PROTEOMICS

GETTING STARTED WITH PROTEIN PURIFICATION

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Getting Started with Protein Purification

Protein purification is a multistep process which exploits biochemical and biophysical characteristics of the target protein such as its source, relative concentration, solubility, charge and hydrophobiciy.
Ideal Purification involves maximum recovery of the desired protein, with minimal loss of activity combined with maximum removal of other contaminating proteins.

While designing a purification protocol one should aim for 1) High recovery 2) Highly purified end product 3) reproducibility within the lab and or other labs and also when scaled up or down 4) economical use of reagents 5) convenience with regard to time.

Protein Purification is routinely assessed by summarizing the results of each purification step as Specific activities, Total units, Total Protein, and yields. Activity units are calculated based on the assay system that is used to track the target protein. This is known as the Purification Table and gives a good track record of the protocol.

Steps Involved in Purification

1. **Making Cell free extract - Starting material may come from bacterial, yeast or cells in culture, animal tissue like liver or skeletal muscle or plants tissues.**
2. **Cellular Disruption - Need to have a method by which you can disrupt the cells to efficiently release the target protein into the aqueous supernatant so as to enable further steps. Extraction should minimize degradation of the target by enhancing its stability. Homogenization of cultured cells is performed in a hypotonic soln. Temperature of this whole process has to be maintained at low temp 0-4 C and sample on ice always.**
3. **Extraction Buffer Composition : 20-50mM Phosphate Buffer pH 7-7.5 or 100mM Tris-HCl pH 7.5, 100 mM NaCl, EDTA 1-5mM pH 8, B-Mercaptoethanol (2-ME) (5-20mM), Sucrose and protease inhibitors.**
4. **Protease Inhibitors – Intact cells contain elements for their own destruction which are compartmentalized and released in response to specific physiological signals which usually happens when extract is being prepared. On destruction of this delicate balance proteolytic enzymes mix with cellular contents leading to degrading of the protein by proteases or enzymes that cleave peptide bonds and need to be inactivated and removed. Eg. Leupeptin, EDTA-Na2, Pepstatin A, Aprotinin, PMSF (Phenyl methylsulfonyl Fluoride), Benzamidine HCl, Soybean.**

Methods of Cell Disruption

1. **Bead Mill Homogenizer - Form of grinding cells works well with yeast, spores, bacteria and plant tissue. Large number of glass beads are vigorously agitated with the starting material at 3000-6000 oscillations per min. Disruption is by shearing and crushing action as beads colide with cells. Beads settle down on centrifugation and can be reused after washing and autoclaved too. Sample size is restricted to 3 ml or less. Eg. Mini Bead Beater, BioSpec Products, Bartlesville, OK. B.Braun Biotech also provides with beads and an instrument.**
2. **High Pressure Homogenizers – Induces cell lysis by forcing cell suspensions through a narrow orifice (hole) under high pressure. Cells are sheared as they pass through. Efficiency is directly proportional to pressure applied. Eg. French press.**
3. **Nitrogen Decompression – Suited for mammalian cells and some plant cells. Animal tissue requires some pretreatment to form a homogenous suspension capable of passing through a discharge valve so it is placed in a stainless steel vessel sealed and pressurized to 2000 psi using compressed nitrogen gas placed in a ice bath. Gas is allowed to dissolve into the aqueous media and the intracellular volume of the cells and then allowed to flow out of the pressure vessel. When this pressure goes from high to atmospheric pressure, the dissolved gas in the cells comes out of solution and the cells explosively decompress and disruption occurs by shearing as the sample solution comes out of the outlet valve. Eg. Parr Instruments Moline, IL ; Konetes, Vineland , NJ.**
4. **Ultrasonic Disintegrators – Commonly known as sonicators breaks apart cells by generating intense sonic pressure waves in a liquid suspension. Branson sonic power Danbury, CT.**
5. **Pestle and Tube Homogenizers**
6. **Freezing and Thawing**
7. **Dehydration – With acetone or ethanol powdered tissues is mixed in a blender and filtered.**

Clarification of the Extract

- Particulate contaminants removed by centrifugation or filtration.
- To realize an 80% recovery of the clear supernatant always use 2 volumes of extraction buffer to keep the protein in correct concentration.
- Protein of interest can also be enriched by **differential centrifugation** as in for example using gradients to purify specific fractions such as nuclear extracts of lymphocyte extracts and even sub cellular fractionation such as lysosomes or mitochondria or microsomes and ribosomal pellets.
- Subcellular Markers – Can be used as an aid to check during assay of specific marker proteins in the subcellular fractions. Lactate Dehydrogenase, GST, Carbonic anhydrase (Cytosol), 5' Nucleotidase (Plasma Membrane), b-Galactosidase, Acid Phosphatase (Lysosome, Golgi) Galactosyltransferase (Golgi) etc.
- Protein Quantitation – Bradford Method and BCA (Bicinchoninic acid)

Protein Quantitation

- Bradford (1976) developed a method replacing the Lowry method (1951) involves the addition of an acidic dye Coomassie Brilliant Blue G-250 to the protein solution which binds to basic and aromatic amino acids resulting in a shift of the absorbance from 465 (brown) to 595 (bluish). Method is based on a production of standard graph using a standard protein such as BSA.
- BCA (Bicinchoninic acid) – Smith (1985) found that when protein is placed in an alkaline system containing Cu+2 a colored complex forms between the peptides bonds of the protein and the copper atoms. Bicinchoninic acid (BCA) is a compound that forms a complex with cuprous ions in an alkaline environment resulting in a stable highly colored chromophore with an absorbance at 562 nm. BCA assay is better than Bradford and Lowry as it shows greater tolerance towards commonly interfering compounds such as detergents in buffers containing proteins.
- NanoOrange Protein Quantitation – Fluorescence based assay – these type are more sensitive with lower background and wider dynamic range than absorbance based methods.

NanoOrange

- **One method involves non-fluorescent reactive dyes that couple with protein amines to form fluorescent adducts, such as fluorescamine. (Bohlen et al 73).**
- **Second method uses dyes that exhibit fluorescence enhancement upon non-covalent interaction with hydrophobic regions of proteins or detergent coated proteins (Haugland, 1996).**
- **NanoOrange is essentially nonfluorescent in aqueous soln but becomes intensely fluorescent upon binding to detergent-coated proteins or hydrophobic regions of proteins. (Jones et al., 2003).**
- **As little as 100 ng / ml could be detected in 200 ul vol in a fluorescence microplate reader or a fluorometer.**
- **Fluorescence intensities at 485 nm excitation and 590 nm emission settings can be used for reading the plate.**
- **NanoOrange can be bought from Molecular Probes Eugene, OR.**

Manipulating Proteins in Solution

- **Stabilization and Storage of Proteins – Extract containing protein of interest should be treated with care to ensure stability and maximum retention of activity both during purification protocol or for long term storage. Widely used method is inclusion of Glycerol in buffer solutions from 5- 50% have been used with higher conc. Mainly for storage purpose. Higher % glycerol during purification steps as a stabilizer is more time consuming due to high viscosity of glycerol.**
- **High Salt conc also stabilize enzymes and generally inhibit proteases. Most enzymes are thus supplied in 50% ammonium sulfate and similarly "Wet Pellet" can be stored.**
- **Dilute enzymes lose activity quickly hence store at least at 1 mg / ml conc. Sometimes BSA can be added to dilute solutions upto 1 mg/ml to ensure stability and also it will not interfere with the activity of the protein.**
- **Frozen Storage – During freezing many events occur – first free water freezes and ice crystals grow which can be destructive to membranes and organelles. Least soluble solute will then precipitate and if the solute is one component of the buffer the pH will markedly change before complete solidification takes place. Thus keeping protein conc high is essential to prevent any pH change.**

- **If temperature below -50 C is reached as quickly as possible known as "SNAP FREEZING" then degradative processes are kept at a minimum. -10 to -15C is no good and when thawing the rule is faster the better. Repeated cycles of freezing and thawing is also to be avoided. Store purified proteins in small aliquots and preferably in high concentrations of glycerol such as 50% and in this condition the sample does not freeze. Enzymes are usually supplied thus.**
- **Protecting Cysteine Residues – Cysteine residues are susceptible to modification especially oxidation during purification or storage. Proteins in living cells are in a reduced atmosphere and protected by sulfhydryl-containing groups like glutathione. During high oxygen tensions disulfide bonds are formed, partial oxidation to sulfinic acid irreversible oxidation to a sulfonic acid. Metal ions are are removed from solution with inclusion of a chelating agent EDTA. Sulhydryl containing reagent such B-Mercaptoethanol or dithiothreitol (DTT) can be added 1-5mM conc.**

Concentrating Proteins from Dilute Solutions

- **Proteins in solution may not be always in a perfectly optimal state for purification step – Wrong buffer, may have too much salt or too dilute.**
- **If solution is too concentrated then dilution will not be a problem. However, if it is present in large volume it must be concentrated. Meaning removal of water and there are several methods such as Precipitation methods (> 1mg /ml conc), Ultrafiltration, Dialysis, Evaporation, Adsorption and Lyophilization.**
- **Method chosen should be based on speed, protein stability, familiarity and convenience. Once the volume is reduced to a reasonable size then next step can be proceeded with.**
- **Adsorption – Concentration of dilute protein can be achieved by using an ion exchanger. The protein of interest has to be totally adsorbed and does not matter if others are too. One step purification can be achieved in a small volume.**
- **Precipitation – Solubilities of proteins are decreased by high conc of neutral salts (Salting out) and the protein of interest can be salted out of crude extract with enrichments of 2-10 fold and it can be in a reduced volume. Ammonium sulfate is an excellent precipitating agent and it is non denaturing method to conventrate a protein.**

- **Ultrafiltration – Reverse osmosis is a process by which solvents and solutes up to a certain critical size are forced through a barrier membrane by a higher pressure on one side of the membrane than the other. Both the solvent and solutes move through in the same direction. It can used to concentrate dilute protein solutions.**
- **Thus you would find two fractions one the bigger fractions found in the ‘ RETENATE’ and smaller molecules passing through the membrane and found in the ‘ULTRAFILTRATE’.**
- **Ultrafiltration membranes come in a variety of pore sizes usually referred to as the Nominal Molecular Weight Cutoff (NMWC) for the membrane. Defined as the minimum mol wt globular protein which will not pass through the membrane. Devices are available from 3-100kDa and are suited for small vol upto 5 ml.**

Lyophilization

- **Also known as Freeze Drying is a sublimation process where water and other frozen solvents are removed under vacuum in a progressive and careful dehydration and results in the drying of the solute components of the solution, proteins and any buffer components that were not previously removed by dialysis. Routinely the protein solution is dialyzed first to remove salts or some buffer components.**
- **Using a bath consisting of dry-ice and acetone the protein solution is placed in a special flask and shell frozen by slowly rotating the flask in the ice bath to maximise the surface area. Once frozen the flask is mounted on the lyophilizer and the valve is opened putting the contents of the flask under vacuum and left there for 2 hr to overnight depending upon volume. Make sure the contents are not thawed. If there is any liquid in the flask refreeze the contents and fix it back. Once the outside of the flask reaches room temp the process of freeze drying is complete. Phosphate buffers are not ideally suited while ammonium bicarbonate are preferable.**

DIALYSIS

- **Dialysis is a separation process that takes advantage of osmotic forces between two liquids or a liquid into a solid. Used for removing excess low molecular weight solutes and simultaneously equilibrating the sample in a new buffer and as means of concentrating a dilute solution.**
- **Dialysis tubing is a semi-permeable membrane usually made from cellulose acetate available in a wide range of membrane dimensions and NMWC allowing molecules below a certain mol wt to freely equilibrate on both sides of the membrane.**
- **Soln can be concentrated by dialysis against a high mol wt hygroscopic substance. Soln is placed in a tubing and coated with an inert high mol wt hygroscopic substance such as Polyethylene Glycol (PEG mol wt 20,000) which pulls water out of the bag. Dry Sephadex can also be used. A 10 ml soln can be brought down to 1 ml in less than an hour by coating the bag with Aquacide.**

Precipitation Techniques

- **1. Salting out with Amm. Sulfate – Reference table available to decide what amount should be added with regard to % saturation required and percentage of proteins to be precipitated. As said earlier it helps in reducing the volume.**
- **2. Precipitation with Acetone - Water miscible organic solvents such as ethanol and acetone can be used to precipitate proteins and occurs when the pH of the solution is close to the pI of target protein. Larger proteins will precipitate at lower conc of organic solvents than a smaller protein. Most proteins will precipitate at 20-50% v/v acetone and should be carried out at 0 C.**
- **3. Precipitation with PEG – Commonly available PEG 2000, PEG 4000, PEG 6000 are used. But difficult to remove from protein fraction. Low amounts are compatible for removal and use**
- **4. Selective Denaturants – Principle is to create conditions in which the protein of interest remains stable and soluble while the other components of the extract are denatured and precipitate. Behaviour can be tracked by heat inactivating in increments of 5C between 35-65C for a specific time period and checking activity. Rapidly cooled and centrifuged to remove precipitated material. pH of the solution must be monitored to ensure reproducibility.**
- **5. Methanol-Chloroform Precipitation**
- **6. TCA – Trichloroacetic acid Precipitation.**

- References :

1. Protein Analyses and Purification – Ian M. Rosenberg