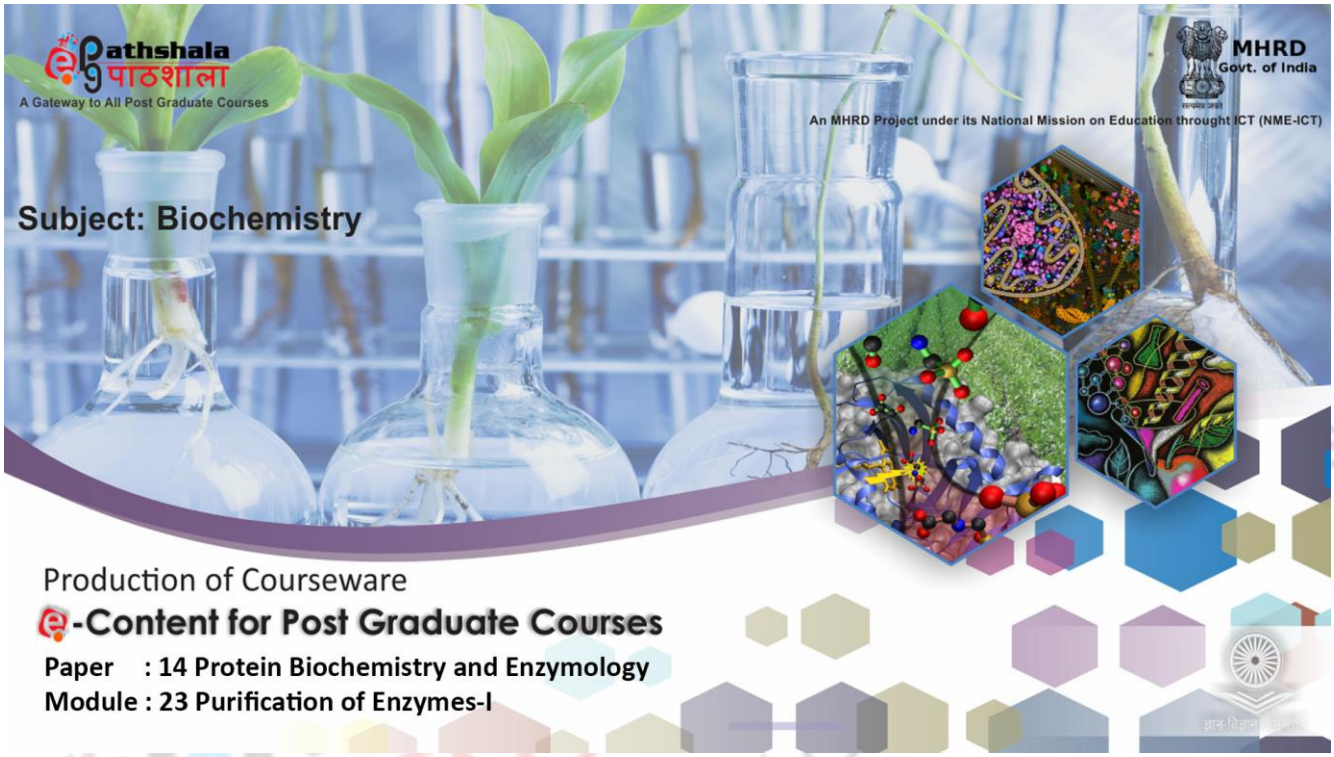

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Paper : 14 Protein Biochemistry and Enzymology

Module : 23 Purification of Enzymes-I

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MHRD Govt. of India

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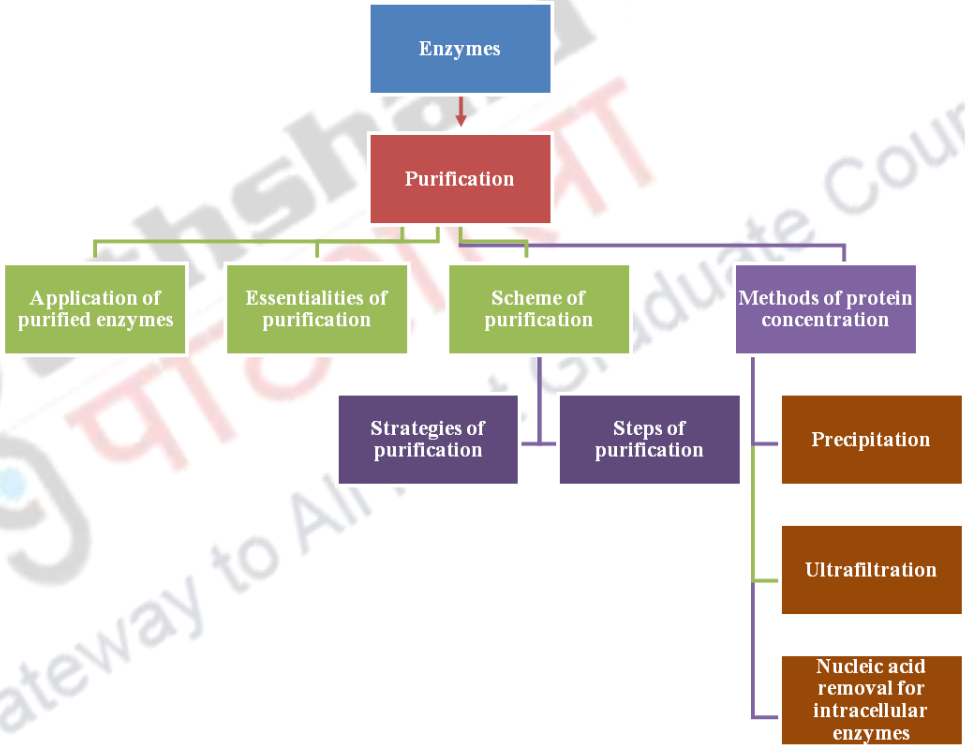
Description of Module	
Subject Name	Biochemistry
Paper Name	08 Protein Biochemistry and Enzymology
Module Name/Title	23 Purification of enzymes-I

1. Objectives

1. Understanding need for purified proteins/enzymes
2. Explain the approach taken for any purification procedure
3. Explain scheme and strategy of protein purification
4. Understanding the importance and methods of protein concentration before purification.

2. Concept Map



3. Description

3.1 Protein purification

Protein purification is an essential component for the characterization of protein properties, structure, function and related interactions, for example conformational alterations, substrate specificities, specific activities as well as interaction with other ligands. The study of proteins and their function is imperative from the perspective of understanding of both cells and organisms. The importance of proteins in emphasized by their role as biocatalysts for cellular metabolic processes, as integral cellular structural components both

within and outside the cell, as cellular receptors for conveying relevant and necessary information to the cell, as intracellular signaling components or as components of the genetic machinery of the cell. Thus, it is often reasonable to purify an isolated protein so that one is able to study the protein in isolation. Additionally, purified proteins also find applications in various areas as shown in Figure 1.

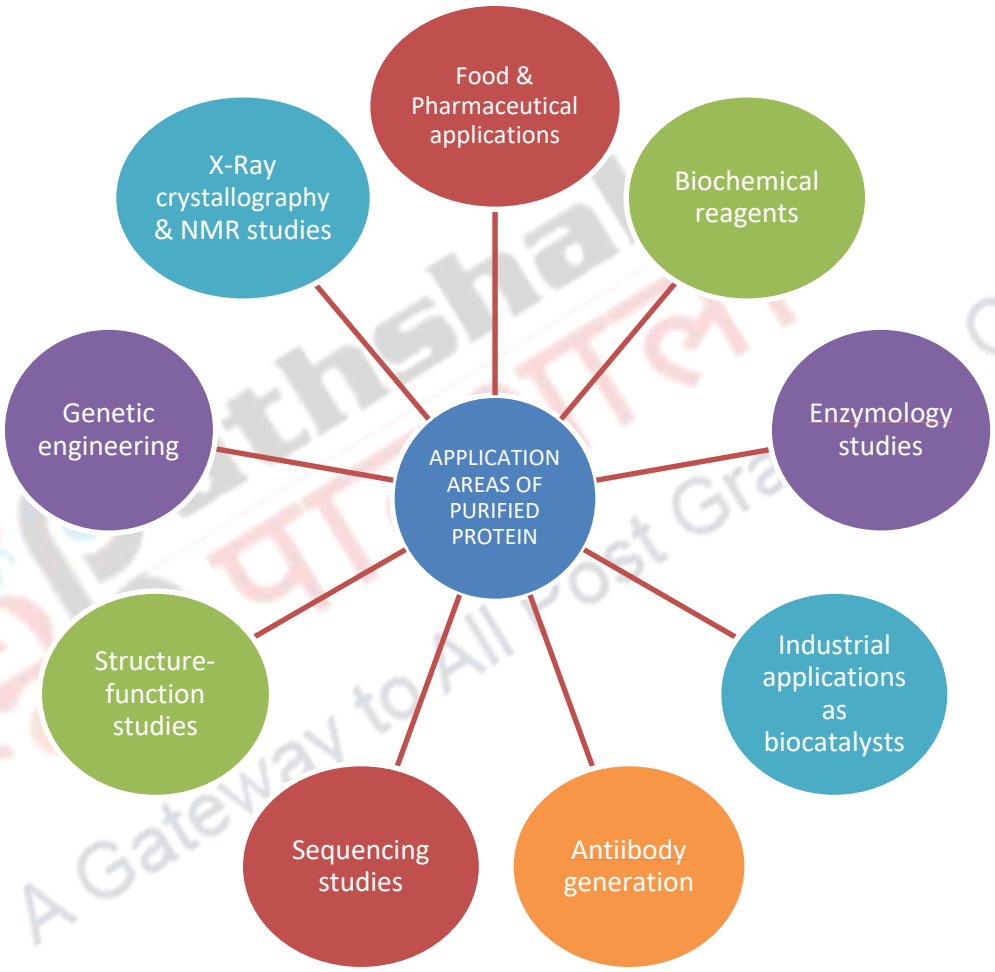


Figure 1. Areas where pure protein is required

The degree of protein purity required depends to a great extent on the end use of the protein. For example, in some applications such as an enzyme additive in washing powder, a relatively impure sample is sufficient. However, for other applications, for example in the food and pharmaceutical industry, a high level of protein

purity is essential and the desired protein must be purified over a number of steps. This is thus achieved through several protein purification methods, carried out in a sequence of purification steps.

As we will see further, purification procedure is not restricted to any specific number of steps or stages. Each step is targeted to bringing about some fold of purification in the protein to be purified. This is also accompanied by some extent of product loss most of the times. An ideal protein purification strategy would be the one which leads to the highest level of purification in fewer numbers of steps and also simultaneously ensures that there is minimum product loss. The selection of steps in the protein purification protocol depends largely on the properties of the target protein such as size, charge, solubility etc.

3.2 Essentialities of purification

3.2.1 Sources

One of the key essentialities of protein purification is that there must exist a reasonable source from which the protein could be derived. The source should be both cheap and readily available.

3.2.2 Assays

An assay is essential for quantitative determination of a particular activity present in the protein within a particular fraction. Assays can be diverse ranging from colorimetric assays (change in absorbance) or a change in molecular weight of a protein or ability to stimulate cell differentiation, cell proliferation or cell death. Since an assay is required to be repeated many times, it is essential that the assay procedure is simple and not much time consuming.

3.3 Scheme of protein purification

The first step in purifying proteins is preparation of a crude extract. Crude preparations of extracellular proteins are obtained by centrifugation wherein the cellular biomass is removed. In case of intracellular (inside the cell) proteins, the cells will be required to be lysed and the lysate so obtained will contain a complex mixture of all the proteins from the cell cytoplasm, additional macromolecules and internal cellular components, nucleic acids as well as cell debris etc. Intracellular crude protein extracts are then prepared by removal of cell debris by centrifugation and removal of nucleic acid, following which a supernatant containing protein of interest is recovered. The soluble protein containing fraction (extracellular or intracellular) is concentrated to obtain an enriched protein fraction. The protein extract can be further concentrated before subjecting it to any chromatographic purification procedure so as to obtain an enriched protein fraction. This can be achieved by adding various types of precipitants such as ammonium sulfate, organic solvents or

polyethylene glycol (PEG). The partially pure fraction may then be subjected to one or more purification strategies until purification is achieved. The basic scheme of protein purification is shown in Figure 2.

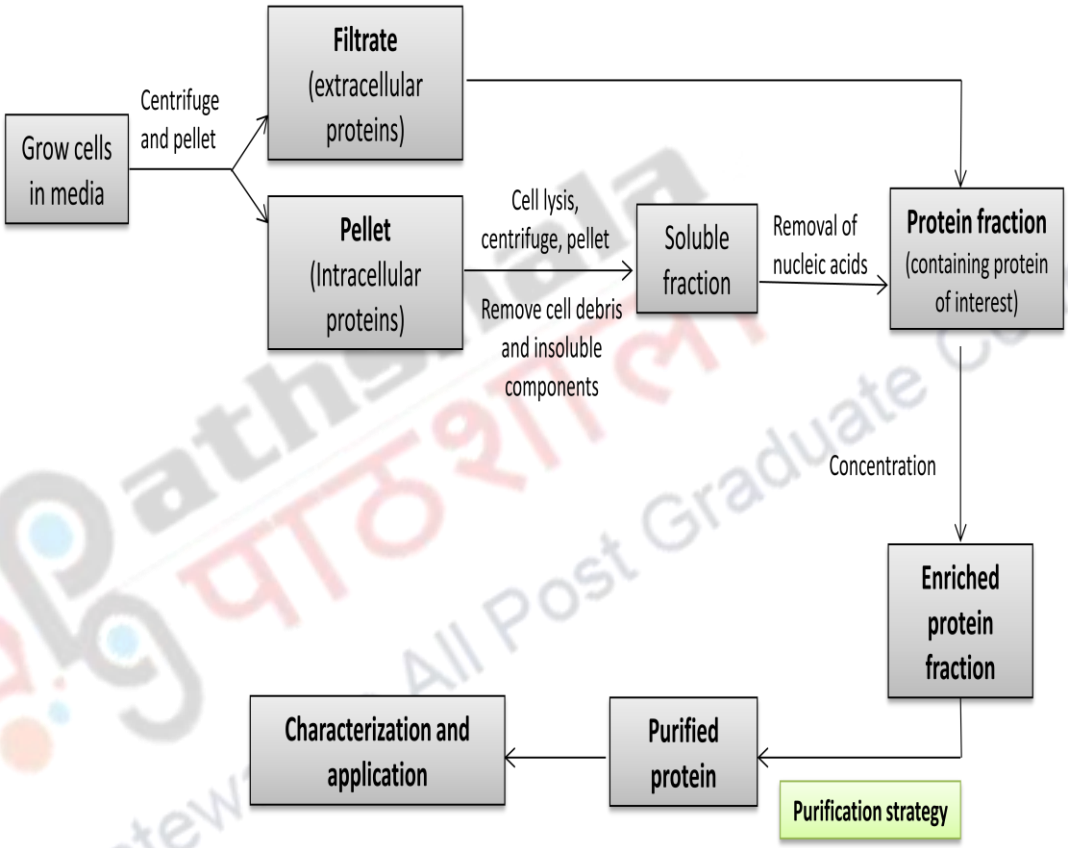


Figure 2. Scheme of protein purification

3.4 Purification strategy

To enable simple planning and execution of protein/enzyme purification protocols, a three step protein purification strategy has been developed. This is known as the CIPP strategy-Capture, Intermediate Purification and Polishing. This strategy is used widely both on laboratory and industrial scale to ensure faster process, shorter time required and a good economy. Together, this enables a cost effective and robust production of target production.

- **The Capture Phase**- In this phase, isolation, concentration and stabilization of the target protein is achieved.
- **The Intermediate Purification Phase**-In this phase, bulk impurities like removal of other unwanted proteins, nucleic acids, endotoxins which are closely related to the protein are removed.
- **The Polishing Phase**-In this phase, most of the major impurities have already been removed. This stage removes any trace amounts of specified impurities or any wanted forms of the target protein that may have been formed during purification.

The basic objectives of each phase in the CIPP purification strategy is shown in Figure 3.

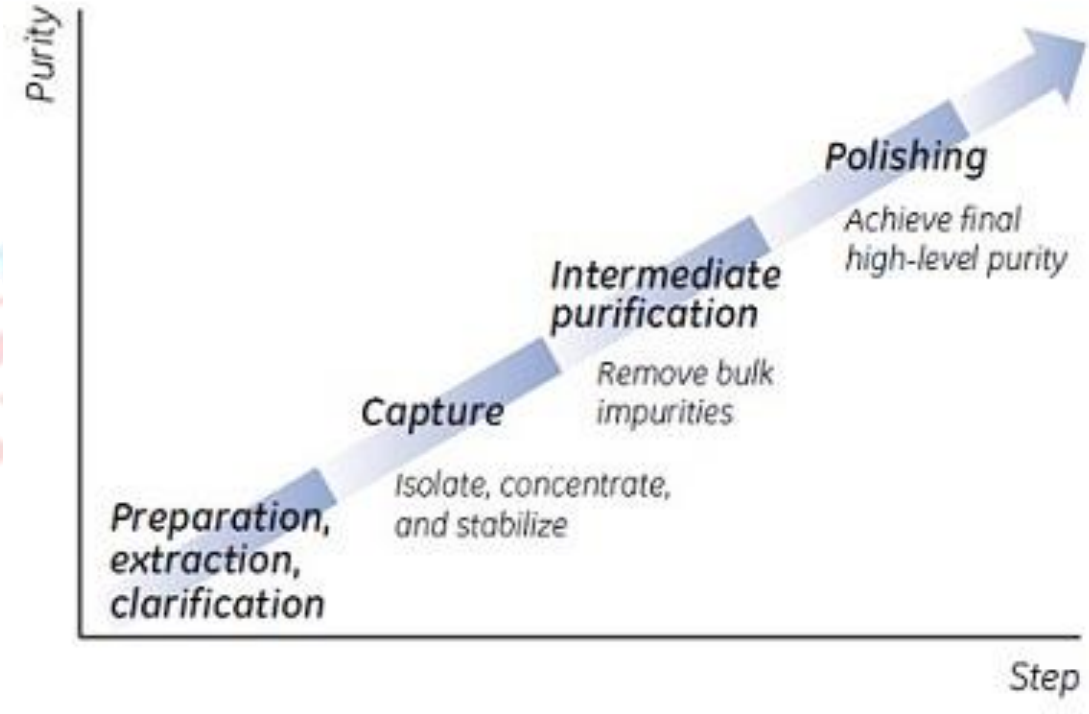


Figure 3. The CIPP strategy

Although this strategy consists of three broad phases, it does not imply that every purification process will contain three steps. Each phase may include one or more purification steps wherein each step may be used to address a specific objective within a purification process. The number of steps will primarily depend on the

intended use of the protein. It should however be kept in mind that as the number of purification steps are increased, the overall protein recovery and activity will decrease, and purification time and cost involved will go up. Therefore, efforts should be made to optimize a purification strategy that gives best results with maximum yield of the purified protein and fold purification.

3.5 A multistep procedure

Purification is a multistep procedure. The various steps in the purification process is aimed removing non-protein components and impurities from the protein of interest, freeing the protein from the matrix that confines it, and finally separating out the desired protein in its pure form. Figure 4 gives outlines the various steps in a purification process.

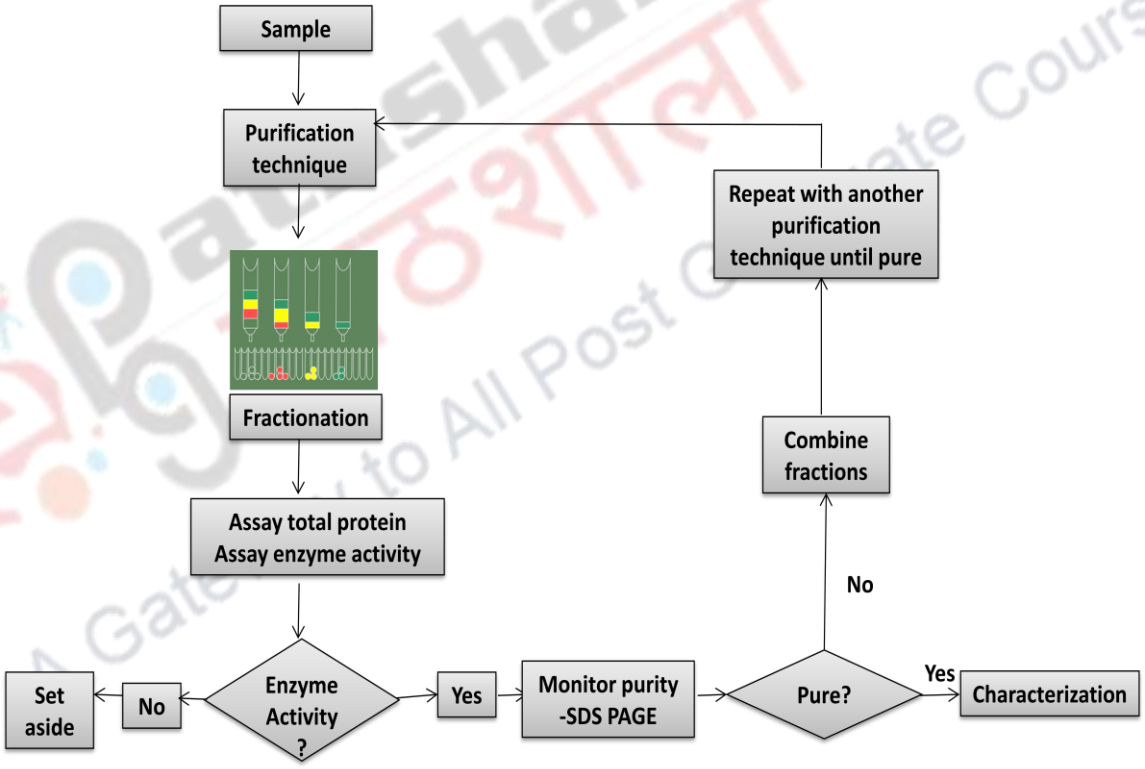


Figure 4. A multistep purification procedure

The soluble fraction containing target protein is concentrated by precipitation with salt, following which, it is subjected to fractionation by passing them through chromatographic columns for further purification. This

could include separation based on charge on protein surfac (Ion-exchange chromatography), separation based on binding to affinity columns (affinity chromatography), on size of protein molecule (gel filtration chromatography), separation based on hydrophobic interactions of protein with the column matrix (hydrophobic interaction chromatography), fractionation by isoelectric focusing or any other method available. The fractionation serves two purposes.

- Removal of added contaminating material and,
- Enrichment of the protein fraction containing the activity of interest.

Once such a fractionation is accomplished, the fractions are assayed for activity to detect the activity of interest. This activity will only be present in some fractions only, which may contain the target proteins. These fractions are then qualititatively analyzed for purity using methods like SDS Page. If not completely pure, this enriched fraction (containing protein of interest but not completely pure) is further put through another round of purification and the process can be repeated till a pure protein is achieved.

3.5 Concentration of proteins prior to purification

Concentration of the soluble fraction containing desired protein to be purified can be achieved through:

- Precipitation
- Ultrafiltration

3.5.1 Precipitation

Precipitation is a commonly applied technique for concentration of proteins present in an extract. The following methods of purification are usually employed:

i. Salting out with ammonium sulphate

ii. Selective precipitation with an organic solvent

- **Salting out with ammonium sulphate**

Salting out is an effective means for purification which explores the reduced solubility of proteins present in a solution of very high ionic strength causing certain proteins to precipitate. Figure 5 below shows salting in and salting out processes

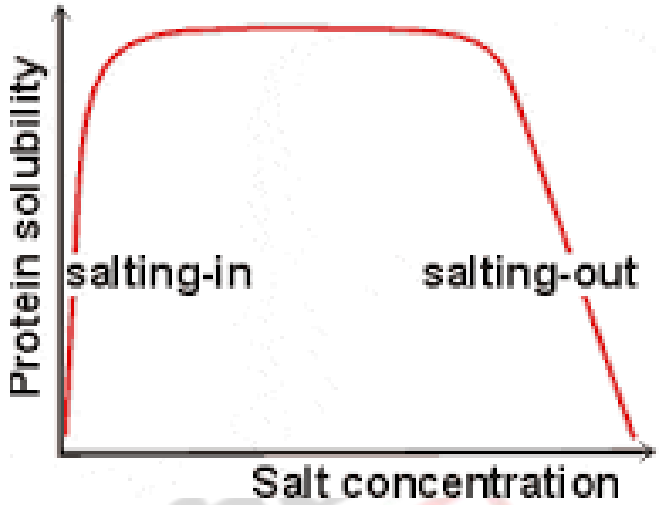


Figure 5 Salting in and salting out

Each protein molecule in solution is uniformly layered by an essential "layer of hydration" by water molecules which enable the molecule to repel each other and stay in solution. As more and more salt is added to the protein, the solubility of the salt added gradually tends to become higher than protein. Owing to the increased affinity of salt molecules for water over protein molecules, the hydration shell around the protein molecule is thus gradually displaced by the increasing ionic concentration in the solvent. In other words, the protein molecules are thus "stripped off" their hydration layer, allowing hydrophobic interaction between proteins (interactions between hydrophobic patches on protein surface) to predominate which leads to aggregation of the protein molecules and precipitation. It is important to note that salting out occurs at high salt concentration. Use of salt at very high concentrations also cause a further increase in surface tension, inducing the protein to aggregate, resulting in salt-precipitation.

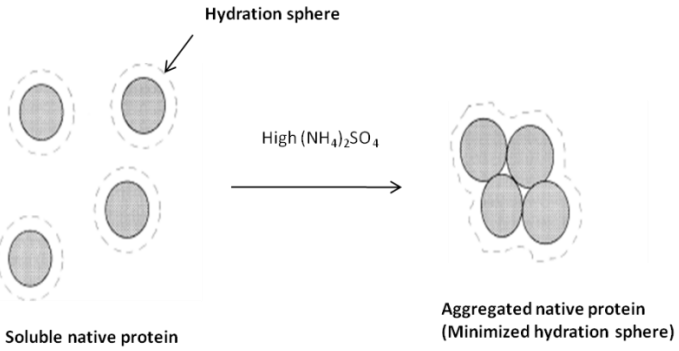


Figure 6. Effects of salt on protein precipitation

Precipitation of proteins by salt does not usually lead to a highly purified protein, but it can assist in removal of some unwanted bulk proteins in a mixture and also in concentrating the sample.
Ammonium sulphate is convenient and effective because it is highly soluble, cheap, less toxics and stabilizes most proteins/enzymes. Fractionation of protein mixtures by the stepwise increase in the ionic strength of the salt being used for protein precipitation can prove to be an effective strategy of obtaining partially purified enzymes.

Hofmeister Series

The effectiveness of the different ions towards protein precipitation was established by Franz Hofmeister in 1888 and the ordering of cations and anions arranged in order of their effectiveness is called Hofmeister series.

Cations: $N(CH_3)_3^+ > NH_4^+ > K^+ > Li^+ > Mg^{2+} > Ca^{2+} > Al^{3+}$ > guanidinium

Anions: $SO_4^{2-} > HPO_4^{2-} > CH_3COO^- >$ citrate > tartrate > $F^- > Cl^- > Br^- > I^- > NO_3^- > ClO_4^- > SCN^-$

Between cations and anions, the anions have the greatest effect on protein precipitation.

The starting molecules decreasing solubility of the non-polar molecules and strengthen hydrophobic interactions, thus salting out the system. Contrarily, the latter molecules tend to form strong ionic interactions with the protein that disrupt hydrogen bonding, thus contributing to the denaturation of the protein.

- **Precipitation by organic solvents**

Sometimes polar organic solvents such as methanol, ethanol, propan-2-ol and acetone are used for precipitating some enzymes/proteins.

The mechanism of precipitation is almost similar to that of salting out, as explained earlier. Use of solvents such as ethanol or methanol (which are miscible in nature) to a solution may cause proteins to precipitate. This is because the organic solvent molecules gradually displaces water from the protein surface thus reducing the solvation layer around the protein and binding the water molecules, so displaced, as hydration layers around the organic solvent molecules. The protein molecules, with reduced/no hydration layers thus tend to aggregate due to predominating protein-protein hydrophobic interactions as well as electrostatic forces. Miscible organic solvents also act by reducing the dielectric of the medium and consequently reducing the solubility of proteins, leading them to precipitate out of the solution.

Dialysis

After precipitating the protein fraction (using above approaches) and re-dissolving it in buffer, it is essential to remove the ammonium sulphate from the protein sample before subjecting it to subsequent steps during purification. One of the most widely used methods to achieve this is to dialyse the solution.

Dialysis leads to the separation of protein molecules from other small molecules, such as salt, by using a semi-permeable membrane (for e.g., cellulose membrane) with pores. The principle behing dialysis is usually diffusion, which is explained further. This membrane contains micro pores through which smaller molecules and ions (present along with the protein sample) move out (from region of higher concentration) and emerge in the dialysate outside the bag (to their region of lower concentration), across a concentration gradient, till equilibrium is achieved. To balance this, water/buffer molecules traverse into the dialysis bag, across a concentration gradient. However, the protein molecules which have sizes that are significantly greater than the membrane pore diameter, therefore cannot traverse out of the dialysis tubing and remain retained inside the dialysis bag. Figure 7 shows a typical set up for protein dialysis.

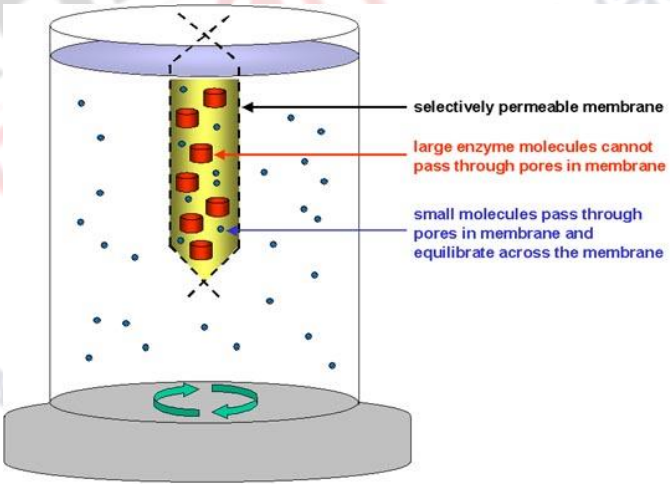


Figure7. Dialysis of proteins

As shown in Figure 7, the protein/enzyme solution is placed in a dialysis bag and immersed in a large volume of buffer that is stirred and maintained at about 4°C. During dialysis, the salt molecules will tend to diffuse out of the dialysis bag. If the buffer is changed several times, more or less all the salt will be removed from the protein solution. It is important to note that dialysis will result in an increase the volume of the enzyme solution, because water molecules from the buffer enter into the bag. It is therefore

necessary to leave some space/gap at the top of the membrane tube as shown in Figure 7, to prevent it from bursting.

3.5.2 Ultrafiltration

Ultrafiltration is another method for protein concentration in which water and low molecular weight materials are removed from the protein by passing it through a membrane in an inert atmosphere and under pressure. The protein is retained and does not pass through the membrane, leading to its concentration with little loss of enzyme activity. Membranes with molecular weight cut-offs from 1000 to 100,000 kDa are available which can be used under pressure up to 2 MPa.

Figure 8 represents the simplest ultrafiltration cell. The protein solution is contained in a cylindrical stirred cell where the membrane is placed at the base of a cell and the cell is equipped with a magnetic stirrer. An inert gas, for example, nitrogen is passed continuously through the stirrer which can force the water and low molecular weight materials through the membrane. Ultrafiltraion procedures for concentration of laboratory column eluates are commonly used, however their large scale use is not much explored.

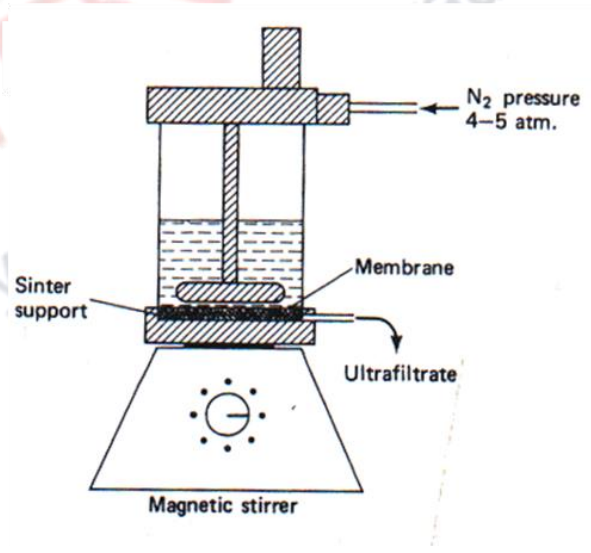


Figure 8. A stirred ultrafiltration cell

3.6 Nucleic acid removal

The intracellular enzymes obtained after cell lysis often contain nucleic acids which give rise to increased viscosity and interfere with enzyme purification procedures. Thus removal of such nucleic acids before proceeding to next purification steps becomes pertinent.

Nucleic acids can be removed by precipitation or degraded by externally added nucleases. In some cases, sufficient nuclease activity is priorly present in the lysed cell extract which can naturally degrade the nucleic acids present.

Positively-charged materials like polyethyleneimine, cetyltrimethyl ammonium bromide (a cationic detergent), streptomycin sulphate and protamine sulphate, which form complexes with the negatively-charged phosphate residues of the nucleic acids are used as precipitating agents for removal of nucleic acids (Figure 9). The nucleic acids complex with these compounds and the precipitates can be removed via centrifugation.

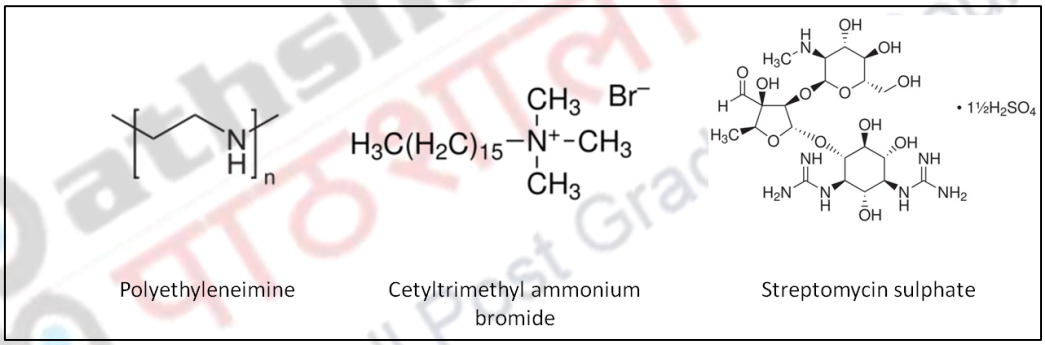


Figure 9. Nucleic acid precipitants

However, all of the above precipitants are expensive and sometimes toxic. Treatment with bovine pancreatic nucleases is another cost-effective and safer alternative for nucleic acid removal.

4. Summary

1. Protein purification is a multistep procedure comprising of several steps starting from the crude extract to the purified protein;
2. The CIPP strategy for purification enables simple planning and execution of purification protocols and cost effective production;
3. Crude proteins can be concentrated using salting out, solvent precipitation or ultrafiltration, before proceeding to chromatographic steps.
4. Nucleic acid removal is an additional crucial step for intracellular enzyme extracts before purification.