

ENZYMOLGY

Enzyme purification

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Keywords

Pestle & mortar; Waring blender; Potter Elvehjem homogenizer; Ultrasonicator; Ammonium sulfate fractionation; Ion-exchange chromatography; Gel filtration chromatography; Chromatofocusing; Affinity chromatography; Polyacrylamide gel electrophoresis; Isoelectrofocusing; Fold purification; Molecular weight; Sulphydryl groups, N- & C-terminal groups

Introduction

Enzymes are biological catalysts and are protein in nature with an exception of ribozyme, an RNA catalyzing the RNA splicing in eukaryotes. Almost, all the metabolic reactions, in the living systems, are catalyzed by the enzymes. Enzymes are catalytically active in isolated form too. In other words, living cells are not essential to get the enzyme activity. This property of enzymes has been exploited by the biochemists to study enzymes *in vitro*. While studying enzymes *in vitro*, it is believed that whatever we are getting *in vitro*, the same is *in vivo*. Although in almost all the cases, it is true but still there may be exceptions.

Although, mostly enzymes are found within the cells, there are few enzymes especially in microbes which are secreted in the medium by the microbe and are called extra-cellular enzymes. In order to study an enzyme *in vitro*, it is essential to isolate it from the cell. It is preferable to purify too at least up to some extent before studying its characteristics. It is pertinent to mention that some times, there is change in the conformation of the enzyme molecule after isolation and due to that, it is not necessary, whatever is studied *in vitro*, the same property is exhibited *in vivo* too. For isolation of the enzyme, cell has to be ruptured using a suitable isolation medium and suitable rupturing technique. However, it is also important to select tissue, organism for isolating the enzyme.

Criteria for selection of tissue/ organism

Although, there is no hard and fast rule for selecting tissue and/ or organism for the isolation of an enzyme, it is always preferable to select a source enriched in that particular enzyme. First, the worker should check from the literature whether the enzyme occurs universally (in animals, plants as well as microbes) or confined to a particular Kingdom. It is believed that working with microbial and animal enzymes is easier compared to plant enzymes since plants are generally rich in phenolics, which on exposure with air get converted into quinones and quinones bind with enzyme protein and makes it inactive. Special care has to be taken while working with plants. On the other hand, it is easier to get a plant tissue provided plants are grown in plenty in the surrounding compared to get animal tissue or a pure microbe. For animal tissue, either one will have to sacrifice the animal in the laboratory or will have to bring the tissue from a slaughterhouse. In case of microbes, one will have to grow microbe in pure form on a suitable growth medium under aseptic conditions after getting inoculum of the microbe. Upto 1980s, there were comparatively fewer reports of enzyme studies from plant sources and especially purification was being considered to be very difficult job. However, considering the fact that only few studies have been done from plant sources compared to their abundance in nature, many scientists preferred working with plant tissues and also became successful in purifying plant enzymes upto homogeneity (free from other protein(s)). During this period, there became drastic development in the techniques. Nowadays, enzyme purification is not considered to be a difficult job. After deciding whether one wishes to work with the enzyme from animal, plant or microbial source, one tries to select enriched source in that category. Some people also keep in mind about the availability of the tissue throughout the year otherwise the tissue will have to be stored in a deep freezer (generally at -80°C).

Enzyme solubilization techniques

Since almost all the enzymes (with few exceptions) are heat labile and not much stable at room temperature, the entire process of enzyme isolation, purification is carried out at $0-4^{\circ}\text{C}$ using a cold room. However, enzyme work can also be done without a cold room if

precautions of cold conditions are followed. All the isolation medium components should be in chilled condition (which is done by putting them in a refrigerator overnight). The component of the homogenization technique like pestle and mortar, bowl of the Waring blender should also be in chilled condition. While homogenizing in a pestle and mortar, it should be surrounded by the ice flakes. In case of Waring blender bowl, many people also wrap a cloth wet with chilled water.

Enzyme may be present in the cytoplasm or may be localized in an organelle (applicable only in eukaryotic cells, there are no distinct organelles in prokaryotes). Even in the organelle, the enzyme may be present in the matrix of the organelle or may be bound with the membrane. If the enzyme is localized in a particular organelle, it is preferable first to isolate the organelle in an intact form and afterwards conditions may be applied to rupture the organelle. In this process, the enzyme protein will not get contaminated with the proteins present in other organelles. If it is a soluble enzyme present in cytoplasm, then generally the complete cell is ruptured without taking care of getting organelles in intact form.

If one has to isolate the organelle in an intact form, one will have to use an isotonic isolation medium and there should not be any detergent in the medium capable of rupturing the organelles. It is important to select a suitable cell rupturing technique and an optimal isolation medium.

Selection of the isolating medium

In case of animal tissues and microbes, many people just use distilled water as isolating (homogenizing) medium. However, generally a buffer of a suitable ionic concentration and pH is preferred in order to maintain the pH and ionic concentration in the medium. A certain ionic strength of the buffer is essential for maintaining the buffering capacity. However, much higher ionic strength is also avoided since some times, high ionic concentration may be inhibitory to the activity of the enzyme. All the enzymes are pH sensitive. Every enzyme is stable in a particular pH range only. Every enzyme shows enzyme activity in a particular pH range only. However, mostly for isolation, pH of the medium is little different than the pH at which enzyme activity is measured. The pH of the buffer is maintained according to the nature of the enzyme. Selection of the pH should be such that isolated enzyme should be in fully active form. In case of plants, presence of the buffer in the isolating medium is more important since there is accumulation of acids in plant cell vacuoles and these acids get released in the medium after rupturing. The released acids will influence (decrease) the pH of the medium and if buffer has good buffering capacity, it will counteract the change in pH by the acids. However, sometimes, for isolation of the enzymes which act in acidic pH range like acid phosphatase, it is preferable to use water instead of buffer since release of the acids will not decrease the pH of water lesser than the optimum pH of the enzyme. In addition to buffer or water as the case may be, other components are also used as per need. The other commonly used components in the isolating medium are described below:

If the enzyme under study requires free sulfhydryl group(s) in its structure for exhibiting activity or in case of plant tissues especially if that tissue accumulates phenolics, many workers prefer to use a reducing agent. The commonly used reducing agents are cysteine, 2-mercaptoethanol (β -mercaptoethanol), sodium metabisulfite, dithiothreitol, dithioerythritol, reduced glutathione etc. and all are used generally in the concentration range 10 to 50 mM. In some cases, sodium metabisulfite has been used even up to 100 mM. The reducing agent may get oxidized by air, therefore, it is always dissolved freshly (except 2-mercaptoethanol

which is commercially available as concentrate, 14.5 M, in the liquid form). After rupturing of the cell, phenolics (if present) get oxidized to quinones. The quinones bind with the proteins to make protein-quinone complex which makes the enzyme protein inactive. The reducing agent keeps the phenolics in the reduced form. Sometimes, only reducing agent is not sufficient to keep the phenolics in the reduced form. Under the conditions, it is preferred to use a non-ionic detergent like Triton-X-100 in the isolating medium (generally up to 1%). The Triton-X-100 dissociates protein quinone complex. Triton-X-100 also ruptures the cell organelles. If organelles have to be kept intact, then under the conditions, it is preferred to use phenolfixing agents like poly vinyl pyrrolidone (PVP), which is used generally at a final concentration of 1% (w/v). PVP fixes the phenolics and therefore, phenolics are not oxidized to quinones. Sometimes, proteases get released from the organelles on rupturing the cells and chew the enzyme protein. To prevent this a protease inhibitor like phenyl methyl sulfonyl fluoride (PMSF) in 10 to 20 mM concentration is added in the isolating medium. A metal chelating agent like ethylene diamine tetra acetate (EDTA) or ethylene glycol bis (2-aminoethyl ether), N, N, N', N' tetra acetate (EGTA) (in the concentration range 10 to 50 mM) is added in the isolating medium to prevent the denaturation of the enzyme protein by heavy metals already present in the tissue/ cell. Sometimes, presence of metal ions like Mg^{+2} , Mn^{+2} , Zn^{+2} , etc. is essential in the isolating medium for getting the enzyme in the active form. If both metal chelating agent and metal ions are required in the isolating medium, amount of the metal ion is put more compared to the amount of the metal chelating agent. If one wishes to rupture only the cell wall and cell membrane keeping the cell organelles intact, isotonic conditions have to be maintained. Generally, 0.25 M mannitol or 0.5 M sucrose is used in the isolating medium. These are considered comparatively to be the inert substances on which enzymes do not act. If one is studying invertase enzyme, then sucrose is not used, since it will be degraded by the enzyme. There may be a special requirement in the isolating medium for a particular enzyme like the presence of an effector molecule. Therefore, composition of the isolating medium has to be optimized before starting the actual work.

Techniques used for enzyme isolation

As mentioned above, generally enzymes are isolated in the cold condition (at 0 to 4°C). For the purpose, homogenizing medium as well as container should be in the chilled condition. It is preferable to homogenize the tissue in a cold room. The following are the commonly used techniques for enzyme homogenization:

Pestle and mortar

Pestle and mortar is a moderate technique for tissue homogenization. Mechanical breakdown occurs during the process. Sometimes, grinding is done in the presence of purified sand or glass beads for aberration. Pestle and mortar is considered to be a moderate grinding technique and rupturing of the cell organelles does not occur if isotonic grinding medium without detergent is used.

Blenders

Waring blender (commonly called as mixie) is comparatively harsh technique of grinding the tissue compared to pestle and mortar and is mostly used for homogenizing the harder tissues (generally the plant tissues). If the worker is interested in isolating intact cell organelles, then Waring blender is not a preferred technique. Waring blender is first operated at low speed for

few seconds and then at medium speed(s) for few seconds before bringing it at high speed. Time of grinding at various speeds is decided according to the nature of the tissue being ground. If homogenization has to be done for a little longer time, then it is generally done after few seconds interval after every minute of grinding at high speed to avoid heating during operation of the Waring blender.

Ultra- Sonicator

This technique of rupturing the cells is generally used for microbial/ bacterial cells. Ultra-sonicator generates low as well as high wavelength ultrasonic waves. For the purpose, a suitable probe depending on the volume of the homogenizing medium is selected and connected with the ultra-sonicator. The container having cells and homogenizing (isolating) medium is put in chilled condition by covering the container with ice. There is much generation of heat during ultra-sonication, therefore, ultrasonic waves are thrown in the sample after few seconds interval, every 10 to 15 seconds ultrasonication.

Vir-Tis homogenizer

This is considered to be a mild technique and generally used for homogenization of soft tissues such as animal tissues. Here a motorized pestle with teeth like aberrations is used. With Vir-Tis homogenizer, generally no rupturing of cell organelles occurs during grinding provided isotonic medium with no detergent is used.

Potter Elvehjem homogenizer

This is also a mild technique and is used for homogenization of soft animal tissues. Potter Elvehjem Homogenizer is a simple equipment having a pestle like glass rod with teeth like aberrations on its tip. There are down aberrations in the tube too on which teeth of the rod are fitted during up and down process of the rod. Up and down process of the pestle is done manually by hand or by mechanical device.

Razor blade

It is comparatively very mild technique. It is generally used only for isolation of intact cell organelles for the purpose of studying the intracellular localization of the enzyme proteins. In the technique, razor blade is used for chopping the tissue in the presence of isolating medium. Although the technique is good for the isolation of intact organelles, but it is unable to rupture all the cells. Therefore, there is low recovery of the enzyme due to left out of un-ruptured or partially ruptured cells. These un-ruptured or partially ruptured cells are removed as cell debris after centrifugation.

Extrusion method

This method relies on the principle that forcing a cell suspension at high pressure through a narrow orifice will provide a rapid pressure drop. This is a powerful mean of disrupting cells especially from bacteria.

Lytic enzymes

Cell wall and cell membrane lytic enzymes like cellulase, pectinase, xylanase, pectin methyl esterase, lysozyme etc. can be used for rupturing the cells. Enzymes being costly are not commonly used for making cell free preparation for isolation of enzymes. In plant tissue culture, lytic enzymes are used to prepare protoplast.

Freeze-Thaw

With certain susceptible microbes and eukaryotic cells, repeated freezing and thawing results in extensive membrane lesions with release of periplasmic and intracellular proteins.

Acetone powder

Drying with acetone is a good method for rupturing the cell membrane. Using acetone, powder of the tissue may be prepared which may be stored in a Deep freezer for a long time. It forms a convenient starting material from which the enzyme may be extracted with the isolating medium, whenever required. However, one has to take much precautions of low temperature (generally -20°C), otherwise, acetone may denature the enzyme protein.

Isolation of enzymes from sub-cellular organelles requires rupturing of the organelle. Generally for the purpose, organelle is isolated in intact form thus removing the contaminating proteins of the cytoplasm and other cell organelles. Afterwards, cell organelle is ruptured in the presence of a suitable detergent like tween, teepol, digitonin etc.

Methods of enzyme purification

The purification of a particular enzyme involves removal of other substances (proteins as well as non-proteins) present in the preparation.

Purification of an enzyme protein is generally a multi-step process exploiting a range of biophysical and biochemical characteristics such as its relative concentration in the source, solubility, charge, size (molecular weight), hydrophobicity/ hydrophilicity of the target protein. In general, design of the purification technique/ protocol should be focused on: (i) high recovery, (ii) highly purified enzyme protein, (iii) reproducibility of the methods, (iv) economical use of the chemicals (reagents) and (v) shorter time for complete purification.

Proteins are relatively labile and get denatured at high temperatures and variation in pH. Each protein has its own physico-chemical characteristics. The techniques selected for enzyme purification should be moderate and native conformation of the enzyme protein should not change as a result of purification. During purification, degree of purity and percent of recovery should be checked after each step of purification. It is not always necessary to purify the enzyme protein to homogeneity since enzyme purification is a costly affair and also the time consuming. Therefore, it is necessary to have an idea of the degree of purity necessary for the intended use of the targeted enzyme.

In general, it has been observed that enzymes are more unstable in dilute solutions. Therefore, while designing the purification procedure, initially emphasis is given on concentrating the protein concentration in the sample rather than purification. After

concentration, emphasis is given to purification (removal of unwanted proteins) and lesser loss of enzyme activity of the targeted enzyme.

Commonly, the first step in enzyme purification is based on fractionation of proteins on the basis of solubility of proteins in aqueous solutions of salts or organic solvents.

Fractionation of the proteins on the basis of solubility in aqueous solutions of salts or organic solvents

The solubility of a protein is the result of polar interactions with the aqueous solvent, ionic interactions with salts and repulsive electrostatic interactions between alike charged molecules. The properties of water may be changed by changing the ionic concentration and pH. The addition of miscible organic solvents, other inert solutes and polymers with temperature variation can be manipulated to cause selective precipitation. Isoelectric precipitation may also be used since protein is least soluble when net charge on it is zero.

Salting out

Generally, salting out of proteins using ammonium sulfate is used as the first step in the enzyme purification. However, other salts like sodium sulfate may also be used but ammonium sulfate is most common. On addition of salt, dehydration in the microenvironment of the protein molecules occurs. A large number of water molecules bind with the salt reducing the amount of water available to interact with the protein molecules. Precipitation of proteins using salt also removes non-protein impurities present in the enzyme homogenate (crude extract). At a particular concentration of the salt, unbound water will keep the protein in the soluble form. Generally, solid salt is added in the range of 0 to 30%, 30 to 60% and 60 to 90 % with continuous gentle stirring keeping the pH constant (near neutrality or slightly alkaline by the addition of dilute ammonia dropwise). Care is taken that no local precipitation of protein occurs. On the other hand, care is taken that no denaturation of protein should occur due to stirring. Stirring is done either with the help of a glass rod (if volume is not too much) or with the help of a motorized mechanical stirrer. After addition of the salt, the suspension is stored for few hours in cold condition for complete precipitation. The precipitate is collected by centrifugation in cold condition and thereafter the precipitate is dissolved in suitable medium (which may be same as the isolating medium or may be little different). For calculation of the amount of ammonium sulfate, standard tables showing grams of ammonium sulfate per litre are available in many manufacturer's booklets, books, periodicals etc. It is pertinent to mention that amount of the ammonium sulfate differed at 0°C and at 25°C. A 100% saturated solution of ammonium sulfate at 25°C requires 767 gm ammonium sulfate per litre whereas at 0°C requires 697 gm per litre. The dissolved precipitate is centrifuged to get clear solution. Afterwards, the protein solution is generally desalted either by dialysis or by using Sephadex G-25 gel filtration chromatography.

The following equation explains the salting out phenomenon:

$$\text{Log } s = \beta - K_s \cdot \mu$$

where s is the solubility of the protein, β is the intercept constant, K_s is the salting out constant, μ is the ionic strength. A relationship of logarithm of solubility of protein with ionic strength is shown in Fig. 1.

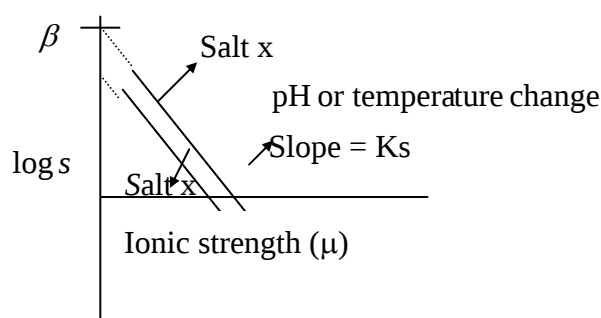


Fig. 1: Salt- solubility relationship (salting out)

As shown in Fig. 1, solubility of the protein decreases with increase in the ionic strength. The solubility of the protein depends on the following factors: (i) pH; (ii) temperature and (iii) type of the salt used.

K_s is the characteristic of the salt. It's value for a particular salt remains same irrespective of the pH and temperature used. As shown in Fig. 1, slope of the line will be the K_s value. Intercept value at the vertical axis is the intercept constant, β . Its value changes on change in pH and / or temperature.

Protein fractionation using organic solvents

Generally, acetone is used for fractionation of the enzyme protein. While using acetone, extreme care is to be taken otherwise acetone will denature the enzyme protein. Chilled acetone at -20°C is used and continuous stirring is done to avoid denaturation of the proteins. Like salt fractionation, here also fractionation of proteins is done by using different concentrations of acetone like 10%, 20%, 30% and so on. The suspension is stored for few hours before centrifugation at -20°C to collect the precipitate. The precipitate is dissolved in suitable medium.

Protein precipitation by organic solvents is explained on the basis of Debye Huckel theory. According to this theory, protein molecules in solid state are considered as held together by columbic forces between opposite charges on adhering molecules. The following formula is used for columbic force:

$$F = e \cdot e' / D \cdot r^2$$

F is the columbic force, e and e' are the opposite charges, D is Dielectric constant of the solvent, r is the distance between the charges. Organic solvents like acetone lower the Dielectric constant of water and as a result, there is increase in columbic force.

Protein fractionation using nonionic polymers

Nonionic polymers can be used for precipitation of enzyme proteins. Polyethylene glycol (PEG) is commercially available in different molecular weight ranges like 6000, 40,000, 360,000. Lower molecular weight PEG is more soluble in aqueous solvents compared to high molecular weight, therefore, PEG of 6000 molecular weight is preferable. Different ranges of PEG (like 0-3%, 3-10%, 10-20%, 20-30%, 30-40%, 40-50%) are used for fractionation of proteins. The precipitated clear protein solution can be loaded on ion exchange column like

DEAE cellulose as PEG does not bind to ion exchanger. Ammonium sulfate fractionation can also be carried out after PEG fractionation. Ammonium sulfate precipitates proteins whereas PEG remains in the soluble form.

Protein fractionation by heat treatment

In many cases, the enzyme protein to be purified is fairly stable at temperature like 55°C or 60°C. At this temperature, many unwanted proteins get denatured and precipitated out with little or no loss of enzyme activity. The protein solution is kept for 5 to 10 minutes at this temperature and afterwards immediately chilled by keeping in ice. In few cases of allosteric enzymes, the enzyme is stable at high temperature in the presence of an effector molecule. However, this method is not in much use since there is always a possibility of getting the conformation of the enzyme protein changed at higher temperature.

Chromatographic separation of the enzyme proteins

Chromatography is a separation technique based on partitioning of the proteins between moving phase and a stationary phase. The technique allows separation to be modified by changes in packing chemistry and elution buffer. Fully automatic high performance liquid chromatography equipments with more speed, resolution, sensitivity, reproducibility and recovery are available commercially.

For enzyme purification, commonly used chromatography techniques are: (i) Ion exchange chromatography; (ii) Adsorption chromatography; (iii) Gel filtration chromatography and (iv) Affinity chromatography.

In general, the procedure of carrying the work is same in all types of chromatography. First, the enzyme protein sample to be purified is applied onto the pre-equilibrated column and thereafter, the sample from the column is eluted with buffer with a series of steps of different solute concentrations, with a gradient of solute or with a specific ligand for the desired enzyme protein. The effluent eluted out from the column is collected as a series of fractions using a fraction collector, tested for enzyme activity and protein.

Ion exchange chromatography

The basic principle involved in ion exchange chromatography is binding of charged proteins on the ion exchanger by electrostatic attraction (ionic bonds) between charged groups on the proteins and opposite charges on the exchanger. Conditions like pH are set in such a way that opposite charges be there between the proteins and ion exchanger. Unbound proteins are removed from the column by washing with the same medium used for pre-equilibrium. Bound proteins are eluted by passing buffer of higher ionic strength (using salts like sodium or potassium chloride) or by using buffer of different pH. It is preferred to make a linear gradient of the salt or pH, instead of step-wise elution. Gradient elution is considered better since with gradient, there are more chances of removal of unwanted proteins. Fractions of the effluent are collected and analyzed for the desired enzyme activity.

Two types of ion exchangers are in common use for separation of enzymes: Anion exchangers and cation exchangers.

The most commonly used anion exchanger is diethyl amino ethyl cellulose (DEAE cellulose). Some other are amino ethyl cellulose (AE cellulose), triethyl amino ethyl cellulose (TEAE cellulose) and guanido ethyl cellulose (GE cellulose). The most commonly used cation exchanger is carboxy methyl cellulose (CM cellulose). The other examples of cation exchangers are phospho cellulose (P cellulose) and sulfo ethyl cellulose (SE cellulose). These exchangers have cellulose matrix, which is considered to be inert. The other matrices used in exchangers are Sephadex and Sepharose.

There may be a condition when enzyme protein of interest is not bound on the exchanger and unwanted proteins are bound. Although it is not considered to be a preferred way, however, if sufficient purification and recovery is obtained, the condition may be used. Under the conditions, some times, it is called negative chromatography. On an average, we apply 2 to 5 mg proteins per ml packed bed of the exchanger. In ion exchange chromatography, amount of the protein applied on the column is more important than the volume of the sample. Regarding the dimensions of the packed exchanger bed, it is preferred to have 1:10 diameter to length ratio.

Adsorption chromatography

The basic principle in this type of chromatography is binding of the proteins on the matrix by physical adsorption on the surface of insoluble matrix (through weaker bonds like hydrogen, van der Waals bonds). Afterwards, proteins are eluted from the column matrix by using a suitable elution buffer either having change in ionic concentration or pH. The commonly used matrices in adsorption chromatography are: (i) calcium phosphate gel; (ii) alumina gel and (iii) hydroxylapatite gel.

In this type of chromatography, gel to protein ratio is important for physical adsorption. It is preferable to carry a trial experiment in centrifuge tubes. A constant amount of the gel is put in each tube and different amounts of protein sample are added in each tube so that ratio of 0.1 to 2.0 in different tubes be obtained. After addition of sample, it is mixed with the gel and allowed to bind for few minutes. Afterwards, tubes are centrifuged and enzyme activity is determined in different tubes supernatants. If enzyme activity is present in a supernatant, it means binding of the enzyme protein (of interest) on the gel did not occur. From this trial experiment, one can determine, what will be the optimum gel to protein ratio so that enzyme protein of interest gets adsorbed on the gel surface. Afterwards, accordingly, size of the packed gel in the column be decided. For elution, generally either buffer of high ionic strength or buffer with salt like NaCl or KCl is used.

The gels used in adsorption chromatography are commercially available. The gels may also be prepared in the laboratory. It is found that older gels are more effective in separation compared to newly prepared gel. In the laboratory, calcium phosphate gel is prepared by addition of sodium tri phosphate to a diluted solution of calcium chloride and pH is adjusted to 7.4. A precipitate of calcium phosphate formed is washed to remove excess ions. Alumina gel is prepared by the addition of a hot solution of aluminum ammonium sulfate to a solution containing ammonium sulfate, ammonia and water at 60°C. The solution is cooled, the precipitate of alumina formed is washed with water to remove excess ions. Hydroxylapatite gel is prepared by addition of calcium chloride and di sodium hydrogen phosphate to a solution of one molar sodium chloride. The precipitate of hydroxylapatite formed is treated with alkali and heated to boiling for about 40 to 50 minutes. Afterwards, it is cooled and washed with water to remove excess ions.

Gel filtration (Molecular sieve) chromatography

The basic principle is based on the size and shape of the proteins. Here, gel particles have sponge like porous matrix as a structure with controlled dimension. The gel particles are swollen and equilibrated with appropriate medium and afterwards is packed in the chromatography column. Gel particles are spherical in shape. The molecules (proteins) to be separated enter in the porous matrix of the gel particles and too large molecules are not entered in the porous matrix and are eluted out from the column. Every gel is characterized by exclusion limit that means the proteins of more than that molecular weight will not enter in the matrix and eluted out as such (without separation). Void volume is considered as the space between the gel particles in the packed column. It is determined by passing blue dextran, which has very high molecular weight. Molecules with masses below the exclusion limit of the gel are eluted from the column in order of their molecular mass (weight) with the largest eluting first. Larger molecules have lesser of the interior volume of the gel available to them than the smaller molecules.

The commonly used gel filtration gels are of dextran, agarose, polyacrylamide. These gels are having registered trade names of the manufacturers. For example, dextran gels having registered trade name 'Sephadex' are in much common use. Gel filtration chromatography (with matrix having much lesser exclusion limit such as Sephadex G-25) is also used for desalting purpose. Since in Gel filtration chromatography, separation is based on molecular weight (if shape of all the molecules is same), this chromatography has been commonly used for determination of molecular weight of proteins.

Affinity chromatography

The basic principle involves bio-specific interaction of the enzyme protein of interest with an immobilized ligand, which may be substrate, analogue of the substrate, inhibitor, activator. Inert materials like agarose, polyacrylamide, glass beads, cellulose etc have been used as supporting medium (matrix). The ligand is attached so that its enzyme interaction function is not impaired. Subsequently, elution is done by treatment resulting in dissociation of the desired enzyme ligand complex. Nowadays, affinity matrices (ligand immobilized with the matrix) are commercially available.

Immuno-affinity chromatography is also an affinity chromatography where antibody of the protein is used as ligand. The basic principle of antigen antibody interaction in this chromatography is applied. Although it is a good technique for purification of a protein, it is not in common use for enzymes since generally enzyme gets inactivated after binding with the antibody.

Besides, another affinity chromatography called Dye Affinity Chromatography is also used for enzyme purification. Specific dye bound matrices are available commercially which are used in Dye Affinity Chromatography. One such popular dye matrix is Green A. Binding affinity of the dye is ranging from 1 to 15 mg protein per ml gel. In dye affinity chromatography too, elution is done by using higher ionic strength (presence of salt in the elution medium).

Chromatofocusing

Chromatofocusing was first described by Sluyterman, *et al.* in 1977. They proposed that a pH gradient can be made on an ion exchanger by taking the use of the buffering action of the charged groups present on the ion exchanger. If a buffer initially set to a pH, is passed through a column of ion exchanger set to another pH, a pH gradient is formed just as if two buffers of different pHs are gradually mixed using a linear gradient maker. If such a pH gradient is used to elute the proteins bound on the ion exchanger, the proteins elute in order of their isoelectric pH (IpH). Focusing effects developed during the process result in band sharpening, sample concentration and good resolution. Linear pH gradients are found to be better for resolution of proteins. Commercially amphoteric buffers are available in different pH ranges and also the poly buffer exchangers of different pH range.

Electrophoretic techniques

Now-a-days, electrophoretic techniques are not in common use for enzyme purification. Electrophoretic techniques are more commonly used for analytical purpose for checking the purity of the enzyme protein. The characteristic mobility of the enzyme proteins in electric field is the basis for the electrophoretic purification techniques for proteins. Most commonly used electrophoretic technique is Polyacrylamide Gel Electrophoresis (PAGE). In PAGE, proteins are separated on the basis of charge as well as size. Polyacrylamide gel has the advantage that pore size can be controlled by varying the concentration of acryl amide and N, N'-methylene bis acryl amide.

Polyacrylamide gel is prepared by the free radical induced polymerization of acrylamide and N, N'-methylene bis acryl amide in a suitable buffer. Ammonium persulfate used in polymerization provides free radicals. The addition of N, N, N', N'-tetra methyl ethylene di amine (TEMED) accelerates the polymerization and cross linking of the gel. Some people also use riboflavin as polymerizing agent instead of ammonium persulfate. Polyacrylamide gel electrophoresis is done either as continuous system (only one type of gel is used and the same buffer is used in the gel preparation and in the electrode compartments) or as discontinuous system. In discontinuous system, a large pore gel called as stacking gel is layered on the top of separating gel and each .gel layer is made with a different buffer and electrode compartments buffer is different from gel buffers. The discontinuous system has been found better than continuous system due to better separation. The gel / separating b(resolving) gel in discontinuous system is generally 5 to 12 % gel prepared with a buffer of nearly pH 8. It is a smaller pore size gel and separates the proteins during electrophoresis. The stacking gel has a lesser concentration of polyacrylamide (in the range of 3%). The pH of the stacking gel is kept generally 2 units lower than the pH of the separating gel. However, it is pertinent to mention that there is no such hard and fast rule about it. The stacking gel allows the proteins in the sample to be concentrated into the stacked band before entering into the separating gel. To watch the movement, generally bromophenol blue is used as a marker dye. However, when enzyme purification is carried out using electrophoresis, bromophenol blue dye is not mixed in the enzyme protein sample since it will inactivate the enzyme protein. If enzyme purification has to be done, generally bromophenol blue is used in a separate well and electrophoresis is run in the cold condition either by doing electrophoresis in a cold room or by passing chilled water through the electrophoresis jacketed apparatus. Use of jacketed apparatus is preferred. After separation of protein sample, slices of the gel may be cut and each slice after crushing into a suitable medium is assayed for the desired enzyme protein and afterwards in the preparatory gel, slice at the corresponding distance is

cut and enzyme protein may be eluted simply by crushing the gel in a suitable buffer. Elution may also be done by using electro-elution apparatus in cold condition.

Isoelectrofocusing

In isoelectrofocusing, protein sample is electrophoresed through a gel having stable pH gradient. The pH in the gel increases linearly from anode to cathode. Each protein in the sample will migrate in the pH gradient and will get stacked at the position corresponding to its isoelectric point since at isoelectric point, the protein becomes immobile in the electric field due to no net charge. Each protein is focused into a narrow band at its pI that may be as thin as 0.01pH unit. The pH gradient is prepared in the gel by mixing a mixture of low molecular mass oligomers bearing aliphatic amino and carboxylic groups that have a range of isoelectric points. In the electric field, these carrier ampholytes segregate according to their isoelectric points such that the most acidic gather at the anode with the progressively more basic closer to the cathode.

Now-a-days, carrier ampholytes are commercially available and are famous by their trade names (like ampholine, pharmalyte). Vesterberg was the first scientist who synthesized carrier ampholyte involving coupling of propanoic acid residues to polyethylene polyamines accompanied by reacting polyethylene polyamines with acrylic acid at 70°C. Ethylene amine can be condensed with propylenediimine to obtain a polyamine. The resulting polyamine is reacted with propane sulfone and chloromethylphosphonic acid by alkylation. Commercially, ampholytes have been synthesized by condensation of glycine, glycyl-glycine and amines of selected pKs with epichlorohydrin. D and L isomers of epichlorohydrin and amino acids are incorporated into the reaction mixture to increase the heterogeneity of the mixture.

Identification of the desired enzyme protein and elution from the gel may be carried out in the same way as described above under the polyacrylamide gel electrophoresis.

Miscellaneous

Ultrafiltration

Although ultrafiltration is used to concentrate the sample, ultrafiltration is also capable of removing low molecular weight substances. In ultra-filtration, protein sample is fed into a cell fitted with a membrane, which retains high molecular weight proteins while solvent and low molecular weight molecules (depending on the type of the membrane used, for example, membranes with different retention capacity say 10,000; 30,000 molecular weight proteins are commercially available) are filtered through the membrane. In ultra-filtration, positive or negative pressure using nitrogen gas is put into the solution or solvent collecting chamber, respectively to maintain flow across the membrane. For retaining and concentrating low molecular weight proteins, smaller pore size ultra-filtration membranes are commercially available. Use of successive large to small pore size membranes may result in enzyme purification as well as concentration of the sample.

Dialysis

Dialysis is used for removal of low molecular weight contaminants, salt etc. In dialysis, important points are nature of the dialysis membrane and its ability to allow the free flow outward of low molecular mass components, relative surface area of the membrane to the volume of the solution under dialysis, relative volume of the solution inside the dialysis tubing (the retentate) to that of the solvent outside (the diffusate), the concentration of diffusible substances outside the membrane which will oppose the further outflow. The dialysis membranes used are thin films of highly polymerized substances which swell in aqueous solvent resulting in a type of molecular sieve. Through the pores in the molecular sieve, low molecular weight substances will pass while high molecular weight proteins will be retarded. The dialysis membrane must be chemically inert and without fixed charge groups. Animal membranes, collodion, colloids deposited in porous pots, cellophane tubing etc are the commonly used membranes for dialysis.

Crystallization

After considerable degree of purification, enzyme protein may be tried for crystallization. However, crystallization is considered to be a difficult task. Generally ammonium sulfate or other salts such as sodium sulfate is added to the concentrated enzyme protein sample till slight turbidity. Thereafter, the preparation is kept to stand in cold condition. After storage for 24 to 48 hours, crystals of the enzyme protein may appear. It is pertinent to mention that crystallization is not an evidence of purity. The first crystals may contain other proteins too. On re-crystallization (after dissolving the crystals in a suitable medium and repeating the process the same way), specific activity of the crystals may increase.

Criteria of purity of the enzyme protein

There are number of techniques used for determining the purity of an enzyme protein. Here, purity of the enzyme protein means that it is a single protein free from any other protein and also non-protein contaminant. Each technique is based on the different physical property of the proteins. It is pertinent to mention that each technique may show the presence of an impurity rather than its absence. A single test (technique) carried out for showing the enzyme purity is not enough. A combination of techniques should be used for showing enzyme purity. Still, one should mention that the enzyme purity has been checked using these techniques.

Detection of non-protein contaminant(s)

Generally, a major non-protein contaminant found in a protein preparation is nucleic acid(s). The presence of nucleic acid in the protein may be checked by using a spectrophotometer. The ratio of OD_{280} to OD_{260} should be between 1.8 and 2.0. If it is less than 1.8, contamination of nucleic acid (or any other substance having absorbance at or near 260 nm) is there.

Detection of protein contaminants

Specific activity change

Enzyme protein purification can be estimated by calculating specific activity after every purification step. The specific activity must increase after every purification step. When enzyme protein is pure, there will not be increase in the specific activity after a purification step. In fact, some times, due to inactivation of a fraction of the enzyme protein, there may be some decrease in the enzyme activity. It is an indirect procedure to make an idea of the purity of the enzyme protein. However, it cannot be taken as granted that if after a particular purification step, there is no increase in the enzyme activity, enzyme protein preparation is pure (homogeneous).

Chromatographic methods

The chromatographic techniques used for the purpose are gel filtration chromatography, ion exchange chromatography, affinity chromatography. These techniques are simple methods for assaying the level of purity. In general, these methods are less sensitive compared to electrophoretic methods described below. However, these methods have the benefit of being non-destructive and therefore sample may be recovered. In gel filtration chromatography, as described above, molecules are separated on the basis of molecular size and shape. Impurities are detected either as separate peaks in the chromatogram or as a shoulder on the peak of interest, or as a strongly skewed peak. In ion exchange chromatography, there is interaction between the protein and the ion exchange resin. Presence of no absorption at OD₂₈₀ of the washing fractions and presence of a single protein peak without any shoulder in the chromatogram suggests the purity of the enzyme protein. However, one should be sure that all of the applied protein has been eluted from the column. In affinity chromatography, interaction between the protein and gel matrix is on the basis of biological property. On elution, if there is a single protein peak without any shoulder then it indicates purity of the enzyme protein. Here also, one should be sure that all the protein applied has been eluted from the column.

Electrophoretic methods

Polyacrylamide gel electrophoresis has been considered to be a better technique for checking the purity of the enzyme protein. Polyacrylamide gel electrophoresis may be carried out in three different ways: (i) non-denaturing (native) polyacrylamide gel electrophoresis, (ii) sodium dodecyl sulfate denaturing polyacrylamide gel electrophoresis and (iii) isoelectrofocusing.

Non-denaturing (native) polyacrylamide gel electrophoresis

Generally discontinuous type polyacrylamide gel electrophoresis is carried out for checking the purity of the enzyme protein. For the purpose, electrophoresis can be run at room temperature and after electrophoresis runs, protein bands are detected using Coomassie Brilliant Blue dye or more sensitive silver staining. If there is single band, it indicates that the enzyme protein is pure. As in polyacrylamide gel electrophoresis, separation is on the basis of charge as well as size of the proteins, it is preferable to run electrophoresis at different gel concentrations (5%, 6%, 7.5% or so) and also at different pH (pH 6.5, 7.5, 8.5 or so). It is considered that if two proteins having different charge and/or size are not separated under

particular conditions of gel concentration (pore size) and pH, they may get separated under different conditions. If under all the conditions (at each gel concentration using different pHs), a single band is obtained, protein is considered to be pure. To judge purity of an enzyme preparation, the electrophoresis is run under cold conditions with sample applied at two places in the gel. After gel run, half gel is processed for protein stain using Coomassie Brilliant Blue and another half for activity stain either directly by incubating the gel in the enzyme assay medium and afterwards staining suitably for detecting the product formed or by doing slices of the gel. The activity band should correspond with the protein band.

Sodium dodecyl sulfate denaturing polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS) is an anionic detergent and is capable of disrupting macromolecules whose structure is stabilized by hydrophobic interactions. It binds to the hydrophobic regions of the protein and separate them into their component subunits. It also imparts a negative charge on the protein, therefore, proteins move and separate in the electrophoretic field entirely on the basis of their molecular weights (provided all the proteins have same shape). If on SDS polyacrylamide gel electrophoresis, a single band is obtained after protein staining, the preparation is considered to be pure. Sometimes, even the preparation is pure, more than one band may be obtained on this electrophoresis. It may be due to heterogeneous nature of the protein (if the enzyme protein is oligomeric in nature and is not having identical subunits). Therefore, it is recommended that one should also check the purity using non-denaturing conditions.

Isoelectrofocusing

Proteins have a net positive charge in an acidic condition. With a gradual increase in pH, positively charge groups on the protein decrease with increase in the negatively charge groups. At a particular pH, the net charge on the protein becomes zero (at this pH, negative charges and positive charges are equal). This pH for that protein is called isoelectric pH (isoelectric point). At this particular pH, that protein will not move in an electric field. In isoelectrofocusing, with the help of carrier ampholyte, a stable pH gradient is prepared. Due to pH gradient, protein moves into a range of increasing pH. Finally, the protein encounters the pH where the net charge on it is zero and stop moving. The sensitivity of isoelectrofocusing is very high. Two proteins having a difference in pI of 0.01 can be separated on it. Therefore, if on performing isoelectrofocusing of the enzyme sample, one gets a single sharp band, sample is considered to be pure. In this technique, point of application of the sample does not matter and diffusion is not a problem. Once a final stable focusing is reached, the resolution is retained even if the experiment is continued for longer time.

Immuno-electrophoresis

The technique uses the specificity of the reaction between an antigen and corresponding antibody and molecular sieving of the gel in which the reaction takes place for analysis of the sample. It is a modification of Ouchterlony method of double diffusion.

First, antibodies in an animal like rabbit are developed against the enzyme sample whose purity has to be checked. Afterwards, an agar gel in a suitable buffer is prepared on a glass plate (like a microscopic slide) and appropriate well (hole) is punched in the middle of the

gel. The sample is applied in the sample well and electrophoresis is run using 4-8 volts / cm current for 1-2 hours. During electrophoresis, protein(s) will move either towards anode or cathode depending on the charge present on them. Care is taken (by using a marker dye) that no protein should move out of the gel. A trough is made in the length of the gel and filled with the antiserum raised against the sample and gel is incubated overnight at room temperature in the humid chamber to allow the diffusion of the antigen as well as antibody towards each other. This leads to precipitin arc formation at the site lateral to the position where sample is separated. The major advantage of this technique is increased resolving power due to combination of electrophoresis with immune specificity. If there is only one arc, it will indicate that only one protein is in the sample.

Centrifugation methods

Sedimentation velocity and sedimentation equilibrium centrifugation are used to check the enzyme protein purity. None of these methods are really suitable for detecting minute amount of contamination. It is the reason that ultra-centrifugal methods are not commonly used for checking the protein purity. Besides, for checking, analytical ultracentrifuge is required which is not available in most of the laboratories.

Sedimentation velocity method is relatively a simple method used for checking the protein purity and also used for determining the molecular weight of the protein. Presence of impurity (ies) in the sample is detected either as distinct species sedimented or by a discrepancy between the expected molecular mass and that determined experimentally. A limitation of this method is its insensitivity to small differences between the molecules.

Sedimentation equilibrium method is also used to check the protein purity and also for determining the molecular weight of the protein. If the sample is pure, the concentration dependence of the apparent molecular weight is independent of rotor speed. If this is not there, sample heterogeneity is suspected.

End groups determination

Determination of N- (amino) and C- (carboxyl) terminal amino acids in the enzyme protein sample can also be used to determine enzyme protein purity. If it is a single protein, it will give only one amino terminal and one carboxyl terminal amino acid. However, this method also cannot be considered to be full proof since, few proteins have been found to have blocked N- terminal amino acid or there may be a protein or peptide having cyclization. In that case, neither N- terminal nor C-terminal amino acid will be detected. Besides, there may be more than one protein having the same N- and C- terminal amino acids. Methods used for end group determination are mentioned below under the characterization.

As mentioned above, no single method can be considered to be a proof of enzyme purity. One must check more than one methods and mention the methods used. However, still among all the methods, SDS polyacrylamide gel electrophoresis and non-denaturing polyacrylamide gel electrophoresis are considered to be better than other methods and are in common use.

Preparation of purification table

Although, there is no much hard and fast rule for preparation of a purification table, one should show steps used for purification of the enzyme protein. In each step, amount of the total enzyme activity, total protein, specific activity, fold purification, percentage recovery are mentioned. Volume of the sample in each step can also be mentioned. A typical arbitrary purification table of starch phosphorylase is shown in Table 1.

Table 1: Purification of starch phosphorylase from *Cuscuta reflexa* filaments

S. No.	Purification step	Enzyme activity (nkats)	Protein (mg)	Specific activity (nkats/mg)	Fold enrichment	Recovery %
1.	Initial extract	2140	2532	0.85	----	100
2.	(NH ₄) ₂ SO ₄ fraction (30-70%)	1978	452	4.38	5.2	92
3.	DEAE cellulose chromatography	1292	44	29.4	34.5	60
4.	Sephadex G-200 chromatography	928	7.8	118	139	43
5.	DEAE – sepharose chromatography	673	3.4	198	233	31

Fold purification in the purification table is mentioned to show, how many times, specific activity increased in a particular purification step compared to crude extract which is also called enzyme homogenate or initial extract. Fold purification is indicative of the purification status of the enzyme protein. Increase in fold enrichment also depends on relative concentration of other contaminating proteins. It is the reason that those enzyme proteins, which are in major amount in the cell/tissue, will not show much fold enrichment with purification steps.

Characterization of enzymes

After purification, characterization of the enzyme protein is an important part in the study of the enzyme. Some of the physical properties like native molecular weight determination, subunit molecular weight determination indicating whether the enzyme protein is monomeric or oligomeric and if oligomeric whether has identical subunits or different types of sub units, number of sulfhydryl groups determination, absorption spectrum indicating whether it is a simple protein or has a cofactor bound, end groups determination; chemical properties like optimum pH, optimum temperature, energy of activation, and kinetic properties are studied.

Determination of molecular weight of the enzyme protein

There are number of methods for molecular weight determination for proteins. Some methods like light scattering method, osmotic pressure method, depression in freezing point method, diffusion rate method are not in common use whereas gel filtration and ultra-centrifugation methods are commonly used methods. For sub unit molecular weight

determination, sodium dodecyl sulfate polyarylamide gel electrophoresis is most commonly used method. The methods of molecular weight determination are described below (in brief):

Light scattering method

Enzymes are proteins and proteins do not make clear solution in aqueous medium. They form colloidal solutions. Colloids have the property of scattering the light called as *Tyndall effect*. If a beam of visible light is passed through a colloidal solution, a part of light is transmitted and other part gets scattered. The fractional decrease in the intensity of the incident light can be measured and using the following formula, molecular weight of the enzyme protein may be calculated:

$$M = - (\log_e I/I_0) / HCl$$

where I is the intensity of the transmitted light, I_0 is the intensity of incident light, H is the proportionality constant, C is concentration of enzyme protein in gm/ ml, l is the length of the path (in cm) through the scattered solution.

The value of H may be determined using the following formula:

$$H = \frac{32 \pi^3 n^2}{3 N \lambda^4} \left(\frac{n - n_0}{c} \right)^2$$

Where λ is the wave length of the incident light, n_0 and n are the indices of refraction of the solvent and solution, respectively, N is the Avogadro number and its value may be taken 6.02×10^{23} , c is the concentration in gm/ml.

Therefore, one will have to determine the indices of refraction of the solvent and solution separately.

Osmotic pressure method

After determining the osmotic pressure of the enzyme protein solution, molecular weight may be determined using the following formula:

$$\pi v = nRT$$

where π is the osmotic pressure in atmospheres, v is volume in litres, n is the number of moles of the solute, R is the molar gas constant in litre-atmosphere, T is the absolute temperature, n may be taken as g/M where g is the amount of the solute (enzyme protein) in gm and M is the molecular weight.

Depression in freezing point method

On dissolving the enzyme protein in water, freezing point of water will decrease. The following formula may be used for molecular weight determination:

$$\Delta t = K_f M$$

where Δt is the depression in the freezing point, K_f is the molal freezing point or Cryoscopic constant, M is the molar concentration of the enzyme protein. If a solution containing an Avogadro number (6.02×10^{23}) of total dissolved particles either as undissociated molecules

or ions or as a mixture of ions and undissociated molecules in 1000 gm of water (one molal solution) freezes at -1.858°C , then it is called cryoscopic constant. K_f for water is found to be -1.858°C .

Diffusion rate method

Diffusion rate is measured by optical methods such as refractive index, light absorption, fluorescence and Tyndall effect which do not disturb the solution.

Diffusion coefficient represents the number of moles of the solute diffusing across unit area per unit time under a concentration gradient of unity. The following formula may be used for molecular weight determination:

$$D = \frac{RT}{N\pi 6\eta} \sqrt{\frac{4N}{3MV}}$$

Where D is the Diffusion Coefficient, R is the gas constant in $\text{ergs mole}^{-1} \text{deg}^{-1}$, T is the absolute temperature, N is Avogadro number, η is the viscosity of the medium in poises, V is the partial specific volume, M is the molecular weight.

Partial specific volume is the increase in the volume when 1 gm dry substance is added to a large volume of the solvent. For proteins, its value is taken as 0.74.

Ultra-centrifugation method

Using analytical ultracentrifuge, one can determine the sedimentation constant. In fact, it is customary to express sedimentation velocity in terms of sedimentation constant which represents the velocity of particles in a unit cm-gm-sec (cgs) field of force.

If x represents the distance of the particles from the axis of rotation in the ultracentrifuge, then the rate at which they travel is

$$\frac{dx}{dt} = s \omega^2 x$$

ω is the velocity of revolutions/second, ω is 2π revolutions per second or 6.2832 revolutions per second and t is the time in seconds.

To calculate s , suppose if at time t_1 , the boundary of the particles in the colloidal solution is x_1 cm from the axis of the centrifuge and at time t_2 , it is x_2 cm from the axis, then

$$s = \frac{1}{\omega^2 (t_2 - t_1)} \cdot \log \frac{x_2}{x_1}$$

For molecular weight determination, the following formula may be used:

$$M = \frac{RTs}{D(1-Vp)}$$

Where R is the gas constant, T is the absolute temperature, s is the sedimentation constant, D is the diffusion coefficient, V is the partial specific volume, p is the density of the solution.

By equilibrium studies

If during centrifugation in the ultracentrifuge, the protein band remains in equilibrium i.e. no change in the position of the band on prolonged centrifugation, then, molecular weight of the protein may be calculated using the following formula:

$$M = \frac{2RT \log_e (C_2/C_1)}{\left[\omega^2 (1-Vp) (x_2^2 - x_1^2) \right]}$$

Where C_2 and C_1 are concentrations at distances x_1 and x_2 from the axis of the rotation.

Gel filtration chromatography method

As described above, in gel filtration chromatography, separation is on the basis of molecular size and shape. If all standard proteins and enzyme protein are globular in nature, separation will be on the basis of molecular weight. Although any of the gel filtration matrix may be used, commonly used gel filtration matrix is Sephadex G-200. A calibrated column of Sephadex provides a simple way of determining the molecular weight of the enzyme. Calibrated column may be used repeatedly both for molecular weight determination and for routine separation. The packed gel filtration column may be calibrated using standard markers. There are following two commonly used methods for molecular weight determination:

Whitekar method: A semi log graph paper is used. Log molecular weight is plotted versus elution volume of the standard proteins. A straight line is obtained. After determination of the elution volume of the enzyme protein, from the graph, molecular weight may be calculated.

Andrews method: Here also a semi log graph paper is used. Log molecular weight is plotted versus elution volume/ void volume for the standard proteins. A straight line is obtained. After determination of the elution volume of the enzyme protein, from the graph, molecular weight may be calculated.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis for subunit molecular weight determination

In this technique, the sample to be electrophoresed is denatured and coated with detergent by heating in the presence of SDS and a reducing agent (generally 2-mercapto ethanol). The

SDS coating gives the protein a high net negative charge that is proportional to the length of the polypeptide chain. The sample is loaded on the polyacrylamide gel containing SDS and a high voltage is applied causing the protein component to migrate towards the anode. Since all the proteins have a net negative charge that is in proportion to their size, proteins are separated entirely on the basis of their molecular weight as a result of the sieving effect of the gel matrix. The molecular weight of an enzyme protein may be determined by comparing the electrophoretic mobility of the band with that of the standard proteins.

Sulphydryl groups determination

In many enzyme proteins, presence of free sulphydryl groups has been found essential for enzyme activity. Sulphydryl groups in the enzyme protein are estimated by 5, 5'- dithiobis (2-nitrobenzoic acid) (DTNB) titration. For the purpose, first enzyme protein solution is dialyzed against a suitable buffer to remove any reducing agent present in the preparation. To the enzyme solution in a cuvette of the spectrophotometer, DTNB solution is added, absorbance at zero time is measured and afterwards change in the absorbance at 412 nm is recorded till there is no further increase in absorbance. Number of sulphydryl groups may be calculated using molar extinction coefficient of $13,600 \text{ M}^{-1}.\text{cm}^{-1}$. This will give the value of free or exposed sulphydryl groups. Once, there is no increase in the absorbance at 412 nm after addition of DTNB, sodium dodecyl sulfate solution may be added to a final concentration of 1%. If buried sulphydryl groups will be there, increase in absorbance will start and now from this increase in absorbance, number of buried sulphydryl groups may be calculated. It is preferred to plot a graph showing increase in absorbance with time. First, there may be very fast increase with time followed by medium increase and then slow increase. From these values, fast reacting, medium reacting and slow reacting sulphydryl groups may be calculated. If presence of free sulphydryl group(s) is essential for enzyme activity, the activity gets inhibited by DTNB or pCMB (p- chloro mercuric benzoate). Alkylating agents like iodoacetate and iodoacetamide also block sulphydryl groups.

Absorption spectrum

To check whether any cofactor is bound with the enzyme protein, absorption spectrum of the enzyme protein from 200 nm to 950 nm is taken. Simple enzyme protein absorbs at or near 280 nm due to the presence of tyrosine and tryptophan in the enzyme protein. If enzyme protein has got a bound cofactor which absorbs light, an additional characteristic absorption peak is obtained. From the absorption maximum of the peak, one can determine, which cofactor is bound. Some people also use ratio of two peaks for determination of the purity of the enzyme protein.

Amino acid composition

For determination of amino acid composition, enzyme protein is digested with 6N HCl at 110°C for 24 hours under vacuum condition. This treatment will hydrolyze protein into amino acids. Analysis of amino acids is carried out using automatic amino acid analyzer where the separation of amino acids is based on ion exchange chromatography and amino acid is detected using ninhydrin reaction. By running a standard amino acids mixture, by comparison of absorption, one can calculate the amount of individual amino acids. For identification of individual amino acid, time of elution is important. During acid hydrolysis, serine, tyrosine,

tryptophan, cysteine get destroyed. Serine and threonine are determined by hydrolyzing samples for 24, 48, 72, and 96 hours and plotting a graph between the amount of the serine/threonine and time of hydrolysis. By extrapolation, amount of the serine / threonine may be calculated at zero time of hydrolysis. Tryptophan is separately estimated by spectrophotometric method. Cysteine is determined by estimating sulfhydryl groups using DTNB titration.

End groups determination

Commonly, an enzyme protein has one amino-terminal and one carboxyl-terminal amino acid. Amino terminal amino acid can be determined by 1-fluoro, 2,4-dinitrobenzene, phenyl isothiocyanate or dansyl chloride method. Enzymatic method using amino peptidase can also be used. Carboxyl-terminal amino acid determination can be carried out by hydrazine, lithium borohydride or carboxypeptidase method. Having identified the end groups, the remainder of the sequence of the protein can be determined by digesting the enzyme protein with proteases like trypsin and chymotrypsin or by cyanogen bromide cleavage to get fragments. These fragments can be separated using a suitable chromatographic technique. Afterwards, amino acid composition and sequencing of these fragments can be done.

Chemical modification studies

In order to know about the nature of the active site (which amino acid is part of the active site), chemical modification studies may be done. If enzyme protein gets inhibited by a sulfhydryl agent like DTNB, p-CMB, iodoacetate, iodoacetamide, it indicates that sulfhydryl group containing amino acid (mostly cysteine) is part of the active site or for maintenance of proper three dimensional structure of the enzyme protein. Similarly, N-acetyl imidazole is a tyrosine reagent; organophosphorus compound, diisopropyl phosphoro fluoridate (DFP) is a serine reagent; tri nitro benzene sulfonate is lysine reagent; phenyl glyoxal is an arginine reagent. However, it is pertinent to mention that chemical modification studies give an idea only. These studies are not considered to be full proof. For confirmation, oligonucleotide site directed mutagenesis studies are carried out, where after changing a specific suspected amino acid into other amino acid (or a neutral amino acid like alanine), studies are done.

Suggested Reading

1. Enzymes by Dixon and Web
2. Handbook of Enzymes by Anil Kumar
3. Fundamentals of Enzymology: Cell and Molecular Biology of Catalytic Proteins by Nicholas Price & Lewis Stevens
4. Enzyme Purification and Related Techniques : Vol. 22: Enzyme Purification & related techniques by Colowick & Jakoby William B (eds.)
5. Sluytermann, L. A. E. and Wijdenes, J. Chromatofocusing: isoelectric focusing on ion exchangers in the absence of an externally applied potential. In *Proc. Int. Symp. Electrofocusing and Isotachophoresis*. Radola, B. J. and Graesslin, D. eds. Berlin, de Gruyter, 1977. pp 463-466.