

Enzymes

Learning Objectives

This unit describes the classification and functional organization of enzymes, the biocatalysts, present in cells and tissues of plant and animal. Further the mechanisms by which these enzymes functions and their inhibition by different compounds are also described.

Classification and Specificity of Enzymes

Enzymes

Enzymes are the functional units of cell metabolism. Acting in organized sequences, they catalyze the hundreds of stepwise reactions by which nutrient molecules are degraded, chemical energy is converted and transformed, and cell macromolecules are made from simple precursors.

Nature of Enzymes

Enzymes are soluble, colloidal catalysts formed by living cells, specific in action and protein in nature. They have molecular weights ranging from about 12,000 to over 1 million. They are therefore very large compared with the substrates or functional groups they act up on.

They are inactive at 0°C and destroyed by moist heat at 100°C. Enzymes have extraordinary catalytic power, which is generally far greater than that of synthetic catalysts. They have a high degree of specificity for they are biocatalysts which they accelerate specific chemical reactions without formation of by-products, and they function in dilute aqueous solutions under very mild conditions of temperature and pH.

Types of enzyme

Some enzymes consist only of polypeptides and contain no chemical groups other than amino acid residues; E. g. pancreatic ribonuclease.

Other enzymes, however, require for activity an additional chemical component called a **cofactor** or an organic molecule called **coenzyme**.

The protein part of an enzyme is called as **apoenzyme**. •A complete, catalytically active enzyme together with its coenzyme or metal is called **holoenzyme**.

In some enzymes the coenzyme or metal ion is only loosely and transiently bound to the protein, but in others it is called a prosthetic group. Coenzymes and metal ions are stable on heating, whereas the protein part of an enzyme is denatured by heat.

Cofactor

The cofactor may be either inorganic, such as Fe²⁺, Mn²⁺ or Zn²⁺ ions. Some enzymes require both a coenzyme and one or more ions for their activity. In some enzymes the coenzyme or metal ion is only loosely and transiently bound to the protein, but in others it is called a prosthetic group.

Some Enzymes containing Cofactors

Cofactors	Associated enzymes
Fe ²⁺ or Fe ³⁺	Cytochrome oxidase Catalase Peroxidase
Cu ²⁺	Cytochrome oxidase
Zn ²⁺	DNA polymerase Carbonic anhydrase Alcohol dehydrogenase
Mg ²⁺	Hexokinase Glucose 6- Phosphatase
Mn ²⁺	Arginase

	Pyruvate kinase (also requires Mg ²⁺)
K ⁺	Urease
Ni ²⁺	Nitrate reductase
Se ²⁺	Glutathione peroxidase

Coenzymes

Some enzymes need a complex organic molecule called a coenzyme. Coenzymes function as transient carriers of specific functional groups.

Coenzymes Serving as Transient Carriers of specific atoms or Functional Groups

Type of <u>Coenzyme</u>	Items transferred
Thiamine pyrophosphate	Aldehydes
Flavin <u>adenine</u> dinucleotide	Hydrogen atoms
<u>Nicotinamide adenine</u> dinucleotide	Hydride ion (H ⁻)
<u>Coenzyme A</u>	Acyl groups
<u>Pyridoxal phosphate</u>	Amino groups
5'- Deoxyadenosylcobalamine	H atoms and alkyl groups
Biocytin	CO ₂
Tetrahydrofolate	Other one - carbon groups

Nomenclature of Enzymes

Many enzymes have been named by adding the suffix-ase to the name of their substrate.

e.g. Urease catalyzes the hydrolysis of urea

Arginase catalyzes the hydrolysis of arginine.

But many enzymes have been given names that do not denote their substrates

e.g. Pepsin

The same enzyme is known by two or more names or that two different enzymes have been given the same name.

Classification

Enzymes are classified into six major classes according to the reactions they catalyze.

Class 1. Oxidoreductases: These are enzymes which catalyze the reduction or oxidation of a molecule. The oxidation is the reverse of reduction and that an enzyme has to catalyze the forward and reverse reactions to the same degree and hence named "oxidoreductase."



e.g. Malate dehydrogenase

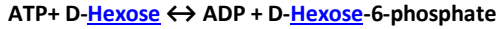


Class 2. Transferases: These enzymes catalyze the transfer of a group of atoms from one molecule to another. The common names are formed as acceptor group transferases. e.g. Hexokinase involves the transfer of a phosphate between ATP and a sugar molecule.

A.R+ B= A+ B.

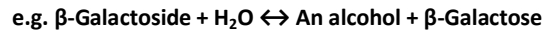
e.g. Hexokinase involves the transfer of a phosphate between ATP and a sugar molecule.

Hexokinase



Class3. Hydrolases: These enzymes catalyse the hydrolysis of various bonds (and their reverse reactions). The hydrolysis of an ester would be an example of such a reaction.

β -Galactosidase



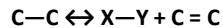
Class 4. Isomerases: These enzymes catalyze the conversion of a molecule into an isomer.

e.g cis-trans interconversion of maleate and fumarate

Class 5. Lyases: Reactions which add a small molecule such as water or ammonia to a double bond (and the reverse, elimination, reactions) are catalyzed by lyases.

X Y

| |

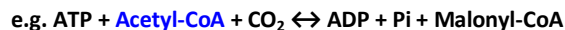


Fumerase

e.g Fumerate + H₂O ↔ L Malate

Class 6 Ligases: These enzymes catalyze reactions which make bonds to join together (ligate) smaller molecules to make larger ones.

Acetyl-CoA carboxylase

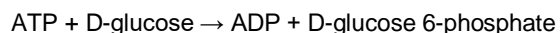


Each of these classes has more specific subclasses and each subclass has sub-sub classes as well. The key to using this classification scheme is to look at the reaction the enzyme catalyzes, decide which type of reaction it is, and apply the appropriate name.

Specific enzyme names are systematically derived by specifying the **substrate** (the molecule being acted upon -- the reactant), the type of reaction, and appending the suffix *ase*.

Each enzyme is assigned a four-digit classification number and a systematic name, which identifies the reaction catalyzed. An example is the naming of the enzymes catalyzing the reaction.

e.g. E.C.2.7.1.1 Hexokinase.



The formal systematic name of this enzyme is ATP: glucose phosphotransferase, which indicates that it catalyzes the transfer of a phosphate group from ATP to glucose. It is placed in Class 2 and its classification number is 2.7.1.1, where the first digit stands for the class 2 name transferase, the third digit for the sub-subclass (phosphotransferases with a hydroxy1 group as acceptor), and the fourth digit for D-glucose as the phosphate group acceptor. The trivial name is hexokinase.

ENZYME SPECIFICITY

Enzymes exhibit different levels of specificity for substrates. The degree of enzyme specificity is determined by the active site. Some active sites accommodate only one particular compound. While others can accommodate a “family” of closely related compounds.

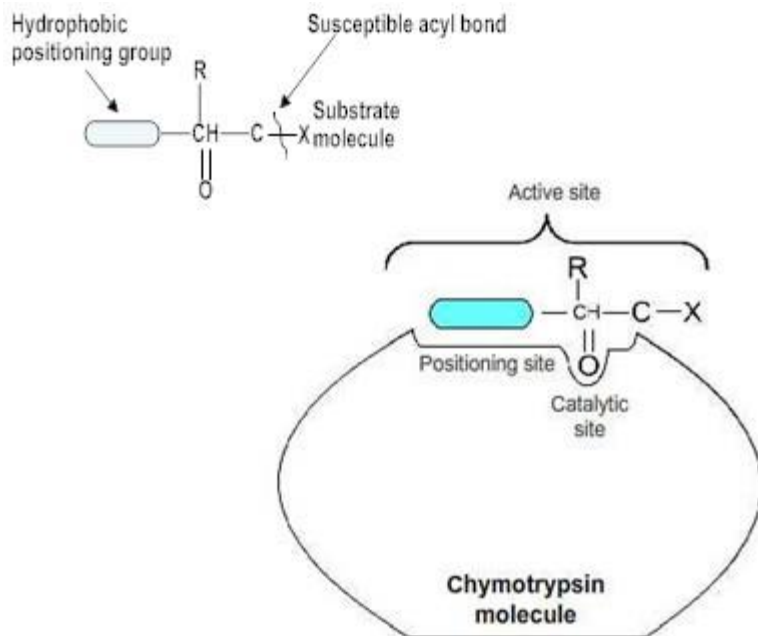
Types of enzyme specificity:

1. **Absolute specificity:** Absolute specificity means an enzyme will catalyze a particular reaction for only one substrate. This most restrictive of all specificity is not common.

Urease is an enzyme with absolute specificity.

2. **Stereochemical specificity:** Such specificity means an enzyme can distinguish between stereoisomers. Chirality is inherent in an active site because amino acids are chiral compounds. L-Amino-acid oxidase will catalyze reactions of L-amino acids but not of D-amino acids.
3. **Group specificity:** Such specificity involves structurally similar compounds that have the same functional groups. Chymotrypsin hydrolyses peptide bond in which the carboxyl group is contributed by aromatic amino acids such as tyrosin, tryptophan etc. Carboxypeptidase is group-specific; it cleaves amino acids, one at a time, from the carboxyl end of the peptide chain.
4. **Linkage or bond specificity:** Such specificity involves a particular type of bond, irrespective of the structural features in the vicinity of the bond. Phosphatases hydrolyze phosphate ester bonds in all types of phosphate esters. Pepsin, chymotrypsin and trypsin on peptide bonds. Linkage specificity is the most general of the specificities considered.

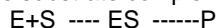
Group specificity



Mechanism of Enzyme Action and Purification

Mechanism of Enzyme action

Explanations of how enzymes function as catalysts in biochemical systems are based on the concepts of an enzyme active site and enzyme substrate complex formation.



The active site is the relatively small part of an enzyme's structure that is actually involved in catalysis.

The active site in an enzyme is a three-dimensional entity formed by groups that come from different parts of the protein chain(s); these groups are brought together by the folding and bending of the protein. The active site is usually a "crevice like" location in the enzyme.

Catalysts offer an alternative pathway with lower activation energy through which a reaction can occur. In enzyme controlled reactions, this alternative pathway involves the formation of an enzyme substrate complex as an intermediate species in the reaction when a substrate binds to the active site of an enzyme. Within the enzyme substrate complex, the substrate encounters more favorable reaction conditions than if it were free. The result is faster formation of product.

Induced fit model

Experimental evidence indicates that many enzymes have flexibility in their shapes. They are not rigid and static; there is constant change in their shape. The **induced fit model** is used for this type of situation.

The induced fit model allows for small changes in the shape or geometry of the active site of an enzyme to accommodate a substrate. This model is a result of the enzyme's flexibility; it adapts to accept the incoming substrate.

This model, gives an explanation for the active site properties of an enzyme because it includes the specificity of the lock and key model coupled with the flexibility of the enzyme protein.

The forces that draw the substrate into the active site are many of the same forces that maintain tertiary structure in the folding of peptide chains. Electrostatic interactions, hydrogen bonds, and hydrophobic interactions all help attract and bind substrate molecules.

For example, a protonated (positively charged) amino group in a substrate could be attracted and held at the active site by a negatively charged aspartate or glutamate residue.

Alternatively, cofactors such as positively charged metal ions often help bind substrate molecules.

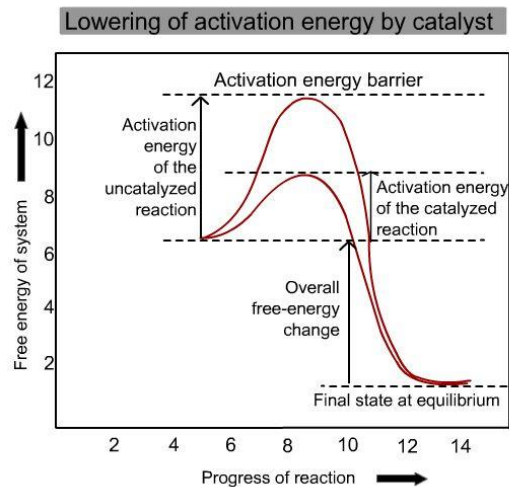
Enzyme kinetics

The energy content of individual molecules in a population at constant temperature varies greatly and may be represented by a bell-shaped curve.

Some molecules are very rich in energy, some are very poor, but most have an energy content near a mean. A chemical reaction, such as A and P, takes place because a certain fraction of A molecules at any given instant possesses more internal energy than the rest of the population, sufficient to bring them to the top of the “energy hill” to a reactive form called the transition state .

The activation energy of a reaction is the amount of energy in calories required to bring all the molecules in 1 mol of a substance at a given temperature state at the top of the energy barrier. At this point there is equal probability for them to undergo reaction to form the products or to fall back into the pool of unreacted A molecules.

The rate of any chemical reaction is proportional to the concentration of the transition- state species. Thus the rate of a chemical reaction will be very high if a large fraction of A molecules is in the energy-rich transition state but very low if only a small fraction of A is in the transition state.



There are two general ways in which the rate of a chemical reaction can be increased.

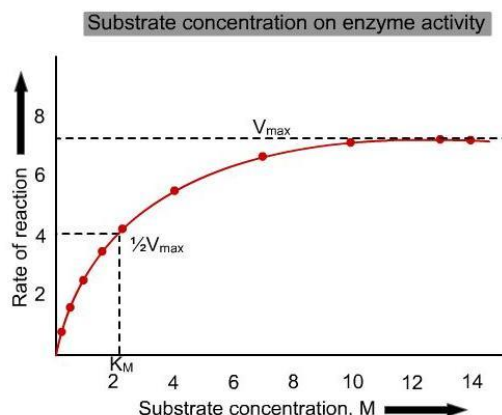
One is to increase the temperature, which increases the thermal motion of the molecules and thus increases the fraction having sufficient internal energy to enter the transition state. Usually the rate of a chemical reaction is approximately doubled by a 10°C rise in temperature.

The second way to accelerate a chemical reaction is to add a [catalyst](#). Catalysts accelerate chemical reactions by finding a lower “pass” over the energy barrier. The [catalyst](#), designated C, combines transiently with the reactant A to produce a new complex or compound CA whose transition state has a much lower activation energy than the transition state of A in the uncatalyzed reaction. The [catalyst](#) releases the free [catalyst](#), which can then combine with another molecule of A and repeat the cycle. In this way catalysts lower the activation energy of chemical reaction, allowing a much larger fraction of the molecules in a given population to react per unit time than the absence of the [catalyst](#).

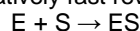
Factors affecting Enzyme catalysed reactions

1. Effect of substrate concentration

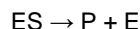
The effect of varying the substrate concentration on the initial rate of an enzyme-catalyzed reaction when enzyme concentration is held constant is as follows: At very low concentrations of substrate the rate of the reaction will increase with an increase in the substrate concentration. At higher substrate concentrations, the rate increases by smaller and smaller amounts. Finally, a point will be reached beyond which there are only vanishingly small increases in the reaction rate with increasing substrate concentration. No matter how high the substrate concentration is raised beyond this point, the reaction rate will approach but never quite reach a plateau. At this plateau, called the maximum rate (V_{max}), the enzyme is "saturated" with its substrate and cannot function faster .



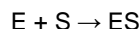
Leonor Michaelis and Maud Menten in 1913, postulated that the enzyme E first combines reversibly with its substrate S to form an enzyme-substrate complex ES in a relatively fast reversible reaction.



The ES complex then breaks down in a second reversible reaction, which is slower, to yield the reaction product P and the free enzyme E



Since the second reaction is the rate-limiting step, the overall rate of the enzyme-catalyzed reaction must be proportional to the concentration of the enzyme-substrate reaction, the enzyme exists in two forms, the free of the catalyzed reaction will obviously be at a maximum when virtually all of the enzyme is present as the ES complex and the concentration of free enzyme E is vanishingly small. This condition will exist at a very high concentration of the substrate, since, by the Law of mass action, the equilibrium of the first reaction will be pushed to the right when we increase the concentration of S



If we increase S to high enough levels, essentially all the free enzyme E will have been converted into the ES form. In the second reaction of the catalytic cycle, the ES complex breaks down continuously and rapidly, to yield the product P and the free enzyme. But if the concentration of S is high enough, the free enzyme E will immediately combine with another molecule of S. Under these conditions a steady state is achieved in which the enzyme is always saturated with its substrate and the reaction rate is maximum.

Thus is the relationship between the substrate concentration and the rate of an enzymatic reaction, it is difficult to say from the closer and closer approach of the reaction rate to the maximum velocity V_{max} exactly what substrate concentration is required to attain V_{max} . However, because the curve expressing this relationship has the same general shape for most enzymes (it is a rectangular hyperbola Fig.13.3). Michaelis and Menten defined a constant, today designated K_M that is useful in establishing the precise relationship between the substrate concentration and the velocity of the enzyme-catalyzed reaction. K_M , the Michaelis - Menten constant, is defined as the concentration of the specific substrate at which a given enzyme yields one-half its maximum velocity. It is mathematically expressed by the Michaelis - Menten equation.

$$V_o = \frac{V_{max} [S]}{K_M + [S]}$$

where V_o = Initial rate at substrate concentration $[S]$

V_{max} = Maximum rate

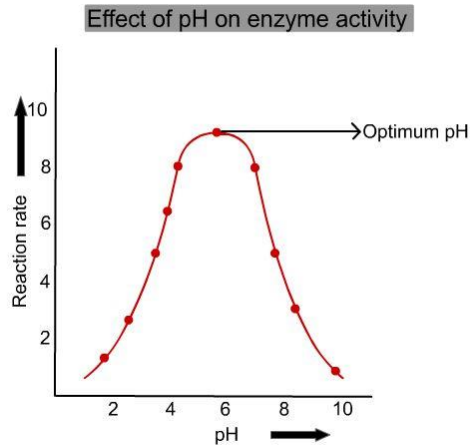
K_M = Michaelis-Menten constant of enzyme for particular substrate

$[S]$ = substrate concentration

This equation was derived by Michaelis and Menten starting from the basic hypothesis that the rate-limiting step in enzymatic reactions is the breakdown of the ES complex to form the product and the free enzyme.

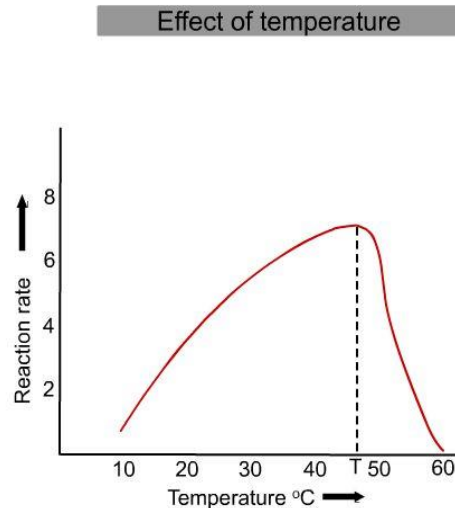
Effect of pH

Enzymes have a characteristic optimum pH at which their activity is generally between 5 and 9. The optimum pH of pepsin:1.5, salivary amylase: 6.8., trypsin: 8.0,and intestinal :7.8. A small change in pH may inhibit enzyme activity. Pepsin work in acid medium and it is in activated by making the medium alkaline. Diagram explains the effect of pH on enzyme activity.



Effect of temperature

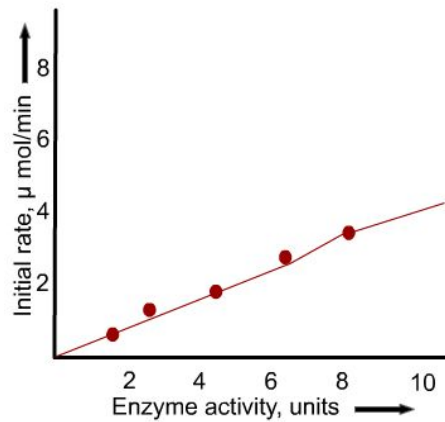
The rise in temperature accelerates the rate of enzyme reaction but at the same time causes inactivation of the enzyme due to denaturation. At optimum temperature the activity of enzyme is maximum. It is around 40°C. If the temperature is lowered the rate of an enzyme reaction is decreased. At 0°C, most enzymes are practically inactive.



Effect of Concentration of enzyme

The rate of enzyme action increases with increase in concentration of enzyme.

Effect of enzyme concentration vs. reaction rate



Quantitative assay of enzymes

The amount of an enzyme in a given solution or tissue extract can be assayed quantitatively in terms of the catalytic effect it produces.

It is necessary to know the overall equation of the reaction catalyzed, an analytical procedure for determining the disappearance of the substrate or the appearance of the reaction products., whether the enzyme requires cofactors such as metal ions or coenzymes, the dependence of the enzyme activity on substrate concentration, i.e., the K_m value, the optimum pH, and a temperature zone in which the enzyme is stable and has high activity.

Enzymes are assayed at their optimum pH, at optimum temperature, usually within the range 25 to 38°C, and with a near-saturating concentration of substrate. Under these conditions the initial reaction rate is usually proportional to enzyme concentration, at least over a given range of enzyme concentration.

Units of measurement

1. Enzyme activity: By international agreement, 1.0 unit of enzyme activity is defined as that amount causing transformation of 1.0 micromole of substrate per minute at 25°C under optimal conditions of measurement. The specific activity is the number of enzyme units per milligram of protein. Carbohydrates, lipids, and proteins are bulk nutrients or macronutrients.

2. The specific activity :It is a measure of enzyme purity: it increase during purification of an enzyme and becomes maximal and constant when the enzyme is in the pure state.

3. Turnover number: The turnover number of an enzyme is the number of substrate molecules transformed per unit time by a single enzyme molecule when the enzyme concentration alone is the rate limiting factor . The enzyme carbonic anhydrase, an important enzyme found in high concentration in the red blood cell, is among the most active of all enzymes, with a turnover number of 36,000,000 per minute per enzyme molecule. It catalyzes the reversible hydration of dissolved carbon dioxide to form carbonic acid an other wise slow reaction: $CO_2 + H_2O \rightleftharpoons H_2CO_3$ ®

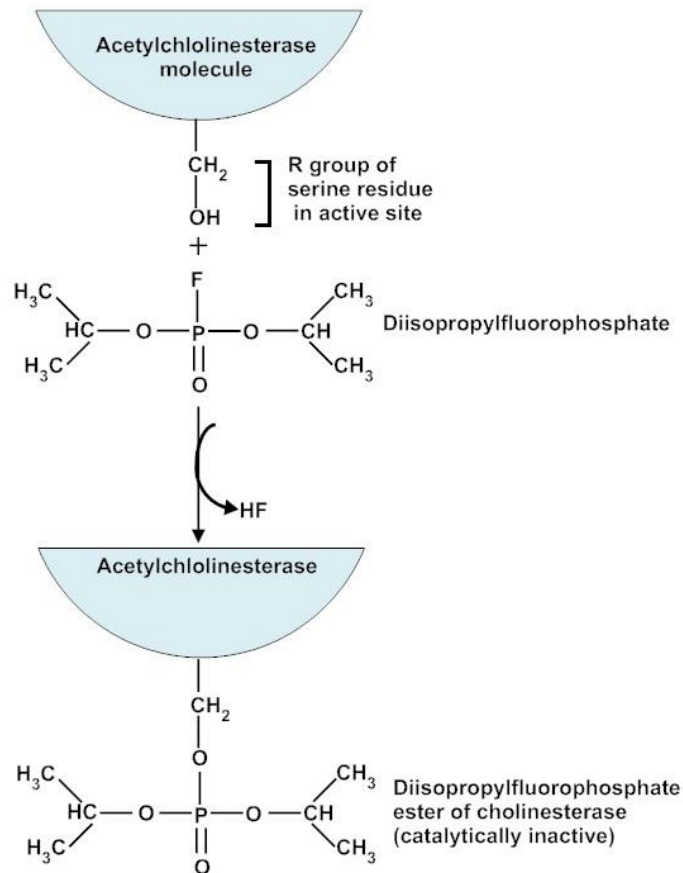
Carbonic anhydrase	36,000,000
- Amylase1,	1,100,000
Galactosidase	12,500
Phosphoglucomutase	1,240

Enzyme Inhibition

Most enzymes can be poisoned or inhibited by certain chemical reagents. There are two major types of enzyme inhibitors: irreversible and reversible.

Irreversible inhibitors: Irreversible inhibitors are those which combine with or destroy a functional group on the enzyme molecule that is necessary for its catalytic activity.

An example of an irreversible inhibitor is the compound diisopropyl fluorophosphate (DFP) which inhibits the enzyme acetyl choline esterase, important in the transmission of nerve impulses. Acetylcholinesterase catalyzes the hydrolysis of acetylcholine, a neurotransmitter substance functioning in certain portions of the nervous system.

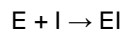


Diisopropylfluorophosphate **Irreversible** **inhibition** **of acetylcholinesterase** **by**

Reversible inhibitors: There are two kinds of reversible inhibitors, competitive and noncompetitive.

Competitive inhibitors

Competitive inhibitors usually resemble the normal substrate in three - dimensional structure. The competitive inhibitor I, simply combine reversibly with the enzyme to form an EI complex



However, the inhibitor I cannot be attacked by the enzyme to form new reaction products.

E.g. [Competitive inhibition](#) of succinate dehydrogenase by the malonate.

This enzyme catalyzes the removal of two hydrogen atoms from each of the two methylene ($-\text{CH}_2-$) groups of succinate to form fumarate. Succinate dehydrogenase is inhibited by malonate, which resembles succinate in having two ionized carboxyl groups pH 7.0 but differs in having only three carbon atoms. However, malonate is not

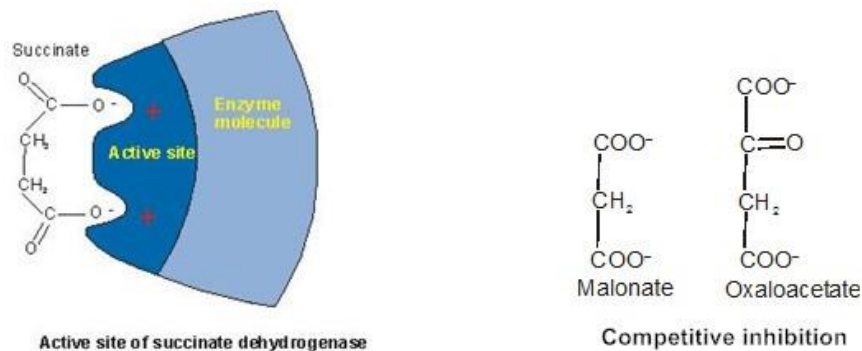
dehydrogenated by succinate dehydrogenase ; it simply occupies the active site keeping it from acting on its normal substrate The inhibition by malonate can be reversed by increasing the concentration of succinate

CH₂-COOH Succinate Dehydrogenase CH-COOH

| + FAD → || + FADH₂

CH₂-COOH HOOC-HC

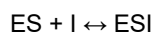
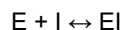
Succinate Fumerate



Another compound that may act as competitive inhibitors of succinate dehydrogenase is oxaloacetate. From this structural relationship it has been concluded that the catalytic site of succinate dehydrogenase has two appropriately spaced positively charged groups capable of attracting the two negatively charged carboxylate groups of the succinate anion. The catalytic site of succinate dehydrogenase thus shows complementarity to the structure of its substrate.

Noncompetitive inhibitor

A non-competitive inhibitor doesn't attach itself to the active site, but attaches somewhere else on the enzyme. By attaching somewhere else it affects the structure of the enzyme and so the way the enzyme works. Because there isn't any competition involved between the inhibitor and the substrate, increasing the substrate concentration won't help. Non competitive inhibitors bind reversibly to both the free enzyme and the ES complex to form the inactive complex EI and ESI:



The inhibitor doesn't affect the ability of the substrate to bond with the active site, but stops it reacting once it is there. The inhibitor causes the shape of the active site. The inhibitor attaches to a side group in the protein chain, and affects the way the protein folds into its tertiary structure. That in turn changes the shape of the active site. If the shape of the active site changes the substrate can't attach to it any more. Some non-competitive inhibitors attach irreversibly to the enzyme, and therefore stop it working permanently. Others attach reversibly.

e.g Heavy metal poisoning

Compounds containing heavy metals such as lead, mercury, copper or silver are poisonous. This is because ions of these metals are non-competitive inhibitors for several enzymes. Silver ions react with -SH groups in the side groups of cysteine residues in the protein chain:

There isn't enough electro negativity difference between silver and sulphur for a full ionic bond and so the bond can be considered as covalent. If the cysteine residue is somewhere on the protein chain which affects the way it folds into its tertiary structure, then altering this group could have an effect on the shape of the active site, and so stop the

enzyme from working. The $2+$ ions from, for example, mercury, copper or lead can behave similarly - also attaching themselves to the sulphur in place of the hydrogen.

Purification of Enzymes

Enzymes are proteinaceous in nature; they can be extracted from living tissues, purified and even crystallized. Under controlled conditions of isolation, they retain their original level of activity and in some cases even exhibit an increased activity. Thus, a purified enzyme can be used to carry on a specific biochemical reaction outside the cell. Enzymes are unstable molecules with a definite physico chemical organization. Even a slight change in this organization reduces the activity of enzyme and sometimes the enzyme is totally inactivated. Therefore, the enzymes have to be isolated under controlled conditions of pH, ionic strength and temperature. Since they are proteinaceous in nature, standard extraction and purification procedures for enzymes are the same as those used for proteins except that the activity of the enzyme is assayed at each of the following four steps of extraction and purification.

Isolation and Purification of Enzymes

Enzymes are proteinaceous in nature; they can be extracted from living tissues, purified and even crystallized. Under controlled conditions of isolation, they retain their original level of activity and in some cases even exhibit an increased activity. Enzymes are unstable molecules with a definite physico chemical organization. Even a slight change in this organization reduces the activity of enzyme and sometimes the enzyme is totally inactivated. Therefore, the enzymes have to be isolated under controlled conditions of pH, ionic strength and temperature. Since they are proteinaceous in nature, standard extraction and purification procedures for enzymes are the same as those used for proteins except that the activity of the enzyme is assayed at each of the following four steps of extraction and purification.

Centrifugation

The enzyme extract is centrifuged to remove cell debris, cell organelles and sometimes other molecular aggregates, leading to partial purification of enzymes. It also helps in characterization of an enzyme, since, depending upon its mass and shape the enzyme will move through a solution at a definite speed and occupy a characteristic position in the centrifuge tube. For most cytosolic enzymes, centrifugation at about 30,000 g for 30 minutes is good enough to obtain a fair amount of activity in the supernatant. However, if the enzyme is located in a specific cell organelle, an extract rich in that organelle is prepared through 'preparative centrifugation'. (Centrifugation for different durations at different velocities allows the cell organelles to sediment according to their sizes). All centrifugation operations are conducted in cold ($0-4^{\circ}\text{C}$).

Precipitation

Enzymes and other proteins are highly charged molecules, and can be precipitated with appropriate charge neutralizing chemicals. Once their charges are broken, they form aggregates and settle down as precipitate.

Acids and Bases

When, an acid or base is added, the enzyme protein can be brought to its isoelectric pH. At this pH, there is no net charge on enzyme molecules and electrostatic repulsion between them is low so that they tend to aggregate. Therefore, adjusting the pH to the isoelectric point of a protein causes its precipitation. Acids and bases, however, often inactivate the enzyme, so that their use for precipitation is not recommended in most cases.

Salts

Salts are used for precipitation in a process called 'salting out'. Salts can change the structure of the solvent, which can lead to large changes in protein [conformation](#) by altering the electrostatic interactions between charged groups on the protein surface. The salt also competes with the protein for solvent molecules and thereby lowers its solution.

Organic solvents

Some organic solvents like acetone, methanol and ethanol are also used for enzyme precipitation, since water miscible solvents decrease the solubility of proteins, leading to precipitation. They are cooled upto $40 \rightarrow 60^{\circ}\text{C}$ before

their use, and precipitation is carried out at 0°C, because precipitation at room temperature causes denaturation of the enzyme, in most cases. Organic solvents are added drop by drop to avoid local concentration.

Water soluble non ionic polymers

Water soluble non ionic polymers such as polyethylene glycol, alginate, pectate, carboxymethyl cellulose, polyacrylic and polymeta acrylic acids, etc. also cause enzyme precipitation. Polyethyleneimine is also widely used as protein precipitant at large scale. They primarily act through the removal of solvent sphere of the enzyme protein.

Extraction of Enzymes

Fresh tissue is crushed into a paste with an extraction medium (often a buffer) in a mortar and pestle, or in a tissue homogenizer, or in a blender or by ultrasonic vibrations (sonication).

The molarity and pH of the buffer is suitably adjusted (which may vary for different enzymes) to achieve maximum solubility and activity of the enzyme.

EDTA (ethylene diamine tetra acetic acid) is often included in the extraction medium to remove heavy metals (which otherwise inhibit enzyme activity), and for disrupting the membranes of cells and cell organelles. Detergents such as Triton-X are also used sometimes to solubilise the membranes.

Many enzyme proteins contain disulfide (S-S) bonds due to the presence of cysteine residues, which are easily broken during enzyme extraction leading to loss of enzyme activity. To overcome this problem are added, thiols such as mercaptoethanol whose sulfhydryl (-SH) group is able to maintain the S-S linkage in enzymes.

If the extract is not homogeneous, the homogenate (extract) is filtered to remove cell debris, fibres etc., otherwise filtration may be avoided. All operations of extraction and purification are generally carried out in cold (0-4°C), since most of the enzymes get inactivated at higher temperatures.

Purification

Purification of the extracted enzyme can be done using dialysis or chromatography.

a) Dialysis for Enzyme Purification

Dialysis is the process that is used to remove small molecules from enzyme. For this, enzyme precipitate obtained in previous step is dissolved in a small quantity of buffer solution in which the enzyme was originally extracted. The solution is taken in a dialysis bag (may be a cellophane tube) and after sealing securely, the bag is suspended in either distilled water or a buffer of known molarity and ionic composition.

b) Chromatography for Enzyme Purification –

Chromatographic separation of proteins is the most common method of enzyme purification. Following four types of chromatography are available for this purpose:

- (i) adsorption or column chromatography;
- (ii) ion exchange chromatography;
- (iii) gel filtration chromatography and
- (iv) affinity chromatography.

b) Adsorption Chromatography for Enzyme Purification –

In adsorption chromatography, the enzyme solution suspected to contain other proteinaceous impurities is passed through a column of inert material packed in a glass or steel tube. Most commonly used column materials include finely divided solids such as charcoal, silica, alumina, calcium phosphate, hydroxyapatite, etc.

The effluent solution is continuously collected in small fractions of 1.0 to 2.0 ml. The protein in each fraction is estimated by measuring the [absorption](#) at 280 nm using a UV spectrophotometer. The enzyme is also assayed in each fraction. Various spleen enzymes such as basic RNAase, acidic RNAase, acidic DNAse, phosphodiesterase, phosphomonoesterase, etc. are often separated from each other using adsorption chromatography.

For large scale chromatographic separation of enzymes, the process is accelerated by using motors and other mechanical devices for packing the column, for loading the enzyme on the column and for eluting the enzyme.

c) [Electrophoresis](#) for [Enzyme Purification](#) –

[Electrophoresis](#) is a technique in which molecules (enzymes, proteins, amino acids, nucleotides and nucleic acids) are separated by differences in their net charge in the presence of an externally applied electric field.

This technique is routinely used in enzyme purification and isozymes separation in the laboratories, although it has found only limited application at large scale, since the technique is time consuming and is a bit expensive.

Final Step in Processing Enzymes - Most of the commercially available enzyme preparations, purified as above, are concentrated and sterile filtered, after purification. This is done to reduce both, the volume and the microbial contamination of the sample. Often, before storage and transport, the sample is freeze dried with additives such as sugar substrates and dextrans.