be considered as customers waiting to be served at either counter, which one can visualize as being at opposite ends of the banking hall.

In electrophoresis, therefore, a low ionic strength is preferred as it increases the rate of migration of proteins. A low ionic strength is also preferred as it gives a lower heat generation. Assuming a constant voltage, if the ionic strength is increased, the electrical resistance decreases but the current will increase. A high ionic strength buffer will therefore lead to greater heat generation, and so a low ionic strength is preferred.

6.19.7 The voltage gradient

The rate of migration will depend on the voltage gradient: There is more voltage gradient in the electric field, protein will move towards the anode (or the cathode) at a faster rate.

6.19.8 Electo-osmosis

Liquid's relative move upon solid medium in an electric field is called electo-osmosis. In applied electric field, electo-osmosis distorts the sample stream and limits the separation. For example, Paper electrophoresis has poor resolution because of electo-osmosis. The surface of paper has -e, so the buffer has +e derived from hydrogen ions because of electrostatic induction. Then +e drive buffer to cathode in electric field,these flows distort the electrophoretic migration of sample by causing a varying residence time. Thus, sample will move more or less than normal.

6.20 Summary

Under this unit we have discussed enzymes and their purification. Enzymes are proteins that act as biological catalysts (biocatalysts). Catalysts accelerate chemical reactions. The molecules upon which enzymes may act are called substrates, and the enzyme converts the substrates into different molecules known as products. Almost all metabolic processes in the cell need enzyme catalysis in order to occur at rates fast enough to sustain life. Metabolic pathways depend upon enzymes to catalyze individual steps. The study of enzymes is called enzymology and a new field of pseudoenzyme analysis has recently grown up, recognising that during evolution, some enzymes have lost the ability to carry out biological catalysis, which is often reflected in their amino acid sequences and unusual 'pseudocatalytic' properties.

Enzymes are known to catalyze more than 5,000 biochemical reaction types. Other biocatalysts are catalytic RNA molecules, called ribozymes. Enzymes' specificity comes from their unique three-dimensional structures. Like all catalysts, enzymes increase the reaction rate by lowering its activation energy. Some enzymes can make their conversion of substrate to product occur many millions of times faster. An extreme example is orotidine 5'-phosphate decarboxylase, which allows a reaction that would otherwise take millions of years to occur in

milliseconds. Chemically, enzymes are like any catalyst and are not consumed in chemical reactions, nor do they alter the equilibrium of a reaction. Enzymes differ from most other catalysts by being much more specific. Enzyme activity can be affected by other molecules: inhibitors are molecules that decrease enzyme activity, and activators are molecules that increase activity. Many therapeutic drugs and poisons are enzyme inhibitors. An enzyme's activity decreases markedly outside its optimal temperature and pH, and many enzymes are (permanently) denatured when exposed to excessive heat, losing their structure and catalytic properties.

Terminal questions

Q.1. Describe the properties of enzymes.

Answer:----------

Q.2. Explain the purification of enzymes

Answer:----------

Q.3 Explain the mechanism of enzyme action.

Answer:----------

Q.3. Write short notes on the followings.

(i) Gel Filtration Chromatography

(ii) Ion exchange chromatography

Answer:----------

Q.4. Explain the principle of affinity chromatography.

Answer:----------

Q.5. Explain the principle of ion exchange chromatography.

Answer:----------

Q.6 Describe the sodium dodecyl Sulphate (SDS) polyacrylamide gel electrophoresis

Answer:----------

Further readings

1. Biochemistry- Lehninger A.L.
2. Biochemistry –J.H.Weil.
3. Biochemistry fourth edition-David Hames and Nigel Hooper.
4. Textbook of Biochemistry for Undergraduates - Rafi, M.D.
5. Biochemistry and molecular biology- Wilson Walker.