2

ENZYME ISOLATION AND PURIFICATION

Introduction

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Introduction

The enzymes are found distributed widely in the living systems and are a major factor in the growth, maintenance and propagation of the living system. In fact any alteration in these "little hinges" may lead to pathological condition and even death. The initial days of development of enzymology were full of controversies about them related to their structure, chemical nature, occurrence and functions. The Pasteur – Liebig controversy has been already mentioned earlier.

Once it was understood that the enzymes have an ability to work in cell free systems, serious attempts were being made about their isolation and purification. Willstatter and his colleagues between 1920 and 1928 carried out the early attempts of purification of enzymes. Dixon and Kodama attempted the purification of xanthine oxidase.

The first enzyme to be crystallized was Urease in 1926 by Sumner. This was followed by the purification and crystallization of proteases like trypsin and pepsin by Northrop. The details will be discussed a little later.

Need Of Isolation Of Enzymes

In general isolation and purification of enzymes is required for the following reasons:

1. It is required for the study about the various aspects of that particular enzyme.
2. It is required for the identification of substrates and the substrate specificity of the enzyme
3. It is required to assess the role of various coenzyme/cofactors on the rate of the reaction.
4. It is required for the researches and as diagnostic components.
5. It is required for therapeutic purposes.
6. It is required for development of commercially exploitable immobilized enzyme systems for the industrial applications, fermentations and value addition processes
7. It is required for leaching of commercially important ores.
8. Pure enzymes are also employed in forensics, food processing and for the synthesis of anti enzymes which have a medicinal value.
9. The inhibition of enzyme studies can indicate potential inhibitors, which may be used to treat metabolic and inheritable disorders.

Before the enzymes are isolated and purified certain general considerations are important; some of which are discussed here.

Extra Cellular Enzymes Are Easy to Isolate Than The Intracellular Enzymes

Irrespective of the source, i.e. plant or animal tissue, extra cellular enzymes are easily collected. Their collection usually does not require the destruction of tissues and therefore reduces the expenses and time required for processes such as homogenization or any other such processes.

Many Enzymes are Present in Various Sources But Some Contain Them in Comparatively Large Amounts:

Most of the enzymes are common to many organisms and are basic requirements for the general metabolism. However different tissues contain different amounts of the same enzyme and a comparison should be made to assess the quantitative contents of the enzyme from which an ideal source can be identified.

The Enzyme Source Has to be Pre-Selected Before it is Commercially Exploited:

The early qualitative and quantitative studies are important before an ideal source is finalized. Many times a host of pilot scale experiments are necessary to determine an ideal source for the enzyme of interest. In some cases the seasonal variations in the yields have to be worked out.

Plant and Microbial Enzymes are Generally Cheaper

Animal tissues are a costlier source for the commercial production of an enzyme isolate. This is primarily because many times the complete animal has to be purchased and slaughtered before a specific tissue or organ can be isolated and utilized as a source of the enzyme. For example rennet required for cheese production required procurement and slaughtering of tender calves to obtain the contents of their fourth pouch which had to be obtained and processed immediately.

A Wide Range of Methods is Available for Isolation and Purification of Enzymes

These vary according to many criteria, which include the source of enzyme, its availability, the economics of the processes, the time factor involved and the availability and requirement of the instrumentation.

The topic of enzyme isolation and purification shall be dealt under following points:

(1) Identification of source of enzymes
(2) General handling procedures for source and crude extracts
(3) Methods for isolation
(4) Methods for purification
(5) Development of assays: qualitative and quantitative
(6) Stabilization and crystallization
(7) Criteria for purity
(8) Methods for preservation of isolated enzymes

(1) Identification of Source of Enzymes

The specificity of the action of enzymes is an important characteristic and their localization in specific tissues and cells or cell organelles is based on their necessity and utility there. Thus it is better to have some basic idea about the role of the enzyme before its actual isolation and purification is employed. It should be understood however that there are some differences within the similar enzymes isolated from different tissues. In general an enzyme may be found in enough amounts where its substrate may exist in sumptuous amounts. The amylases, for example are found in the germinating cereal seeds, while the proteases shall be abundant in germinating pulses.

Table 2.1 Some common sources of enzymes

Enzyme	Common source
Salivary amylase	Salivary juice
Proteases	Germinating pulses
β amylases	Sweet potato
Lipases	Germinating groundnut
α amylases	Germinating cereals
Papain	Papaya fruit
bromelain ficin	Pineapple, Fig.

In the past few decades it has become a common practice to use the microorganisms for isolating enzymes. They have the advantages of

- Growing on cheap media reducing cost of production
- Fast growth leading to lesser time in isolation
- Economic processes of recovery in case of extracellular enzymes.

Some of the commonly used microorganisms and enzymes obtained from them are given in the following table.

Table 2.2 Some Microbial Sources of Enzymes

Enzyme	Microbial source
Amylase	Bacillus subtilis
Acid resistant amylase	Aspergillus niger
Amyloglucosidase	Rhizopus niveus
Cytochrome catalse	Saccharomyces boraldi
Cellulase	Trichoderma viride
Diastase	Aspergillus oryzae
Glucose isomerase	Bacillus coagulans
Glucose oxidase	Aspergillus niger
Invertase	Saccharomyces cerevisiae
Keratinase	Streptomyces fradiae
Lipase	Rhizopus nigricans
Laccase	Coriolus versieolor
Microbial rennet	Mucor species
Naringinase	Aspergillus niger
Penicillinase	Bacillus cereus
Pectinase	Sclerotina libertina
Protease	Streptomyces griseus
5' Phosphodiesterase	Penicillium citrionum

In general the criteria for choosing a particular source for the commercial production of enzyme of interest revolve around the following factors:

It Should Be Cheap

The microbial cells and plant materials are comparatively cheap although some microbial cells may require specially formulated broth or medium for growth. But they are ideal sources for extra cellular enzymes like the amylases, cellulases or proteases. The plant materials, unless it is difficult to cultivate, form a major source. The germinating seeds are many times used to produce amylases and proteases. Animal tissue is comparatively costlier however the slaughterhouse wastes can be effectively used as a source for serum enzymes.

It Should Be Readily Available

A locally available source is readily found when required as compared to one that has to be transported from some other place. The transportation increases the cost, and lessens the available time and if a particular source is rapidly perishable then it will accrue losses in case of transportation and processing delays.

It Should Be Easy to Store And Handle

Many times the raw materials have to be procured in amounts more than that can be immediately processed. In such cases the remaining materials have to be stored for some period before it can be processed. The ideal source in such a case should be easy to store and handle.

It Should Have a Comparatively Longer Shelf Life

When the situation of preprocessing storage of raw materials arises, an ideal raw material has a longer shelf life. This reduces the possible losses that may occur due to its perishability.

IT Should Contain The Enzyme Of Interest In Enough Quantity

The source should contain enough amount of the particular enzyme of interest. This is important to make the isolation and purification processes economical. The comparative studies made by research workers are important guidelines while one chooses a particular source for the enzyme of interest.

It Should Not Generate Any Toxic Waste Products During Its Processing

It has been often seen that when animal tissue based sources are used for enzyme isolation, the byproducts or wastes generated produce hazards if disposal is not proper. This is primarily because a large part of the tissues has to be disposed and may be a source of biohazard due to microbial and chemical action on the tissue waste.

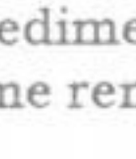
2. General Handling Procedures For Source and Crude Extracts

Once the ideal source is identified and procured, it is many times necessary to pre treat or pre process the same before the actual isolation process is implemented. Many times however the raw material is available in the form of a crude extract or solution, which needs to be processed before isolation and purification of the enzyme. In case the source is a tissue it usually undergoes following processing methods:

(i) Plant Tissue

In general the plant tissues used for enzyme isolation are leaves, however sometimes tender whole plants, bark or roots may be used. The apical tissue or germinal tissue often is the ideal source of enzymes of interest.

Plant Tissue ⟶ *Harvest* ⟶ *Dry* ⟶ *Powder* ⟶ *Use*



Macerate with ideal solvent ⟶ *Filter or Centrifuge* ⟶ *Use*

The tissue is harvested, cleaned and often after the removal of unrequired parts may be processed as such or subjected to shade drying. The partially or fully dried tissue may be then powdered and stored for some time before it is used for the actual isolation process. Sometimes dry powders of seeds and other tissues are used (for example jack bean meal is used for isolation of urease) and in such cases the powders are produced and stored in bottles which can be kept at room temperature for a long period.

(ii) Microbial Cells

The microbial cells are often cultured in liquid media (broths) or on solid media (agar plates) before they are used as a source for the enzymes. If the enzyme is extra cellular, then the broth is centrifuged at a proper "g" value for a specified time to allow the sedimentation of the microbial cells and particulate impurities if any, while the enzyme remains in the supernatant. The supernatant is then used as the crude extract.

Microbial cells ⟶ *Cultivation in enrichment media* ⟶ *Centrifuge* ⟶ *Use Supernatant for extracellular enzymes*

Sedimented Pellet of cells ⟶ *Physiochemical treatments (Enzyme extraction)*

Use extracts for intracellular enzymes (crude)

In case the enzyme is of intracellular localization, the isolated cells of the microorganisms are subjected to homogenization or any feasible physical or chemical method that disrupts its cell wall/ membrane to allow the collection of its cytoplasm as the crude extract.

(iii) Animal Tissue

In case of the animal tissue, which mostly serves as a source of intracellular enzymes, the tissue after isolation has to be kept in cold (icebox) or deep freeze conditions until it is minced and homogenized. The homogenate is often subjected to differential centrifugation before a crude extract is generated. This is mainly to eliminate all sub cellular fractions that may not contain the enzyme and to separate that organelle which contains the enzyme of interest.

(iv) Handling of Crude Extracts

In case of the crude extracts, there are two primary procedures that have to be performed. The first is straining or filtration to remove all particulate matter and

sediments. The second is a primary assay to ensure that the enzyme of interest is present and active. Although a qualitative assay would suffice to determine the presence of the enzyme, many times a pilot quantitative assay is performed and taken as the primary reading on the basis of which the fold purification is determined.

(3) Methods of Isolation

A number of methods have been used for breaking open cells and extracting the cellular contents including the sub cellular organelles. Some of these are briefly described here.

(A) Grinding With Abrasives

Plant tissues or microbial tissue with cell wall is often subjected to grinding with an abrasive material such as alumina or sand. The shear and strain causes the disruption of the cell wall leading to the collection of cell sap as a crude homogenate. Simple pestle- mortar systems or mixers and grinders are used to bring about the desired results.

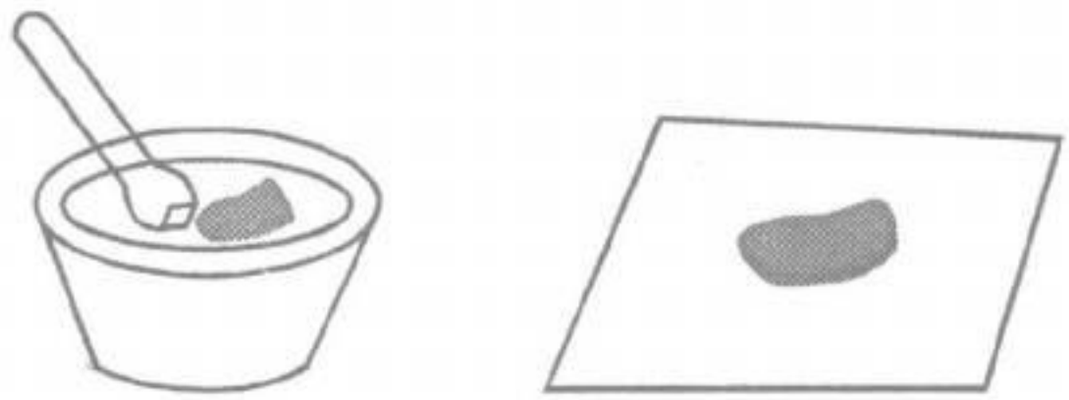


Fig 2.1 Grinding with abrasives

(B) Alternate Freezing And Thawing

This is based on the fact that upon freezing water molecules form ice which expand in size multifold and cause a strain on the cell membrane leading to its disruption. Thawing which causes the melting of ice follows this leading to formation of a crude homogenate.

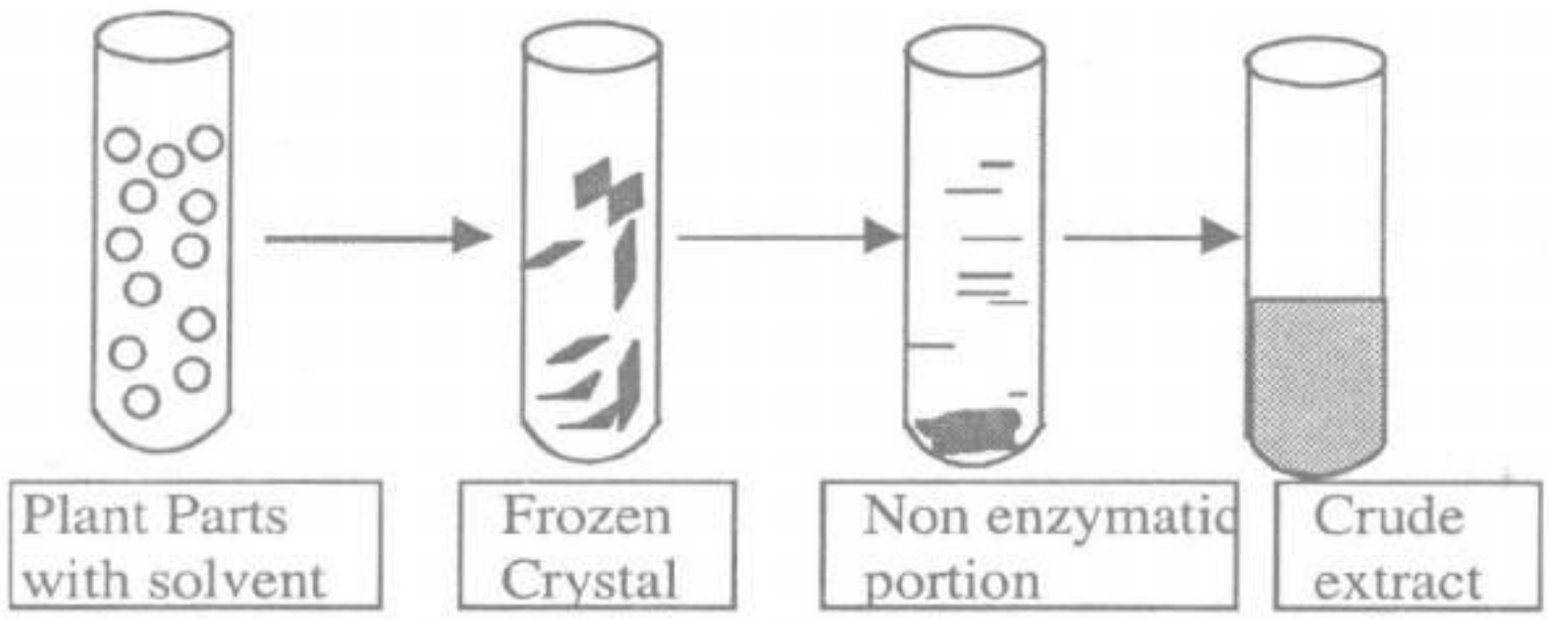


Fig. 2.2 Alternate freezing and thawing

Long periods of Bending

The cells, when subjected to long periods of blending are broken due to the

mechanical forces of collision against the walls of the blender generating a crude homogenate.

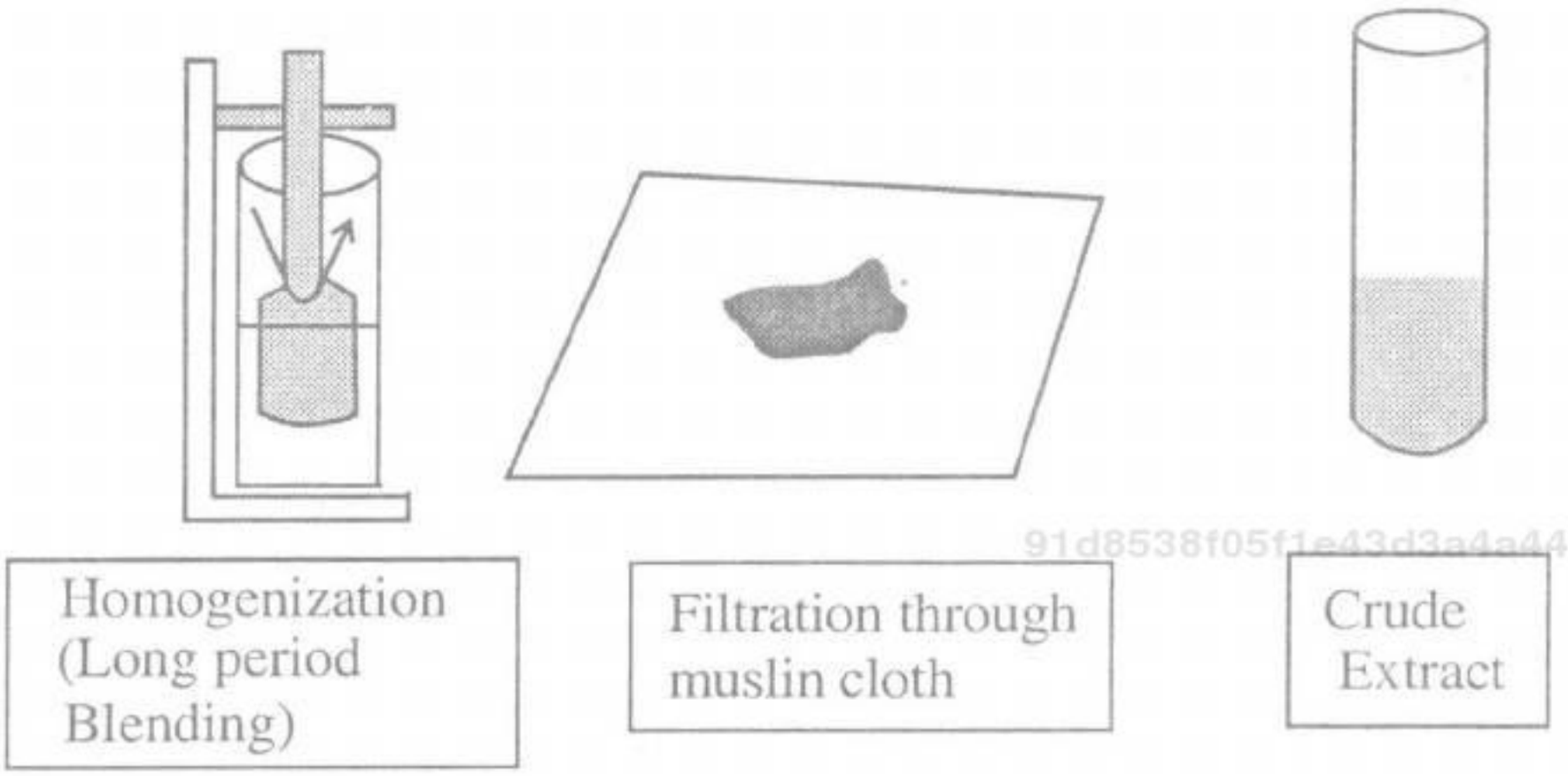


Fig. 2.3 Long periods of blending

(D) Addition Of Glass Beads During Blending

Addition of glass beads or acid washed sand is often employed to increase the mechanical shear which makes the process more effective. Waring blenders are often used in the process. Sometimes instruments with speed adjustments are also employed.

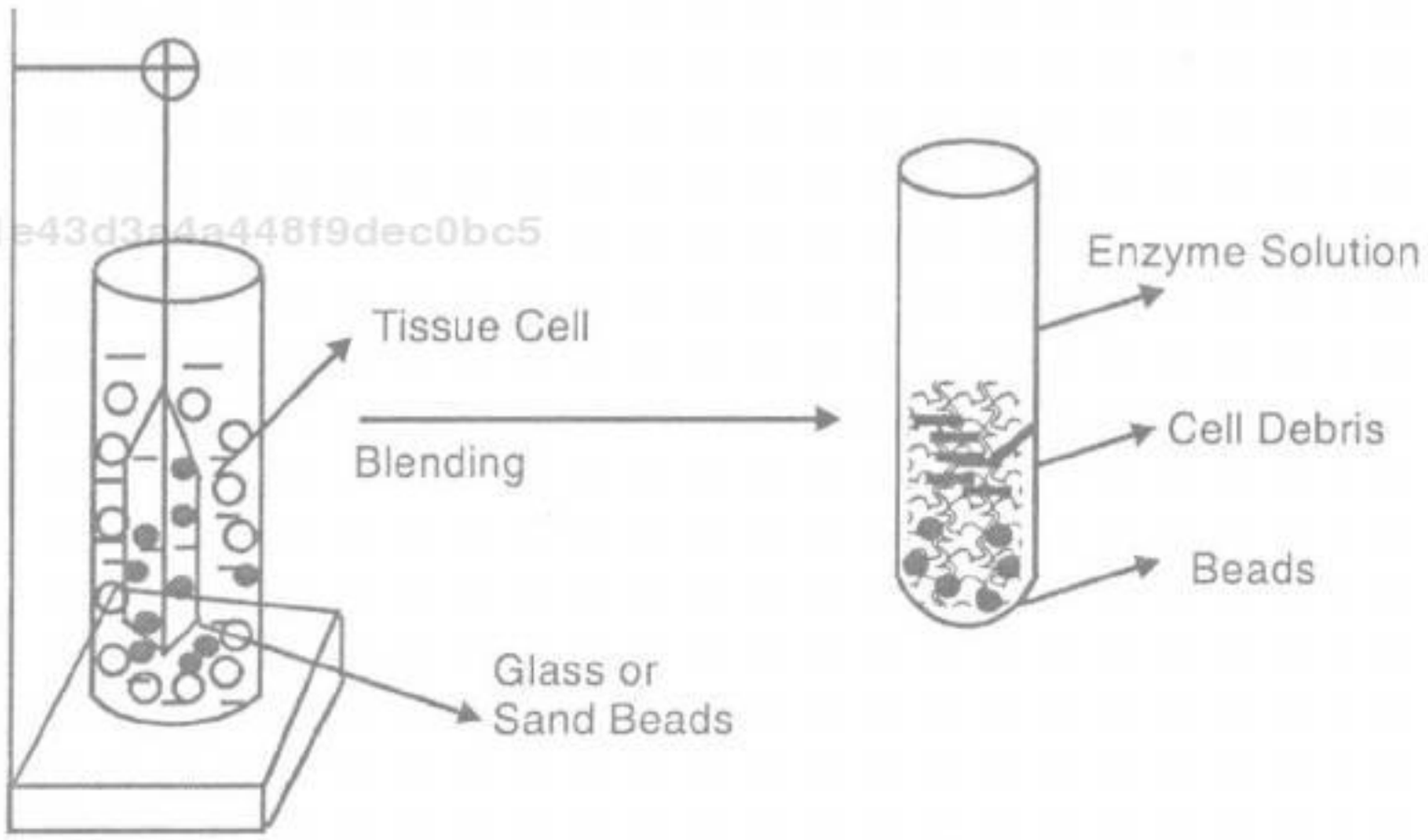


Fig.2.4: Blending with beads

(E) Use Of Appropriate Hydrolytic Enzymes

A different approach has been used with many animal tissues. They are subjected to the action of appropriate enzymes (including lysozyme). Proteases and lipases are conveniently used to cause localized removal of membrane particles leading to formation of holes or pores through which the cell sap and organelles can be brought into solution.

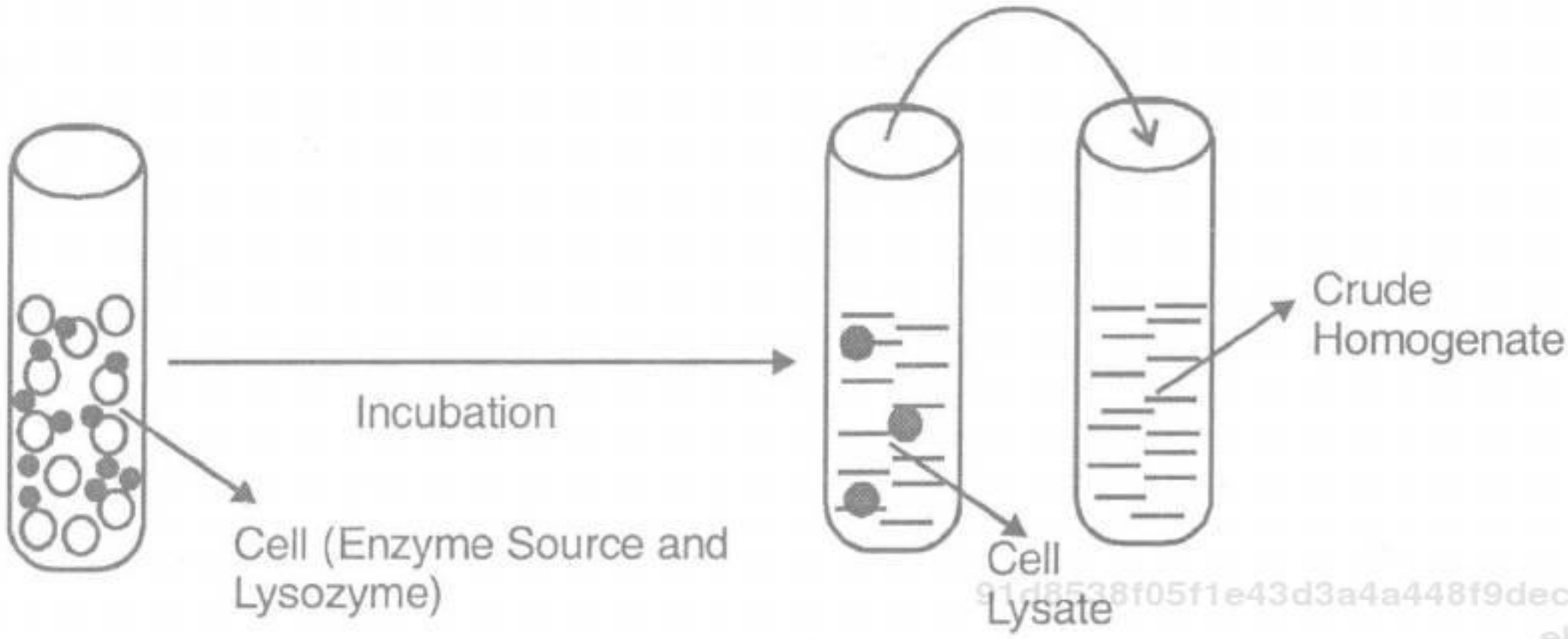


Fig 2.5: Use of hydrolyzing enzymes.

(F) Homogenization by Potter- Elvehjem Homogenizer or a High-Speed Blender

The Potter-Elvehjem homogenizer comprises of a glass tube which perfectly fits on a Teflon pestle which is connected to a high-speed motor. The tissue to be homogenized is minced and mixed with appropriate quantity of isotonic solution and then subjected to homogenization at a very high speed (about 600rpm) for about 5 to 10 minutes.

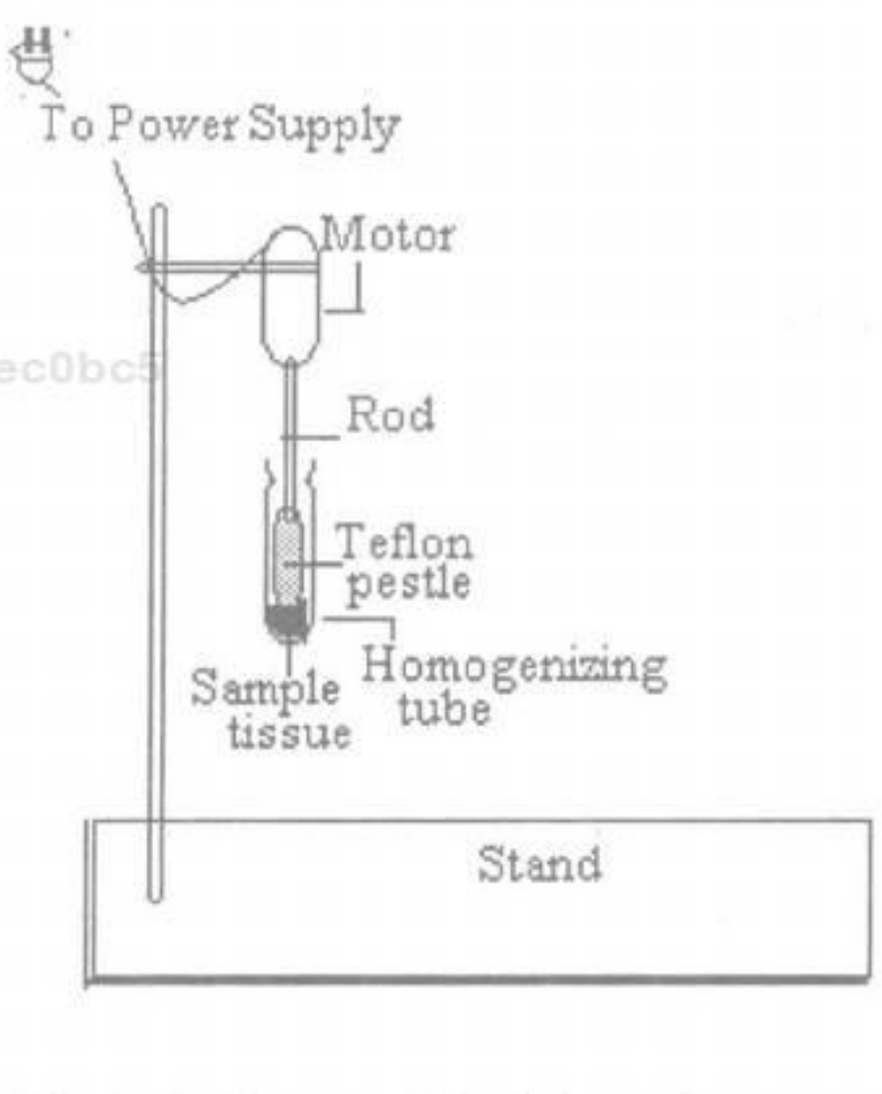


Fig 2.6: Potter Elvehjem Homogenizer.

(G) Use Of Hypotonic Solutions:

Suspension of cells in hypo tonic solutions leads to absorption of water by them and their swelling leading to a turgor pressure that is great enough to cause the cells to burst. Usually sucrose solutions or KCl solutions are employed.

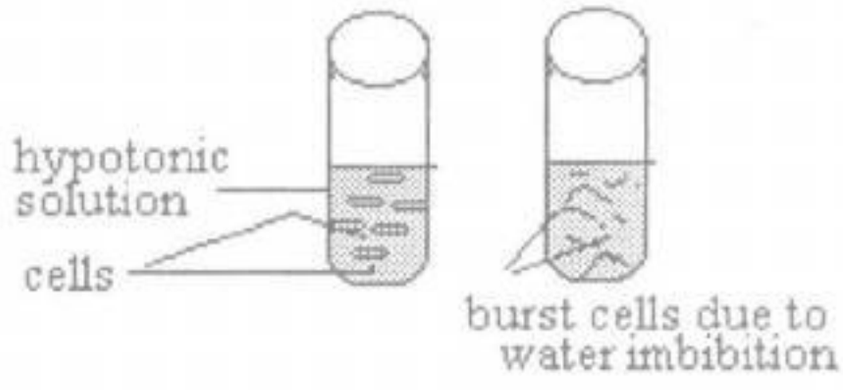


Fig 2.7: Use of hypotonic solution, for lysis of cells during isolation of enzymes.

(H) Ultrasonic Vibrations

The cells are subjected to ultrasonic vibrations that result in their disruption and formation of a homogenate.The cells are subjected to ultrasonic vibrations that result in their disruption and formation of a homogenate.

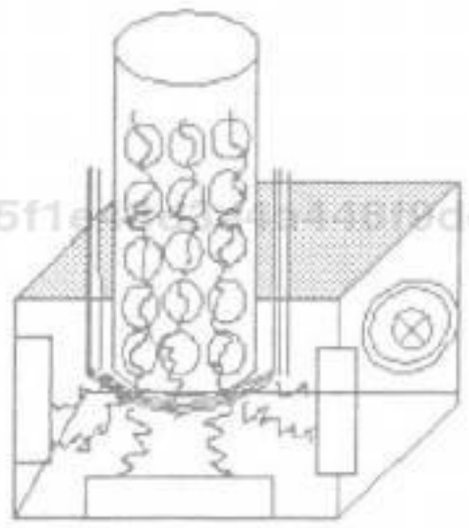


Fig 2.8: Ultrasonicator

(I) Alteration Of pH Of The Solution

Subjecting the cells to an altered pH environment causes the cell membrane proteins to coagulate leading to formation of pores through which the cell sap oozes out as a homogenate.

(J) Organic Solvents

The membranes are lipoprotein bi layers and appropriate organic solvents such as acetone, chloroform, ether, etc. are often employed to dissolve the lipid constituents leading to formation of pores. The cell cytoplasm then flows out as a crude homogenate.

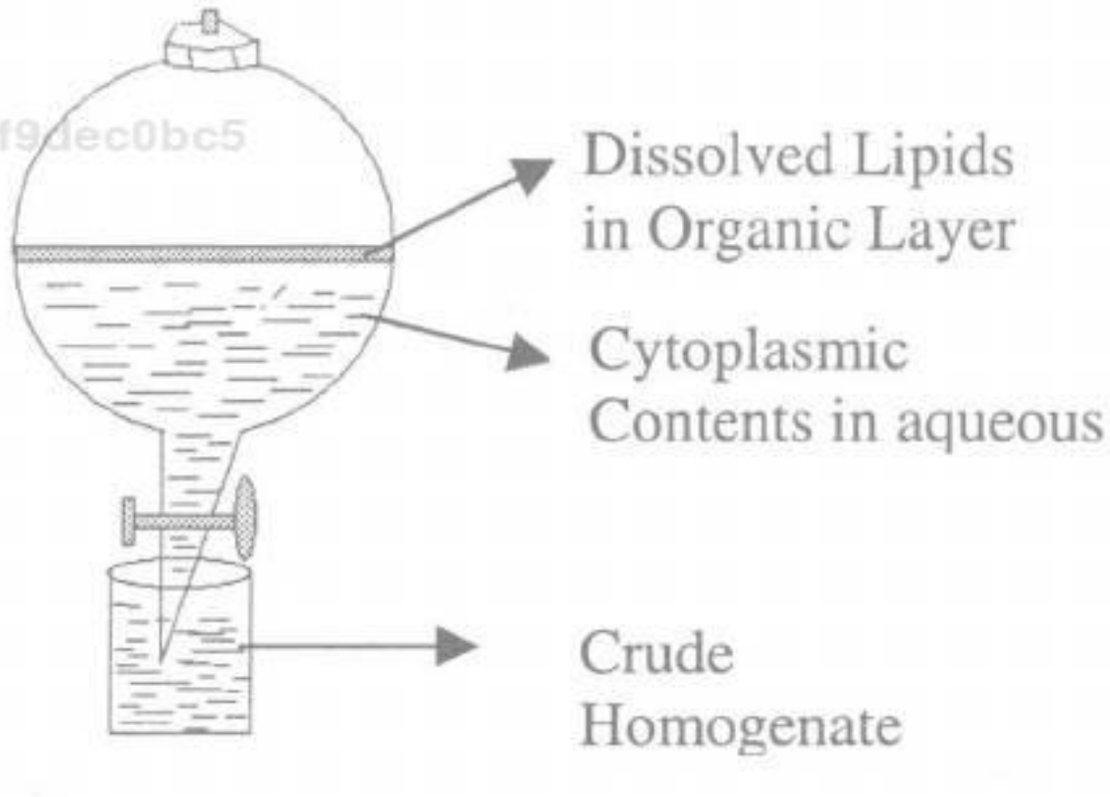


Fig 2.9: Use of Organic Solvents

(4) Methods For Purification

Once the crude homogenate is obtained, the aim of a purification procedure should be to isolate a given enzyme of interest with the maximum possible yield based on fold purification. In addition the preparation should possess the maximum ability of catalysis. In other words it should be free from contamination of other inactive and active proteins.

In the early days of enzyme purification, formation of crystals was taken to be a proof of purity but recent advances have enabled us to determine impurities in crystalline preparations. It is therefore necessary to determine the purity by using other methods and criteria that may or may not include the measurement of catalytic activity.

The general strategy for use of methods of purification and their sequence is dependant upon the source of enzyme, instrumentation available, the time span of the procedures and the economics of the processes. In other words different methods may be chosen by different workers as per their convenience to achieve the purification of a desired enzyme. Some of the methods that are routinely used for enzyme purification are summarized in table 2.3.

Table 2.3 Methods for purification of enzymes

Property	Method	Scale
Size or mass		
	Centrifugation	Large or small
	Gel filtration	Generally small
	Dialysis or ultra filtration	Generally small
Charge		
	Ion exchange chromatography	Large or small
	Electrophoresis	Generally small
	Isoelectric focussing	Generally small
Solubility		
	Change in pH	Generally large
	Change in ionic strength	Large or small
	Decrease in dielectric constant	Generally large
Specific binding sites		
	Affinity chromatography	Generally small
	Affinity elution	Large or small

The term large scale is used to indicate that the amount of protein greater than about 100 mg can be handled at that particular step in the purification procedure. The details of each method are briefly discussed here. It should be remembered however that the choice of methods and sequence is entirely dependent upon the workers using them.

(A) Methods Depending On Size Or Mass

The methods depend upon the general principle that the molecules, sediment at different speeds related to their sizes or masses. Some of the frequently employed methods include:

(1) Centrifugation

Large molecules such as enzymes can be sedimented by the high centrifugal fields (up to 300,000g) generated by an ultra centrifuge. Although the rate at which any particular enzyme will sediment depends on a variety of factors including the size and shape of the molecule and the viscosity of the solution, it is found that in general the

higher the molecular weight, the greater the rate of sedimentation. This method is not widely used in purification procedures to separate one enzyme from another because only small volume (few ml) can be dealt within ultra centrifuges operating at high centrifugal fields. However centrifugation is very widely used to remove precipitated or insoluble material in the course of isolation, for e.g. to remove cell debris after homogenization or to collect enzyme which has been precipitated by the addition of salts.

Density gradient or zonal centrifugation is a widely used and versatile procedure for separating not only proteins and other type of macromolecules but also organelles and viruses. In the most common procedure, a continuous density gradient of sucrose is first prepared in a plastic centrifuge tube by a device that mixes concentrated sucrose solution and water in decreasing ratio as the tube is filled, so that the density of the medium is greatest at the bottom of the tube. The mixture of macromolecules to be resolved is layered on the top of the gradient. Centrifugation of the tube in a horizontal position in a rotor at a high speed causes each type of macromolecule to sediment down the density gradient at its own rate, determined by its particle weight, density and shape in the form of separate bands or zones. Usually centrifugation is stopped before equilibrium is reached. The position of the protein bands can be located optically or by draining of the content of the tube carefully through a pinhole in the bottom and analyzing successive small samples. Alternatively the plastic tube can be frozen and then cut into thin slices for analysis.

It is diagrammatically represented as follows:

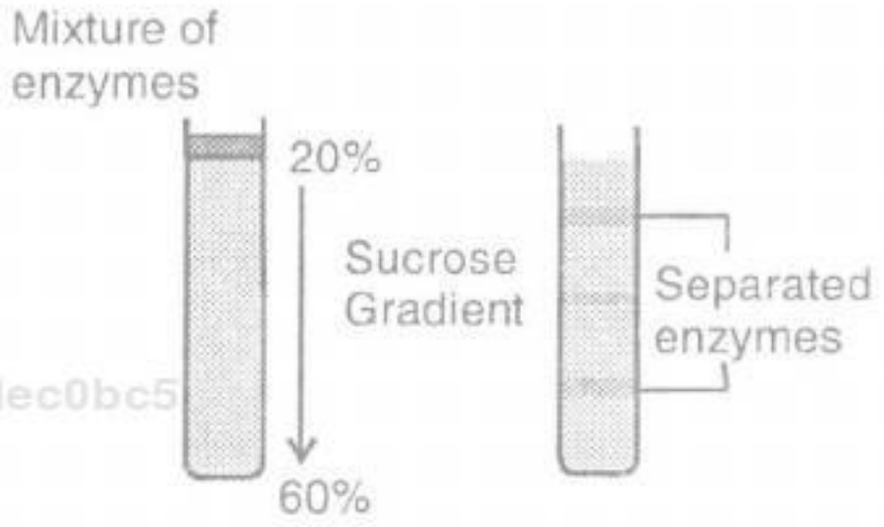


Fig 2.10: Density gradient

(2) Gel Filtration

Gel filtration or gel permeation chromatography is a separation method dependent upon molecular size. The method is also known as molecular sieve, or molecular exclusion chromatography. Its excellent reproducibility, comparatively short time and relatively inexpensive equipment make it far widely used separation method. It is based on the following simple principle,

A column of gel beads or porous glass granules is allowed to attain equilibrium with a solvent suitable for the molecules to be separated. If the mixture of molecules of different size is placed on the top of such an equilibrated column the large molecules pass through the interstitial spaces between the beads. This is because the pores of the gel have smaller diameter than what is needed for the large molecules to enter. Large molecules therefore move down the column with little resistance. The small molecules however can enter the pores and are thereby effectively removed from the stream of the eluting solvent. These molecules are thus retarded. The degree of retardation of a

molecule is proportional to the time it spends inside the gel pore that is a function of the molecule's size and the pore diameter. The molecules having diameter equal to or larger than the pore diameter do not enter the gel and are said to be excluded. The techniques used are column chromatography or thin layer chromatography.

An ideal gel should have the following characteristics:

(i) The gel material should be chemically inert

(ii) It should preferably contain vanishingly small number of ionic groups.

(iii) Gel material should provide a wide choice of pore and particle sizes

(iv) A given gel should have uniform pore and particle size

(v) The gel matrix should have high mechanical rigidity.

Some routinely used gel materials include

(a) Cross- linked dextrans (sephadex):

For proteins and most of the bio molecules, sephadex is by far the most popular of all the gels. Dextrans are cross-linked by epichlorhydrin. A schematic representation of its structure is given in fig 2.11

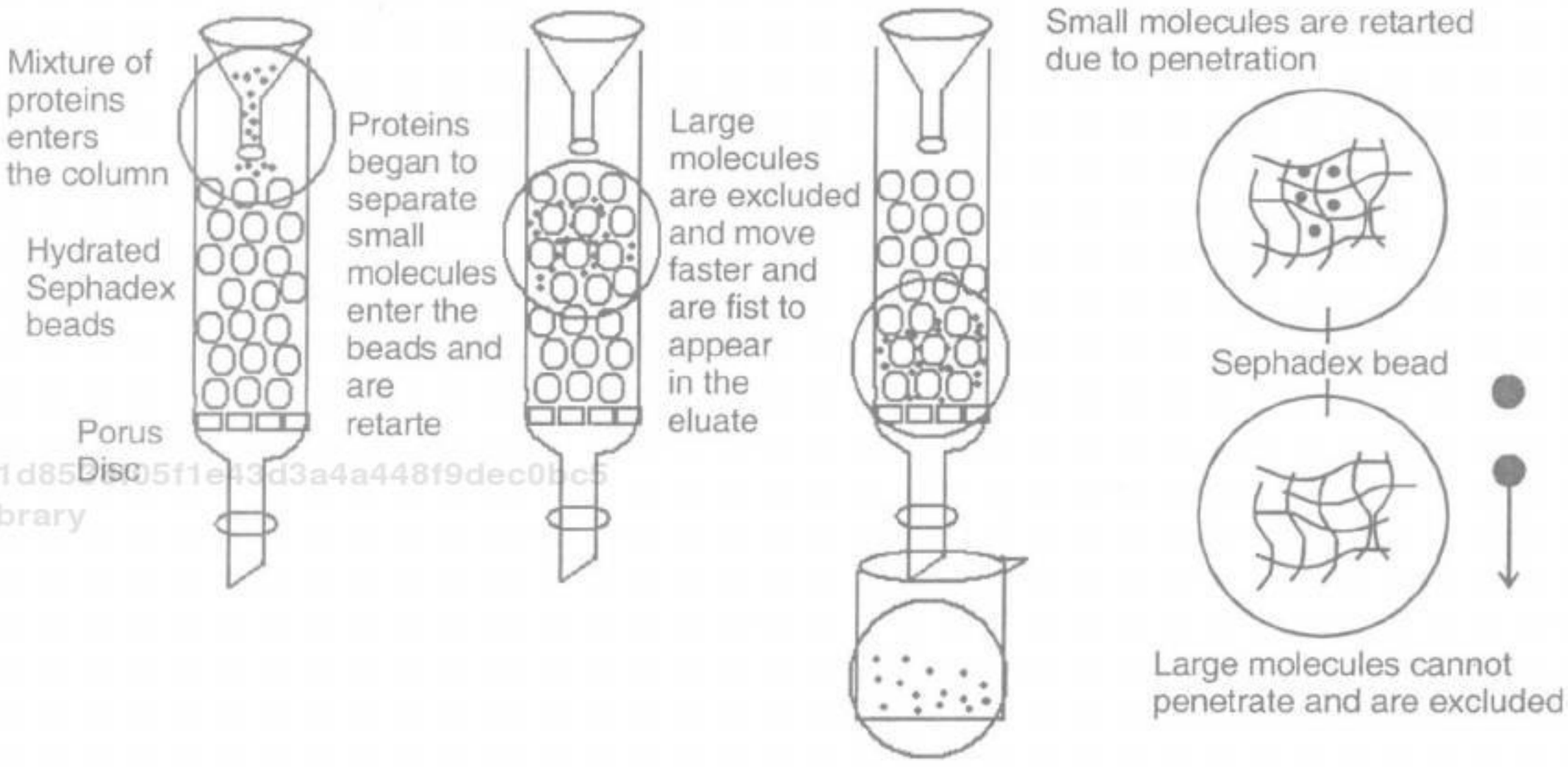


Fig 2.11: Separation by use of sephadex-beads

(b) Polyacrylamide gels:

Polymerization of acrylamide in bead form results in the formation of polyacrylamide. The gels are used to separate molecules having a molecular weight up to 300,000 Dalton. However at such large pore size they lack mechanical rigidity and become compressed in the column. Although they can be used in the pH range of 2-11, they are unstable to bases due to hydrolysis of amide groups. The cross- linking agent is bisacrylamide and the reaction is brought about in the presence of ammonium per sulfate. The gels are insoluble in water and common organic solvents.

(c) Agarose gels:

They are produced from agar. The gels are hydrophilic and almost completely free of charged groups. They have greater pore sizes and can be conveniently used to separate molecules having a molecular weight of a few million Dalton. They are compatible with all aqueous buffers and completely stable within pH range 4-10. Freezing temperatures and temperatures more than 30 degrees alter the gel structure.

(d) Styragel:

The styragel is a cross-linked polymer of polystyrene used for completely non-aqueous separations. It can be prepared in a wide range of porosities and the gel structure is unaffected by temperatures as high as 150 degrees. It can be used with solvents like carbon tetra chloride, trichloro- benzene, cresol, tetrahydrofuran, chloroform and dimethylsulfoxide etc. it is unstable with water, acetone and alcohols.

(e) Controlled pore glass beads:

Borosilicate glass beads are also often used. They give very high flow rates due to their total rigidity. They are treated with hexamethyldisilazane to overcome the problem of protein adsorption. They have a molecular exclusion limit between 3000 to 9 million Dalton.

(3) Dialysis or Ultra filtration:

A dialysis membrane such as cellophane can be used to separate the globular proteins or molecules with a molecular weight of around 20,000 Dalton. The pore size can be changed by various mechanical and chemical treatments. The method is routinely used to separate small organic molecules, salts and organic solvents from the crude extracts. The volume handling capacity is generally low. The process cannot be employed to separate enzymes from each other. Ultra filtration on the other hand is a modification of the dialysis procedure in which small molecules and ions pass through a dialyzing membrane under the influence of applied pressure (usually Nitrogen gas at 4 atm. Pressure). This leads to concentration of enzyme solution, which is useful in reducing the volume of the extract sample during the purification procedure.

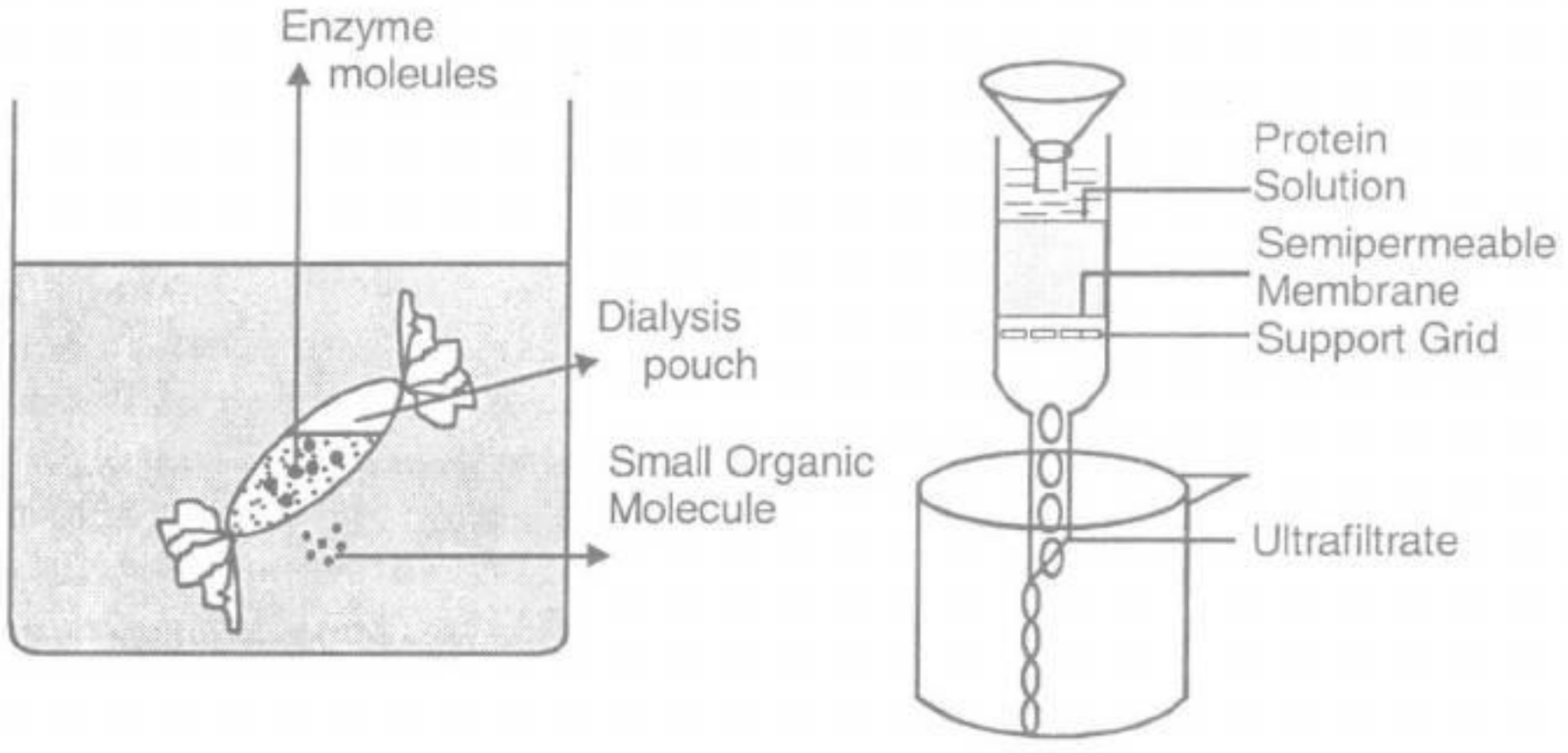


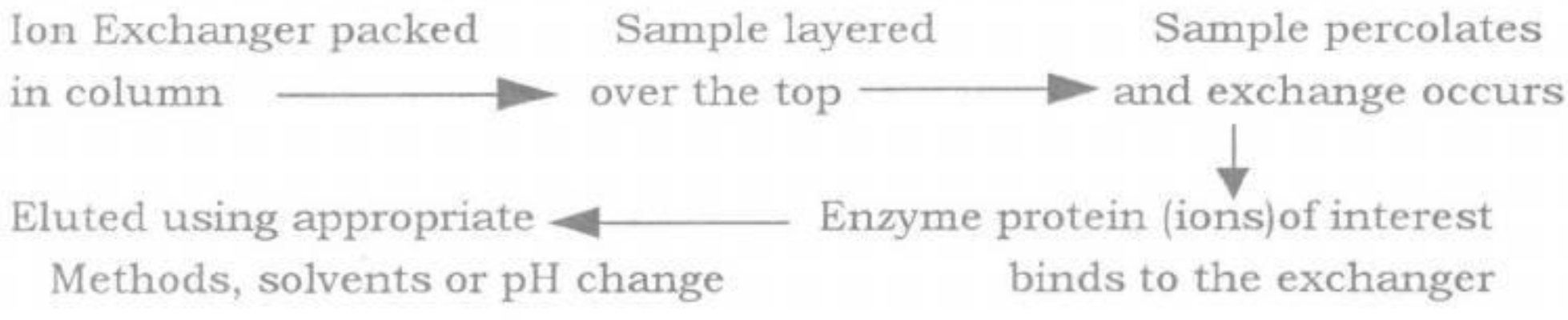
Fig 2.12: Dialysis and Ultrafiltration

(B) Methods Depending Upon Charge:

The methods depend upon the fact that enzymes carry charged side chains which give the molecule a net charge and that the mobility of a charged molecule under an electrical field or in a charged environment depends on the charges carried by it. Some of the routinely used methods include the following:

(1) Ion –Exchange Chromatography:

Ion exchange may be defined as the reversible exchange of ions in solution, with ions, electrostatically bound to inert support medium. The electrostatic force of attraction in turn depends upon the relative charge, the radius of the hydrated ions, and the degree of non-bonding interactions. The technique generally employs ion-exchange columns. There are two types of ion-exchangers, anion exchangers and cation exchangers. The medium is an inert support medium that is covalently bound to a positive(anion exchanger) or negative(cation exchanger) functional groups. The ions electrostatically bound to the exchanger are referred to as counter ions. This technique is extremely useful in the separation of charged compounds and even uncharged compounds that can be tagged with charged group or a variance of pH.



The sample containing the ionic species to be separated is allowed to percolate through the exchanger for such a length of time as will be sufficient for the attainment of equilibrium, E—Y + X ⟷ E—X + Y, where E is an exchanger and X and Y are exchangeable ions. The exchanger and the counter ions are oppositely charged. In case the ion to be exchanged is cation, then the neutral and anionic molecules will not bind at all and shall be washed away out of the column. The exchanged ions (X) bound can then be eluted either by percolating the medium with increasing concentration of Y, or in other case the change in pH, favorable for the elution of X. This principle is also applicable for separation of proteins and nucleic acids which are capable of possessing both positive and negative charges. The amphoteric nature of macromolecules particularly proteins, can be exploited for purification purposes by using ion exchange chromatography. For example the desired protein may be made to behave as a cation by lowering the pH of the protein mixture (the pH is lowered to a limit where most of the other proteins in the mixture behave as anions). This preparation if chromatographed on a cation exchanger will remove many of the anionic protein species. This process will remove many of the unwanted proteins from the starting mixture and resultant mixture is rich for the desired protein. If the pH of this resultant mixture is now increased the desired protein will exist predominantly as an anion (the pH is increased to a limit where most other proteins in the mixture still behave as cations). If the preparation is now chromatographed on an anion exchanger, many cationic proteins will be lost. It should thus be clear that anion and cation exchange chromatography used sequentially can afford a large degree of purification. Routinely used ion exchange resins:

Table 2.2: Ion-exchange resins

Type	Nature	Trade name
Strong cation	Sulfonated polystyrene	Dowex 50 Amberlite IR 120
	Sulfopropyl cellulose	SP- Sephadex
Weak cation	Condensed acrylic acid	Amberlite IRC 50
	Carboxymethyl cellulose	CM-Sephadex
Strong anion	Polystyrene with –CH2NMe3CI	Dowex IAmberlite
	Diethyl(2 hydroxypropyl) quarternary amino cellulose	IRA 400QAE Sephadex
Weak anion	Polystyrene with secondary amine	Dowex 3 Amberlite IR 45
	Diethylaminoethyl cellulose	DEAE-Sephadex
	Diethylaminoethyl agarose	DEAE-Sepharose

(2) Electrophoresis:

Electrophoresis is the migration of charged particles or molecules in a medium under the influence of an applied electric field. Upon suspension in an aqueous solvent almost all particles including bio molecules such as enzymes acquire either positive or negative charges. The acquisition of such charges depends upon the nature of the particle / molecule and the solvent. These groups determine the net charge density of the protein molecule, which makes it move in an electric field in a direction, and at a velocity dependant upon the sign and quantity of this net charge density. Even if two molecules have the same charge, they might not migrate together because if there is difference in their molecular weights they will have different charge: mass ratio. The electrophoretic mobility is affected by following factors:

The Sample:

Charge/mass ratio of the sample dictates the electrophoretic mobility. The mass consists of the size and shape of the molecule.

I Charge: The higher the charge, greater is the mobility, the charge however depends on the pH of the medium

II Size: The bigger the molecule the greater are the frictional and electrostatic forces exerted upon it by the medium. Large particles thus move slower than small particles.

III Shape: Rounded contours generate lesser frictional and electrostatic retardation compared to sharp contours.

Other Factors:

These include the electric field, the medium, the buffer etc. For details the readers are advised to go through books on biophysics or physical chemistry.

The routine techniques employed include:

(A) The Moving Boundary Electrophoresis:

In this technique a buffered solution of macromolecules is placed under a layer of pure buffer solution in a U- shaped observation cell. The pH of the buffer is so chosen that all the macromolecules bear a net negative charge. The movements of macromolecules, consequent to generation of electric field between the electrodes will then be towards an anode. As they do so they migrate from the macromolecule solution to the pure buffer or into the zone of macromolecule free buffer and form a boundary or front. As a result of this there is a sharp change in the refractive index of the solution at this boundary. The refractive index changes along the electrophoretic cell, when measured by appropriate optical devices yield electrophoretic patterns that show the direction and relative rate of migration of the major molecules in the sample. In recent years however it has been superceded by techniques collectively known as zone electrophoresis.

(B) Zone Electrophoresis:

Zone electrophoresis is the name given to the separation technique employing stabilizing media most of which are gels such as agar, starch and polyacrylamide. Upon separation the molecules are immobilized by fixation in different zones. The molecules are then detected by staining them on the supporting medium. Other methods to detect the separated molecules are

1. visualization by ultraviolet light.
2. detection by virtue of enzymatic reaction.
3. detection by radioactivity if the molecules are radiolabeled.
4. Elution of separated components from the medium and further detection.

Zone electrophoresis can also be utilized as a large scale or preparative method whereby large amounts of a component can be purified for further characterization.

Based on the support medium, the techniques are classified as

Paper electrophoresis

Gel electrophoresis

Cellulose acetate electrophoresis

Specialized techniques including the disc gel, gradient gel, high voltage, two-dimensional electrophoresis etc.

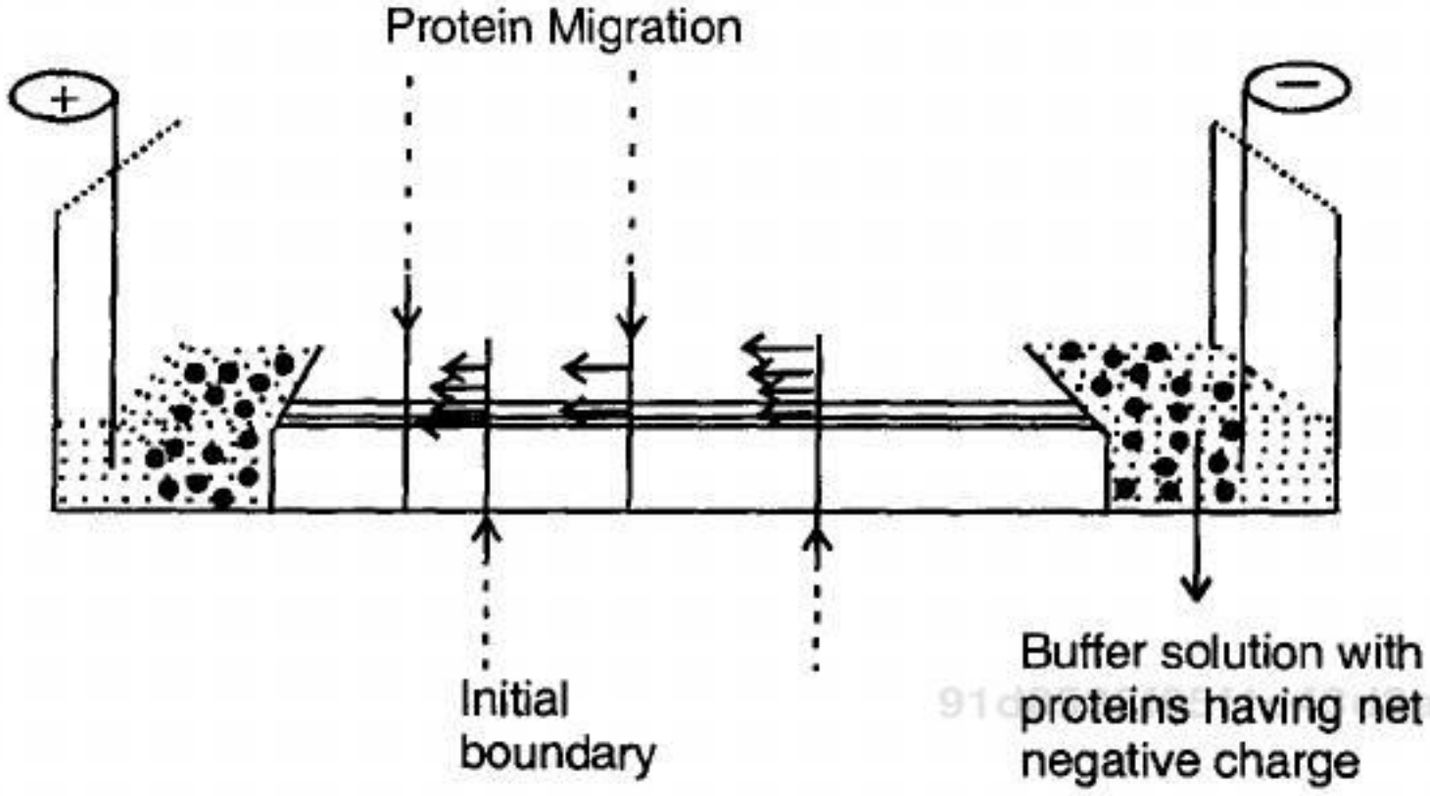


Fig.2.13: Electrophoresis

(3) Isoelectric Focussing:

This technique was discovered by H.Svensson in Sweden and has a high-resolution power. A simple comparison would help establish its superiority. While paper electrophoresis resolves plasma proteins into 6 bands, isoelectric focussing resolves it into at least 40 bands. Protein molecules may have a net positive charge in an acid solution because most amino groups carry a positive net charge and most carboxylic groups are protonated and electrically uncharged. With a gradual increase in pH, the number of carboxyl groups carrying a negative charge increases, while the number of positively charged group decreases. At a certain pH value, the isoionic point, the net charge of the protein molecule is zero. The number and types of protolytic groups and their dissociation constants thus determine the isoionic point of a molecule. Although there is considerable variation in the isoionic point of protein, they are generally in the pH range of 3-11. In conventional electrophoresis, the pH between anode and cathode is constant and the positively charged ions migrate towards the cathode and negative ions migrate to the anode.

In isoelectric focussing, on the other hand, a stable pH gradient is arranged, the pH increases gradually from anode to cathode. A protein introduced into this system at a point where the pH is lower than its isoionic point will possess a net positive charge and will migrate in the direction of the cathode. Due to the presence of the pH gradient, the protein will migrate to an environment of successively higher pH values, in turn, will influence the ionization and net charge of the molecule. Finally the protein will encounter a pH where its net is zero and will stop migrating. This is the isoelectric point of the protein. The consequence of this is that every protein will migrate to and focus at its respective isoelectric point in a stable pH gradient, irrespective of its origin in the apparatus at the time the current was applied. Thus the point of application and volume of the protein solution are not critical. Diffusion, which is an obstacle with every other method of electrophoresis, is not a problem with this technique because focussing effect works against diffusion. Thus once a final stable focussing is reached the resolution will be retained even if the experiment is continued for a long time.

The pH gradient is established with the help of carrier ampholytes. They are special buffer substances that possess following properties:

(i) They should have a certain buffering capacity at their isoelectric point.

(ii) They should have a conductance at their isoelectric point.

(iii) They should have low molecular weight so that macromolecules can be separated from them easily after electrofocussing.

(iv) They should be soluble in water. This hydrophilic character will also prevent their binding to hydrophobic regions of the proteins.

(v) Ideally they should have a low light absorption at 280 nm. This would permit the detection of proteins after electrofocussing by measurements at 280 nm.

Electrofocussing also needs provision for stabilization of separating protein zones against convective flow in the solution. Three ways are in use that include

(a) Density Gradient:

Density gradients suitable for electrofocussing can be made with many uncharged solutes, which can be dissolved in water to a concentration that will increase the density sufficiently. The compounds should not react with proteins and should have a low

content of heavy metals. They should be of high purity. Sucrose is the most preferred compound however other compounds like mannitol, sorbitol, ethylene glycol, dextran and ficoll may be used.

(b) Gels:

In electrofocussing the gel serves only as an anticonvectant and not as a molecular sieve. Obviously the gel concentration should be low to provide large diameter pores. For large proteins, MW exceeding 200 KD, lower concentration of acrylamide might be used in combination with agarose. Acrylamide is the preferred gel for electrofocussing. Agarose starch gels are not preferred as in these gels pH gradient drifts considerably during prolonged experiment.

(C) Zone Convection Electrofocussing

The apparatus is made up of two rectangular boxes, the upper one being the cover. The upper surface of the lower box is corrugated with ridges, separated by depressions corresponding to the ridges of the cover. Thus they fit in with each other leaving a space of few millimeters in between, producing a narrow wave like channel like a series of interconnected broad U tubes. The carrier ampholyte solution is filled in this space with the electrodes situated at the two ends.

A coolant liquid is allowed to flow through the hollow channels built into both the boxes to maintain a constant temperature. When the current is on a density gradient is formed in each depression of the bottom part by the solute. When proteins become immobile at their isoelectric pH, the density increases locally and the proteins settle down in the depressions of the bottom part. They can be separated as fractions after the completion of the experiment without contamination by neighboring fractions.

Separation Of Carrier Ampholytes From Proteins

The average MW of ampholytes is about 800 where as the protein has a MW of 10,000 or more. Dialysis or gel filtration can be effectively used for their separation. Sometimes other methods like ammonium sulfate precipitation; ion exchange chromatography and partition chromatography may be used.

(C) Methods Based On Change In Solubility

The solubility of a compound in a given solvent depends on the balance of the forces between solute and solute and those between solute and solvent. If the former predominate, the compound will be insoluble where as if the latter are predominant, the compound will be soluble. In the course of enzyme purification it is possible to alter the balance between these forces and hence precipitate the enzyme of interest or remove contaminating enzymes. Three of the most important ways of changing the solubility of enzymes are, (1) to change the pH, (2) to change the ionic strength, (3) to decrease the dielectric constant. These methods can be applied on a large scale and are often used in the initial stage of a purification procedure.

(1) Change in pH

An enzyme is least soluble at its isoelectric point since at this pH the repulsive electrostatic forces within the enzyme molecules cease to exist. Under such condition the enzyme molecules tend to precipitate. Adjustment of the pH to the proper value can therefore be used to precipitate the enzyme. It is important to note that the enzyme of

interest should be rendered completely inactive due to this procedure. This process isolates Adenosinetriphosphatase from beef heart.

The method is sometimes employed to remove impurities, as in case of the isolation of adenylate kinase.

Isolation of adenylate kinase from pig muscle: The enzyme, when isolated from pig muscle gives crystals large enough to be used for x-ray crystallography and is exceptionally stable at low pH. The general procedure includes following steps.

1. *Minced muscle is treated with 0.01M KCl and strained through cheesecloth to obtain an extract.*
2. *The pH is adjusted to 3.5 and after 5 min is readjusted to 7.0. The solution is centrifuged.*
3. *Supernatant is loaded on phospho-cellulose column and enzyme is eluted with AMP.*
4. *Fractions are checked for activity and pooled, followed by ammonium sulfate precipitation.*
5. *Precipitate is redissolved in small volume and subjected to gel filtration.*
6. *Crystallization is brought out at 62% ammonium sulfate.All the steps are performed at 0-5 degree.*

(2) Change in Ionic Strength:

The proteins often precipitate on addition of high amount of salts. This is also called as salting out of proteins. The theoretical basis for this is not clearly understood however it is assumed that at high salt concentrations, the concentration of water is greatly reduced leading to a decrease in the solute- solvent interactions and in turn the solubility of proteins. Many salts have been employed for "salt fractionation" or salting out the enzymes. The most widely used and preferred salt is ammonium sulfate due to its following advantages:

- It is cheap
- It is highly soluble in water
- It does not cause any harmful effects on the enzyme to be purified.
- It is a weak acid and the fold purification achieved is about 10 times. It is best used in the initial stages of purification.
- The enzymes glyceraldehyde phosphate dehydrogenase and fructose bis phosphate aldolase have been isolated and purified by using ammonium sulfate fractionation.

Purification of glyceraldehyde phosphate dehydrogenase from rabbit muscle: The enzyme is easy to purify due to its abundant occurrence and contains tightly bound NAD+. It is soluble in high concentrations of ammonium sulfate (up to 70% saturation) which is the basis of the purification procedure given below.

1. The minced muscle is mixed with 0.05 molar EDTA/NH4+ at pH 8.4 and centrifuged at1000g for 20 min.

2. The pH of extract is adjusted to 7.4, solid ammonium sulfate is added up to 50% saturation and centrifuged.
3. The supernatant is added with more ammonium sulfate till saturation is 70% and is centrifuged.
4. Additional ammonium sulfate up to 72% saturation is added and precipitate is collected. It is dissolved in buffer and refractionated with ammonium sulfate
5. The enzyme is allowed to crystallize.

(3) Decrease in Dielectric Constant:

Addition of water –miscible organic solvents will decrease the dielectric constant of a solution and hence increase electrostatic forces. This leads to the alteration in solute-solute and solute-solvent interaction generally leading to precipitation of large molecules such as enzymes. The choice of solvent is crucial, as many enzymes are rendered inactive due to the action of organic solvents. Working at low temperatures is advisable, as many solvents are volatile and hazardous at high temperatures. The commonly used solvents include ethanol, butanol, acetone etc.

(d) Methods Based on Specific Binding Sites:

The characteristic property of specificity associated with enzymes can be employed to purify them. There are two methods based on the stage of use of affinity. (1) Affinity chromatography uses the affinity, at the stage of adsorption of the enzyme of interest, on to an ion exchanger and (2) Affinity elution uses the specificity at the stage of desorption. They are briefly discussed below:

(1) Affinity Chromatography:

The technique exploits the capacity for specific, non-covalent binding of enzymes with other molecules called ligands. Most of the times a substrate analogue (a molecule that resembles the substrate but does not cause fruitful reaction) specific for the enzyme of interest is bound to a solid and inert matrix (like agarose). When the mixture is loaded on the top of the column, only the desired enzyme will bind to the immobilized substrate analogue and shall retard. All other enzymes and proteins will pass down and out of the column.

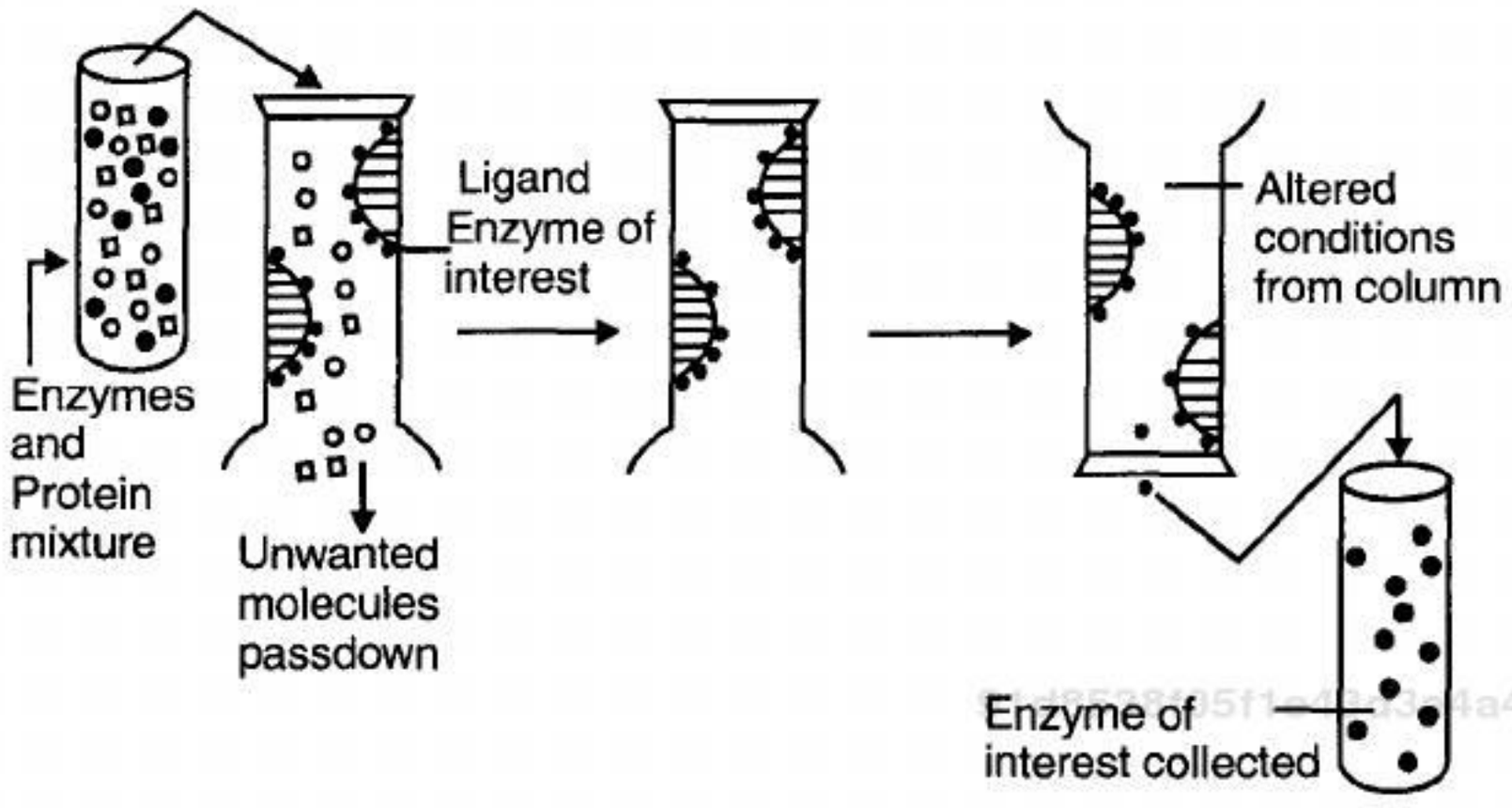


Fig.2.14: Affinity technique, adsorption of enzyme on column.

If the conditions are altered, the enzyme bound to the ligand shall dissociate and be eluted. The fractions can be collected and checked for the enzymatic activity. The enzyme collected by this technique is fairly pure. Some important standard matrix-ligand systems are given below

Table 2.3 Matrix –ligand systems for protein purification.

Matrix- ligand system	Nature of ligand	Used for isolation of
Cibacron – blue –agarose	Dye resembling nucleotides	Kinases, dehydrogenases, DNA polymerases, nitrate reductases, blood coagulation factors
Heparin-agarose	Heparin (natural anticoagulant)	DNA polymerase, bone collagenase etc.
Polynucleotide agarose	Oligo dT	m-RNA, transcription factors, other DNA binding proteins
Lysine agarose	Lysine	Plasminogen, ribosomal RNAs
Protein A agarose	Cell wall protein of *Staphylococcus aureus*	IgG
Lectin- sepharose	Lectins like concanavalin A or wheat germ lectins	Glycoproteins and polysaccharides.

A number of problems are associated with the use of this technique for purification of enzymes. These include:

1. Attaching a suitable substrate analogue to the matrix is a difficult task.
2. Linking of the ligand to the matrix may interfere with the binding property of the enzyme leading to partial or complete loss of specificity. Use of spacer arms may be necessary.
3. The strength of the enzyme-ligand binding is a crucial factor. If it is too weak then retardation of enzyme will not be satisfactory, while too strong interaction will pose the problem of elution.
4. Enzymes catalyzing bi-substrate reactions create special problems. It is necessary to study and pay particular attention to the elution techniques involved. In case of liver alcohol dehydrogenase, it gets bound to AMP analogue bond with the matrix but its elution requires the use of NAD and pyrazole. Pyrazole is a competitive inhibitor of the enzyme.

(2) Affinity Elution:

It is a complimentary technique to affinity chromatography. It is used with partially purified enzyme mixtures and is more advantageous than affinity chromatography as:

a. The complex reactions of binding the substrate analogue are eliminated.

b. The columns are cheaper because ion exchangers are cheaper than affinity matrices.

The best example of use of affinity elution is, the separation of enzymes of the glycolytic sequence in a single multiple operation scheme. A partially fractionated mixture

of the enzymes (formed by saturation by ammonium sulfate up to 45-65 %) is loaded on a CM- cellulose column at pH 6.5 Elution with phosphoenol pyruvate yields pyruvate kinase, while elution with fructose-bis-phosphate yields aldolase. The molarity of the eluting solution is also important.

Variations In Affinity Techniques

The techniques of affinity based enzyme separations are increasingly required however they suffer two major disadvantages:

1. The techniques are costly because of the high cost of affinity matrices, costly ligands and long incubation time along with the complications of process.
2. They could not be used at the initial stages of purification, primarily because a host of contaminants in the extract tend to clog the system rendering it useless.

Variations have however been worked out to counter these problems. Some of them are briefly mentioned below

(a) Affinity Cross-Flow Ultra filtration

The technique uses micro porous membranes with a pore size of 3-5 mm. They are made of cellulose acetate, polytetrafluoroethylene (PTFE) or polyvinylidene. Prepared activated membranes are now commercially available.

When the mixture is incubated with the membrane, it retains the protein-ligand complex while the unwanted material passes through the membrane pores. A change of buffer can be used to release the protein from the complex. The flow rates are usually high and greatly improve the steps such as washing and elution. It is important to note that the ligand used should be very specific and the material of the membrane should have minimum non-specific adsorption.

(b) Affinity Precipitation

The technique uses polymers that are soluble under particular conditions and precipitate on change in any condition like pH, ionic strength, temperature or addition of an agent such as a metal ion.

A proper ligand is bound to the polymer and this ligand – polymer complex is kept in solution. When the mixture of proteins or enzymes is added to this solution the enzyme of interest binds with this and after a proper incubation time a change in any condition mentioned above is brought about causing the precipitation of the complex. The precipitate is separated and subjected to change in buffer leading to easiness in isolation of the desired enzyme. The technique is cheaper as it eliminates the costly matrices and ligand binding reactions; moreover it can directly be used at the first step of purification.

(c) Matrix less Affinity Separations

This is a variation of the earlier technique that does not require any ligand or matrix. This is illustrated from the following example. The wheat germ lectin has an affinity for N acetyl glucosamine. Conventionally to separate the wheat germ lectin one would have to use an insoluble matrix to which N acetyl glucosamine has been covalently bound. But instead of such a matrix, polymer of N acetyl glucosamine, chitin is a natural constituent of the exoskeleton of arthopods. It can be partially deacetylated to form chitosan. Chitosan can act as a polymer that can remain in solution or suspension

based on alteration of conditions. This polymer also has an affinity for wheat germ lectin. The technique is to incubate wheat germ extract with chitosan at pH 5.5where the chitosan exists as a solution. After the incubation time is completed, the pH is changed to 8.5 at which the chitosan- wheat germ complex precipitates. This complex is separated and redissolved by altering the pH to 5.5. After this a simple gel filtration can separate the chitosan and wheat germ lectin.

(E) Some Other Methods Of Enzyme Purification

Although most of the methods mentioned earlier are routinely used for the isolation of enzymes, some other methods have been used. A brief mention of these is given here.

(1) Fractional Precipitation By Heat

This treatment is a valuable tool to eliminate much of the unwanted protein contamination, hence is useful as the primary step in purification. Each enzyme has a characteristic and sharp destruction temperature. By heating the mixture for a definite time to a temperature just below the destruction temperature, it is possible to coagulate much of the unwanted protein that can be centrifuged off and eliminated. The fact that in presence of substrate, an enzyme is able to withstand a higher temperature by about 10 degrees than in its absence can be effectively used to eliminate even more proteins that are contaminants. The system generally comprises of three water baths, one at the desired temperature, other a few degrees higher and one colder. The solution is kept in a round bottom flask, about half filled and is first transferred to the hottest bath, with a continuous swirling to avoid overheating of a particular part. The temperature is monitored with a thermometer and immediately on reaching the desired value, the flask is transferred to the second bath (with the desired temperature) where it is kept for the necessary time period. After this the flask is transferred to cold and the contents are rapidly cooled with the help of swirling. It is then left in cold till precipitation occurs after which centrifugation is done. The process has to be strictly monitored and the time of heating is between 10-15 min.

(2) Fractional Adsorption On Calcium Phosphate Gels

Fractional adsorption is a widely used method and the chief adsorbents include calcium phosphate gel and alumina Cγ gel. The pH is about 5.5 and the electrolyte concentration is low. Usually a mixture treated with dialysis is used for adsorption. This lowers salt concentration and thus reduces interference in adsorption.

The procedure is simple. Small successive portions of the gel are added to the enzyme solution, followed by mixing, spinning down and removal of each portion before adding the next. The procedure is followed by activity tests on small samples withdrawn from the main solution. Calcium phosphate gel has the advantage that initially the other proteins are removed followed by a sudden removal of the enzyme leaving an inactive solution with many proteins.

A slightly alkaline buffer is used for the elution of the adsorbed enzyme from active fractions. Usually phosphate buffer at pH 7.6 is used. If this fails to elute the enzyme it is repeated till further removal of contaminants is done. The final elution is done by using phosphate containing 10% ammonium sulfate.

The volume of the elutant should not be large and high-speed stirrers may be used to increase efficiency.

(3) Additional Methods:

Some other methods, which are not so popular now, include chromatography on hydroxyapatite, hydrophobic chromatography, and precipitation by polyethylene glycol and concentration by freeze-drying. Heavy metal salts can also be used for purification of enzymes. They result in enzyme precipitation and form characteristic crystals. The heavy metals are then carefully removed by using chelating agents. In recent years use of western blotting technique has also become a common tool for enzyme isolation.

In *Western Blotting* the mixture is subjected to SDS-PAGE and then transferred on a membrane from the gel. The membrane is bound with specific antibodies against the desired enzyme protein and therefore after the incubation only the enzyme binds with the antibody. This is then reacted with a second antibody that is tagged with an enzyme that enables a color mediated detection of the complex.

Some representative techniques employed initially for enzyme isolation

These are discussed in brief,

(A) Sumner's Method For Isolation Of Urease

The method which Sumner and collegues have used to obtain the crystals is extremely simple. It consists of the following,

1. Extract finely powdered, fat-free jack bean meal with 31.6 per cent acetone
2. Allow the material to filter by gravity in an ice chest.
3. After standing overnight the filtrate is centrifuged and the precipitate of crystalline urease is obtained.
4. The precipitate is stirred with cold 31.6 per cent acetone and centrifuged again.
5. The crystals can be now dissolved in distilled water and centrifuged free from insoluble and inactive matter that has passed through the filter during the filtration.
6. Of the urease extracted from the meal as much as 47 per cent may be present in the crystals.
7. If one uses coarsely ground jack bean meal that has not been freed from fat the crystals are still obtained, but in traces only.
8. The process described above has been repeated many times since first discovering the crystals and has always had success.

(B) Northrop's Procedure For Isolation Of Pepsin

The procedure primarily uses ammonium sulfate precipitation followed by magnesium sulfate precipitation. In the later stages sodium acetate and sulfuric acid are used. A brief description is given as follows,

1. Contents of the fourth pouch are emptied after slaughter and immidiately filtered at 6degreesC for 48 hours. The filtrate is designated as solution 1.(volume approx. 5 litres)
2. Solution 1 is saturated with ammonium sulfate, decanted and filtered with suction. The precipitate is dissolved in 200 ml of N/500 hydrochloric acid. This is solution 2.

3. Solution 2 is cooled to –10 degrees C. To this 300 ml of cold acetone is added mixed and centrifuged. The supernatant is solution 3.
4. Solution 3 is cooled to –10 degrees C and to this 500 ml of cold acetone is added. The solution is filtered and the ppt is dissolved in 300 ml of N/500 hydrochloric acid. This is solution 4.
5. To the solution 4, 1 volume of saturated magnesium sulfate solution is added and mixed. It is centrifuged and the precipitate is dissolved and 100ml of N/500 hydrochloric acid is added. This gives solution 5.
6. 1 volume of saturated magnesium sulfate is added to solution 5. It is mixed and centrifuged. The precipitate is dissolved and 100 ml of N/500 HCl is added. This gives solution 6.
7. The procedure is repeated again and 70 ml of N/500 HCl is added to obtain solution 7.
8. The procedure is repeatedfor one moretime and 75ml of N/500 HCL is added. This gives solution 8 with a volume of about 80ml.
9. 240 ml of solution 8 equivalent to about 15 liters of gastric juiceis mixed with 1 volume of staurated magnesium sulfate and then centrifuged. The precipitate is about 3 grams.
10. To this precipitate, add 10 ml of N/10 sodium acetate and titrate it to pH 3. Add N/2 sulfuric acid till a dark viscous liquid with a slight ppt is obtained. To this add 1 volume of saturated magnesium sulfate and filter the suspension with suction. The precipitate is dissolved in 8 ml of N/10 sodium acetate solution. The solution is yellow and clear. This is titrated to pH 3 and N/2 sulfuric acid is added. It is allowed to stand for 18 hrs at 6 degrees C. The solution is then filtered with slow suction. The precipitate is dissolved in minimum quantity of water at 45 degrees C. It is slowly cooled and allowed to crystallize. The crystals appear in about an hour as yellow colored crystals.
11. They are kept at 20 degrees C for 24 hours and then filtered. The yield is about 0.1 gm. They may be later dissolved in about 20ml of N/10 sodium acetate and used as enzyme solution.

Choice Of Methods Of Purification

The readers are by now aware that there are many methods that can be used for purification of enzymes. It should be noted that no single procedure is good enough to give 100% pure enzyme isolate. The choice of methods and their sequence of use are dependent on various criteria. They are briefly discussed below:

(A) The Volume Of The Crude Extract

Usually when an enzyme is isolated and purified the initial volume of the homogenate or crude extract is considerably large. It is known that many purification procedures have a volume restriction. Ideally in the initial stages, salt fractionation or solvent fractionation is often employed. For those enzymes that are heat stable the heat precipitation method may be used. Chromatographic techniques in the column mode are sometimes used.

(B) The Instrumentation Required

Many laboratories do not have updated and sophisticated instruments and in such case, the fractionation methods are a practically feasible alternative. These have an advantage of simple instrumentation and cheapness although the purification may not be great. Saturation with ammonium sulfate and precipitation by acetone are the preferred techniques. Dialysis is sometimes used.

(C) The Time Period Available

Some techniques like matrix less affinity separation are rapid and so are the salting out methods. Other methods may be time consuming. The time factor available with the worker is of great importance, based on which he may choose the methods

(D) The Economics Of The Process Involved

Some processes are financially expensive while other are labor intensive. It is necessary to choose methods that balance between the two.

(E) The Purity Required

Sometimes the enzyme isolated need to be extra pure, as in the case of research, diagnostics and therapeutic administrations. Contamination in such preparation may not give expected results. For this more sophisticated techniques based on affinity and specificity of the enzymes are usually employed.

Terms Related To Enzyme Purification

Whether the enzyme has been purified or not is generally determined by checking its activity in the purified preparation. Since most enzymes are proteins in nature the purified preparation is subjected to activity determination and protein quantitation to determine the specific activity and fold purification of the preparation. The terms most often used include,

(A) Yield.

(B) Specific activity

(C) Fold purification

The terms are discussed in brief as,

(A) Yield:

The crude extract is often subjected to fractionation and to check the presence of the desired enzyme in the purified fraction, its activity is compared with that of the crude extract. The yield is then determined using the formula,

$$\text{Yield} = \frac{\text{Units of enzyme activity in fraction}}{\text{Units of enzyme in the crude extract}}$$

(B) Specific Activity

It is given by activity in units per gram of protein in the solution. This is obtained by dividing the activity in units by the total amount of protein in the solution. In terms of the formula used it is written as,

$$\text{Specific activity} = \frac{\text{Units of enzyme activity in solution}}{\text{Total protein content in the fraction.}}$$

(C) Fold Purification

During the purification of the enzymes, different processes are employed. The fold purification is based on comparison between the specific activities of the enzyme in the purified fraction and that of the crude extract. It is written as,

$$\text{Fold purification} = \frac{\text{Specific activity of fraction}}{\text{Specific activity of crude extract}}$$

(5) Development of Assays

While working with the enzyme purification, it is necessary and customary to check the activity of enzymes. In fact enzyme assays are performed with the crude extract as well to determine whether the enzyme of interest is present in the homogenate or not. The assays may use determination of the formation of product or the utilization of substrate and this can change with the enzymes (for example activity of amylase may be followed by disappearance of starch while that of a phosphatase may be followed by measuring the inorganic phosphate generated as a result of enzymatic catalysis.

In general there are two types of assays:

(A) Qualitative Assays

The qualitative assays are not stoichiometric and are only used in the first step to determine that the enzyme of interest is present in the chosen source for isolating it or sometimes as a confirmatory test for the final isolate. These assays are simple to design. A little knowledge of the reaction catalyzed, substrate required and the other requirements (including the proper reaction conditions) is enough for formulating the qualitative assay.

Qualitative assay of amylase: determination of the achromic point.

Amylase can be isolated from saliva, germinating cereal grains or sweet potato(Ipomea batatas). Saliva can be clarified if necessary and directly used as an enzyme solution. The amylase from grains or sweet potato needs fractional precipitation or salting out procedure before it can be used. Readers probably are aware that the amylases act on starch and produce sequentially different products that include various dextrins, reducing oligosaccharides and glucose. The qualitative assay of amylase is based on the fact that with the activity of the enzyme, its substrate starch disappears and while starch has an ability to form a blue color with iodine, the dextrins, oligosaccharides and glucose do not give color reaction with iodine. Thus the determination of achromic point (disappearance of color) can be taken as a proof of amylase activity. The procedure is simple and does not require costly instrumentation.A mixture of buffer, enzyme, NaCl, and starch is made and kept at room temperature or 37 degrees C for incubation. Simultaneously a series of test tubes containing buffered iodine solution are prepared and numbered. Each contains the same amount of iodine. After regular time intervals of 60 seconds a small quantity, say 0.1ml is withdrawn from the reaction mixture and added to a tube containing iodine soluton. It is then

observed for color formation. The first tube shows a distinct blue color due to the reaction of starch and iodine which fades in subsequent tubes. The tube which does not show any change in the iodine color is taken as the achromic point. It should be noted here that NaCl is added as Cl acts as an activator. The iodine solution should be same in concentration and quantity. Alternately a multicavity tile may be used in which periodically the reaction mixture and iodine solution can be added till the achromic point is reached.

Qualitative assay of urease

Urease can be isolated from jackbean meal. The enzyme catalyzes the reaction of splitting urea into ammonia and carbondioxide. The isolation is done by using acetone. The crude enzyme is added to a solution of urea and incubated at 40degrees c for 30 min. the ammonia liberated is qualitatively assayed by addition of Nessler' s reagent. A yellow color is formed.

(B) Quantitative Assays

The quantitative assays are used to measure the activity of the enzyme, within a set of well-defined parameters. The conditions are predefined and all the parameters except the variable are kept constant. These studies generate important kinetic data. The end of the reaction is usually followed by the quantitative estimation of the product (s) formed through colorimetric analysis. Quantitative assays are used to determine the fold purification during the subsequent steps employed for the purification of the enzyme. They are also important for enzymes that are used in research, diagnosis and treatment. The protocols are arranged so that keeping all other requirements constant only the parameter whose effect on the enzyme activity is to be studied is varied. For example in case where the effect of the substrate concentration has to be determined, a set of tubes from blank is taken wherein the substrate concentration is increased by 0.2 ml increments. The volume is made to say 2 ml with an appropriate buffer or distilled water and all other reagents are added. The tubes are then kept at appropriate temperature for the reaction time (usually 15min or 30 min). The color developing agent is added, mixed and the tubes are read at the appropriate wavelength to obtain optical densties from which a graph is constructed. The OD of the unknown solution is similarly worked out and using the graph the amount of product formed is calculated. From this value it is easy to determine the units of enzyme activity.

There are two types of quantitative assays viz. Two point assay and Kinetic assay.

(a) Two Point Assay

It is also called as fixed time assay. The reaction time is fixed in this method and the enzyme activity is expressed as the amount of substrate transformed, product formed or cofactor altered by the measured amount of enzyme solution under controlled conditions. In other words the assay is performed after the end of the specific reaction time.

(b) Kinetic Assay

It is also called continuous monitoring assay. The readings are recorded continuously for the determination of reaction velocity hence multiple readings are obtained. When the substrate concentration decreases the rate of reaction is also decreased. In most clinical enzymological assays this method is adopted.

Quantitative assay of Succinate Dehydrogenase: The enzyme is a marker enzyme for the mitochondrial fraction. It catalyzes the conversion of succinate to fumarate simultaneously reducing FAD to $FADH_2$ If TTZ (triphenyl tetrazolium chloride) is added to the reaction mixture it accepts the hydrogen atoms from $FADH_2$ and forms formazan, a colored compound that is easily estimated at 420nm. Phosphate buffer at pH 7.4 is used.

(6) Stabilization and Crystallization of Enzymes:

The stability of an enzyme poses a major problem since the enzymes may lose their activity under many laboratory conditions. Specific steps are therefore necessary to prevent the denaturation and loss of activity of the enzyme. The general recommendations are on the line of preservation of proteins but the best conditions for each enzyme have to be determined specifically. It can be said that enzymes are optimally stabilized by specific solution conditions of pH, ionic strength, anionic/cationic composition and so on. It should be noted here that the conditions of stability are different than the conditions of optimum activity.

For long-term storage, enzymes should be kept at cryogenic temperatures around –70degrees or under liquid nitrogen. Although most laboratories use conventional freezing techniques that use temperatures between 0 to –20 degrees, the enzymes stocked undergo unintentional freeze-thaw cycles, as higher temperatures are required to keep them frost-free. The stability of the enzyme protein can be greatly enhanced by adding equal volume of glycerol and mixing it properly with the sample. This enables the protein to remain in the liquid phase at low temperatures and prevent their possible denaturation due to freeze-thaw cycles.

Yet another concern is about the microbial contaminants and their degrading activities on the proteins. The samples should be sterile-filtered through micro filters composed of a low protein binding material and placed in sterilized cryogenic tubes to avoid bacterial contamination. A frozen enzyme sample should be thawed only up to the required amounts and used immediately. To avoid wasting of enzyme samples, they should be stored in small amounts. Once thawed the enzyme should be kept at ice temperature (4 degrees) for as long as possible before equilibration to the assay temperature. Certain additives enhance the stability of enzymes for long-term storage at cryogenic temperatures and sometimes for short-term storage in solutions. They include glycerol, sucrose and cyclodextrans. The amounts of required stabilizers are different for different enzymes and have to be worked out specifically. Some enzymes are stabilized in the presence of their cofactors, substrates, or even inhibitors that bind to their active sites to form transient compounds having more stability.

Enzymes may sometimes lose activity due to their non-specific adsorption on the walls of storage devices made of glass or ordinary plastic. It is therefore advisable to use storage containers made of polypropylene or polyethylene. The problem can be countered with the addition of inert carrier proteins at a concentration much higher than the enzyme, which then saturates the protein binding surfaces and leaves the enzyme molecules free and intact. Bovine serum albumin and gelatin have been frequently used with great success by enzymologists world wide for this purpose. Gelatin at a concentration of 1mg/ml satisfies most enzyme stock solutions, as it does not interfere with ultraviolet assays due to its lack of aromatic amino acids.

Crystallization of enzymes was initially taken as a criterion for purity however later it was found that many crystalline enzymes contained up to 50 % impurities. Re crystallization may be often required before a crystal can be said to be pure enough. The most commonly employed method is formation of crystals from ammonium sulfate solutions where the solution is kept standing for long periods up to weeks during which good quality crystals can be obtained. There are two approaches that are commonly employed. The salt is added to a comparatively concentrated enzyme solution until a slight turbidity appears following which the solution is allowed to stand and the concentration of salt is slowly increased by adding a few drops of saturated salt solution after long time intervals. Alternately capillary mediated delivery systems may be used. Dialysis or simple evaporation of the solution may produce similar effects. Another approach is to keep the salt concentration unchanged and modify the pH or temperature so that crystallization is effected. Crystallization is better with enzyme isolates separated with solvent fractionations, probably due to elimination of interfering lipids during such steps. Formation of acetone powders is also used routinely in many laboratories.

Some researchers have used heavy metals to form crystals of enzymes. For example the enzyme, phosphopyruvate hydratase was crystallized as an inactive mercury salt.

In general crystallization of enzymes have been done since 1930s and the enzyme crystals have a number of advantages over solutions. Some of these include:

They can be easily packaged, stored and transported

They have a less chance of microbial attack and spontaneous changes

They can be used conveniently in micro quantities

They can be used for crystallographic studies for getting more information about the enzyme.

(7) Criteria For Purity

The purity of an enzyme preparation is difficult to determine and there is no single method that is good enough, however an enzyme is said to be pure if a combination of tests reveal it to be homogenous. The tests are based on physical properties or catalytic action and specificity of enzymes.

Many of the tests are same as the techniques used for purification. A single band with electrophoresis, or single component sedimentation or with ultracentrifuge. This is because the sedimentation constants of proteins are not uniformly distributed and they lie in close probable values, increasing the chances of contamination by non-enzymatic proteins. As it often occurs many proteins have similar molecular weights or shape, leading to their grouping in a single band or sedimentation coefficient. Even the electrophoretic mobility is not a criterion that might be taken as a 100% proof of purity, as many proteins may have similar electrical charges resulting into same mobility. The sensitivity of the affinity techniques is supposedly the most yet there are many times when contaminants end up in the final preparations. The second disadvantage while working with protein solutions is that no techniques are sensitive enough to detect small amounts of contaminants. The solubility tests are a better alternative. A series of enzyme solutions in regularly increasing increments are mixed with a constant amount of water or a salt solution, shaken and then filtered or centrifuged and the protein in the resultant liquid is estimated. If the amount is plotted against the amount taken then initially the graph is linear (as all the protein added dissolves, a line with unit

slope is obtained) but as further additions are made, the solution becomes saturated and a horizontal line is obtained. If a second, contaminating protein is present, initially the protein that is more soluble reaches a saturation stage after which the less soluble protein continues to dissolve till its saturation point is reached, which is apparent by the bend seen in graph.

A simplified procedure, proposed by Northrop, uses the determination of two points, one at the occurrence of the bend and the other at the far right. Two tubes are taken and in the first amount of protein enough to generate slight turbidity is added. To the second tube 10 times the amount is added, mixed and both the tubes are centrifuged. If the supernatants contain exactly same amounts of protein, then the preparation is considered to be pure. If however there is an increase, it is considered as an impure or contaminated solution.

Re crystallization is often used as it has been observed that the specific activity value reaches a constant after a few re crystallization cycles. It is thus taken that purity is maximal. In case of isoenzymes of a particular enzyme, agar gel separation is often used.

The choice of the type and number of methods used for determination of purity of the enzyme preparation is dependent up on the choice of workers as well as the facilities available and the economical support.

(8) Methods Of Preservation Of Purified Enzymes

Once the enzymes are purified and stabilized, they are preserved till further use. The methods of preservation used depend up on the volume of isolate, the frequency and period of utilization and storage facilities available. By far cryogenic preservation has been the most effective technique. However this facility is not available with most of the laboratories. Some research labs store their isolates at a freezing temperature in deep freezers (-4 to –20degrees C) and such preparations are often added with some stabilizing materials to enhance their keeping quality and time. Many others use acetone powders and crystalline enzymes.

In general some precautions are common to the storage of all isolates. They include:

1. The storage should be in micro aliquots.
2. The materials such as glass, quartz and ordinary plastic should be avoided while choosing containers.
3. The preparation should be micro filtered to remove any possible bacterial contamination before it is preserved.
4. If a part of the micro aliquot is used for an experiment, the remaining solution should not be preserved.
5. Some enzymes, especially the allosteric enzymes are unstable at 0 degree and they lose their sub-unit interactions.
6. Stabilizers are often recommended however the exact amount and the type of stabilizer required needs to be specifically worked out.

General Properties Of Enzymes

Enzymes are remarkable biomolecules that act as catalysts. They are responsible for the biotransformations that include catabolism and biosynthesis of important

compounds in living systems. Although they act as catalysts, they differ in several properties from chemical catalysts.

A comparison between enzymes and chemical catalysts is given in a tabular form as,

Table 2.4 Comparison of enzymes with chemical catalysts.

Property	**Enzymes**	**Chemical catalysts**
Chemical nature	Protein or RNA	Inorganic ions,acids &alkali.
Molecular weight	High	Low
Specificity	Specific in action	Not specific in action
Working Environment	Cell or cell free systems	Reaction mixtures / furnaces
High temperature tolerance	Present in rare cases	Present in all cases
Effect of pH	Range restriction	Not influential
Colloidal properties	Present	Absent
Source	Living cells	Extracellular
Coenzyme/cofactor requirements	May be required	Not required
Commercial exploitation	More	Less
Environmental competability	More	Less
Immobilization	Possible	Rare
Exhaustion of source	Impossible	Possible
Utility spectrum	Broad	Limited
Examples	Hexokinase, pepsin, etc.	Mn, Mg, Zn, Cu, Fe , H_2SO_4 HNO_3 etc.

The various properties of enzymes are briefly discussed as below,

- ***They are synthesized in cells:*** Almost all the living systems except viruses contain enzymes. In fact the cells can survive, grow and bring about all the changes, and transformations within them, with the enzymes. However it is interesting to note that they can be synthesized in reaction mixtures that contain all the components required for their synthesis.

- ***They function as biocatalysts:*** They are required to bring about the various biosynthetic and degradation reactions in the cell. They enable the cell to store materials (e.g.: glucose is stored as glycogen in liver and muscle by the action of specific enzymes), or they can use the stored materials (eg. on breakdown of glycogen, the glucose molecules are reformed). They oxidize substrate to produce energy, (eg. glycolysis, HMP shunt, ETC produce ATP and reducing equivalents that generate chemical energy in the cells) and synthesize new materials as and when required (eg. de Novo synthesis of nucleotides amino acids, sugars etc.)

- ***They are mostly universal:*** Except a few differences, enzymes catalyzing similar reactions are common to all living systems. For example the hexokinase isolated from prokaryotes and from eukaryotes shall catalyze similar reactions.

- ***They are mostly proteins in nature:*** Barring a few exceptions almost all the enzymes are protein in nature, and comprise of naturally occurring amino

acids. The exceptions include the ribo zymes or catalytic RNA molecules. It was earlier believed that all enzymes are protein in nature, however the concept was modified with the discovery of the self splicing RNA from *tetrahymena spp.*

- ***They are also active in cell free systems*:** One of the most remarkable properties of enzymes is that they are active even in the cell free systems. During the developmental stages of enzymology, some scientists including Pasteur believed that the enzymes are inseparable from the cells, resulting into the famous Pasteur – Liebig controversy, which lasted till the evidence shown by Buchner that they are able to remain active in the cell free systems. The present day techniques often use immobilized enzymes, enzyme electrodes etc for diagnosis.
- ***They can catalyze reactions in both directions:*** Many enzymes are reversible in nature, i.e. they can catalyze reactions in both the directions, for example the enzyme lactate dehydrogenase can catalyze the conversion of pyruvate to lactate and vice versa.
- ***They catalyze reactions by lowering activation energy:*** Enzymes combine transiently with the reactants to produce a transition state having a lower energy of activation than the transition state of the un catalysed reaction. Thus they accelerate biochemical reactions by lowering the energy of activation. When the reaction products are formed, the free catalyst is regenerated.
- ***They are highly sensitive to environmental parameters:*** Enzymes function within a limited range of conditions and their activity is markedly affected by various parameters. These include the factors like pH, temperature, substrate concentration, enzyme concentration, inhibitors, activators, ions, radiations, etc. for example a drastic alteration in the pH or temperature leads to the denaturation of the "3D" structure of the active site thereby greatly reducing or completely stopping catalysis.
- ***They may be synthesized in precursor forms:*** Certain enzymes, especially those that act away from the site of their synthesis are usually synthesized in the precursor forms. The proteases are the best examples studied. The precursors are referred as the zymogen forms and have to be activated before they are able to act on their substrates. Examples include trypsinogen, chymotrypsinogen, pepsinogen etc. Their activation is usually brought about by removal of a leader sequence comprising of a few amino acids.
- ***They react specifically:*** Enzymes are specific in action, they react with single or limited substrates. Those that react with a single substrate are called as absolutely specific enzymes (example includes Glucokinase that converts glucose to glucose-6-phosphate) while others could be group specific (example: hexokinase) or broadly specific (example is trypsin that can act on proteins, peptides, esters etc). Specificity has been also found in terms of geometrical or spatial arrangements (D amino acid oxidizes do not act on L amino acids) or optical properties of substrates.
- ***They may be coenzyme/ cofactor dependent:*** Certain enzymes are dependent on inorganic or small organic compounds for their activity. A detailed discussion

is given elsewhere in the book. It is enough to remember here that these components serve various functions including enhancement of substrate affinity, lowering of Km, bridging the enzyme and substrate, affecting the proximity and orientation, playing a role in catalysis etc.

- ***Many enzymes are allosteric in nature:*** The allosteric enzymes are those having a site different than the substrate-binding site. They also have a sub unit structure and show sigmoidal kinetics, due to positive or negative co - operativity. They usually serve as regulatory points in the metabolic sequence. The best-studied example is of Aspartate transcarbamoylase. It catalyses the transfer of aspartate to carbamoyl phosphate in the de Novo synthesis of pyrimidines.
- ***They may be localized within the cell:*** Many times the enzymes that are responsible for a particular metabolic pathway are found in the vicinity of each other, localized in specific region of the cell or a particular sub cellular organelle. Such a togetherness may be due to mere aggregation without any physicochemical bonding of the various components amongst themselves in some instances (for example the enzymes of the glycolysis pathway) while in others they form a multi enzyme complex (examples include the fatty acyl synthetase complex, the keto acid dehydrogenase complexes, and the complexes involved in pyrimidine synthesis)
- ***They may show directional preferences while catalyzing reversible reactions:*** They work equally efficiently in vitro and in vivo. Although some reactions show directional preferences when the enzymes are present in the cell but are freely reversible in the cell free systems, this is primarily due to the specific needs and energy status of the cell. This ability has been greatly helpful in the commercial exploitation of industrially important enzymes. Many enzymes are regularly used in diagnosis and differential diagnosis, therapeutic treatments, industrial fermentations etc.
- ***They can be effectively reused in cell free systems:*** Enzymes after immobilization in solid matrices can be re employed to bring about the catalytic conversion of specific substrates for many number of times.
- ***Sometimes they are better tools than using whole microbial cells:*** This is especially true in single step transformations. Where microbial cells require a stringent control of growth and nutrition parameters and still have a chance of undergoing spontaneous mutations thereby affecting the overall yield, isolated and immobilized enzymes do not lose their efficiency for considerable time.
- ***Some enzymes can act on themselves:*** This is the characteristic feature of the tetrahymena RNA, however many enzymes can act on themselves for their activation.

(For example: pepsin can act on pepsinogen and bring about its activation.)

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