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Protein Purification

Summary

Protein expression is tightly regulated for normal functioning of a cell or organism. To understand protein structure and function in detail, they often need to be separated from other cellular components (lipids, nucleic acids, sugars, etc.) and isolated to homogeneity. After recovering a protein to near homogeneity, it should retain all its native biological characteristics of structure and activity. To achieve this objective, one needs to take into account the physical and chemical property of proteins (size, charge, solubility, hydrophobicity, precipitation, etc.). These common characteristics of the protein can be exploited to separate it from other components of the cell. With the introduction of recombinant DNA technology, protein purification technique has been enhanced and also simplified. Purification protocols vary, depending on the precise nature of the protein. General steps include (i) chromatography, (ii) precipitation and/or (iii) extraction.

A.1 Protein Precipitation

Many cytosolic proteins are water soluble and their solubility is a function of the ionic strength and pH of the solution. The commonly used salt for this purpose is Ammonium Sulphate, due to its high solubility even at lower temperatures. Proteins in aqueous solutions are heavily hydrated, and with the addition of salt, the water molecules become more attracted to the salt than to the protein due to the higher charge. This competition for hydration is usually more favorable towards the salt, which leads to interaction between the proteins, resulting in aggregation and finally precipitation. The precipitate can then be collected by centrifugation and the protein pellet is re-dissolved in a low salt buffer. Since different proteins have distinct characteristics, it is often the case that they precipitate (or 'salt out') at a particular concentration of salt.

Requirements:

- (1) Ammonium sulphate
- (2) Ice tray
- (3) Magnetic bead and stirrer
- (4) Swing-out rotor centrifuge

Protocol 1:

- (1) Clarify the protein solution (in most cases the lysates) by centrifugation.
- (2) Transfer the supernatant into an ice cold beaker with a magnetic bead.
- (3) Note the exact amount of the supernatant (From Table A.1).
- (4) Keep the beaker chilled by placing it in an ice tray.
- (5) Transfer the beaker with the ice tray onto a magnetic stirrer (Fig. A.1).
- (6) Weigh the amount of ammonium sulfate to be added. The amount depends on the volume of the solution and the percentage saturation of the salt needed. Refer to the precipitation chart. In case of protein purification, a step precipitation is carried out.
- (7) Slowly add the ammonium sulphate with stirring. One needs to be careful as the addition of the salt should be very slow. Add a small amount at a time and then allow it to dissolve before further addition.
- (8) Keep it on the stirrer for 1hr precipitation to occur in ice.
- (9) Centrifuge at 10,000g for 15 min at 4°C.
- (10) The pellet contains the precipitated protein which could be dissolved in a suitable buffer for further analysis and purification.
- (11) For a second round of precipitation of a different protein, the supernatant is again used and the above same steps are followed.

A.2 Column Chromatography

This method involves passing the protein through a column filled with resins of unique characteristics. Depending on the type of the resin or beads, purification can be achieved through (i) Ion Exchange, (ii) Size Exclusion or (iii) Affinity Chromatography.

A.2.1 Ionic Exchange Chromatography

This is one of the most useful methods of protein purification. Depending on the surface residues on the protein and the buffer conditions, the protein will have net a positive or negative charge

Table A.1 Amount of Ammonium sulfate required for protein precipitation.

Initial concentration of ammonium sulfate	Percentage saturation at 0°																	
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100	
	Solid ammonium sulfate (grams) to be added to 1 liter of solution																	
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697	
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662	
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627	
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592	
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557	
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522	
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488	
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453	
40					0	29	58	89	120	153	187	222	258	296	335	376	418	
45						0	29	59	90	123	156	190	226	263	302	342	383	
50							0	30	60	92	125	159	194	230	268	308	348	
55								0	30	61	93	127	161	197	235	273	313	
60									0	31	62	95	129	164	201	239	279	
65										0	31	63	97	132	168	205	244	
70											0	32	65	99	134	171	209	
75												0	32	66	101	137	174	
80													0	33	67	103	139	
85														0	34	68	105	
90															0	34	70	
95																0	35	
100																	0	

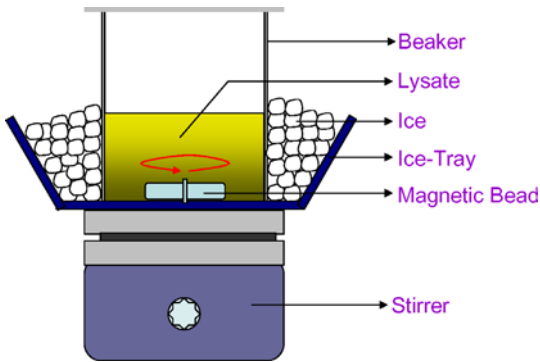


Fig. A.1 Protein Precipitation using ammonium sulfate.

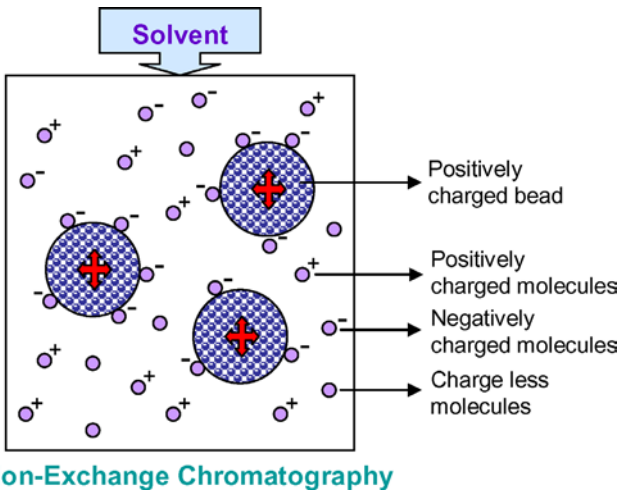


Fig. A.2 Ion Exchange Chromatography. The resins are charged and the protein molecules that bind are of opposite charge.

(Fig. A.2). An ideal buffer should be in the physiological pH range of 6 to 8. At this pH range, most of the proteins have been observed to be negatively charged. Hence, proteins would bind to positively charged molecules of the resin. Change in the buffer pH condition could make the protein relatively positive, thereby allowing it to bind to a negatively charged resin material. Among the most commonly used charged molecules are DEAE and CM. These charged molecules are coupled to an inactive material, often nanoparticle beads, loaded into a column. The protein is

loaded onto this packed column and is allowed to bind. The column is washed and the bound proteins are eluted depending on their tightness of binding, by subjecting them to either increasing concentrations of salt or changes in pH. Proteins with low charge will elute first.

A.2.2 Size-Exclusion Chromatography

In this approach, the size of the protein is taken into consideration. The size of the protein depends on the number of amino acids it contains. This property can be used in protein purification. The column material consists of a porous matrix for proteins to diffuse into (Fig. A.3). The smaller proteins get entangled inside the porous material and hence their mobility is restricted. In contrast, the larger proteins do not get entangled and could just pass through. Hence, in the elution profile, the larger molecules would be the first ones to elute, while the smallest ones will be last to elute.

A.2.3 Affinity Chromatography

As the name suggests, the principle is the use of a moiety or molecule which has high affinity for the protein of interest.

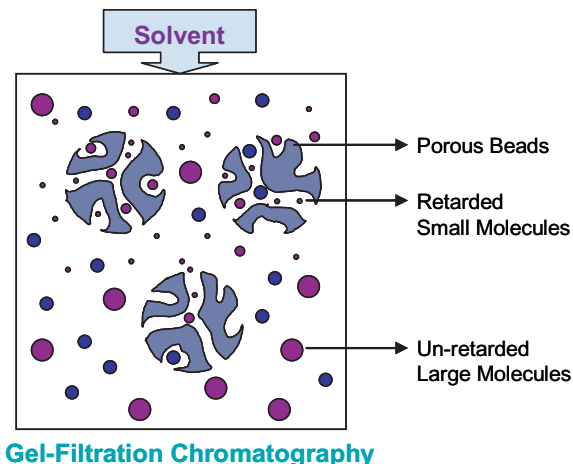


Fig. A.3 Gel filtration Chromatography. The resins are porous and the small molecules get trapped inside the pores whereas the bigger protein molecules exclude out.

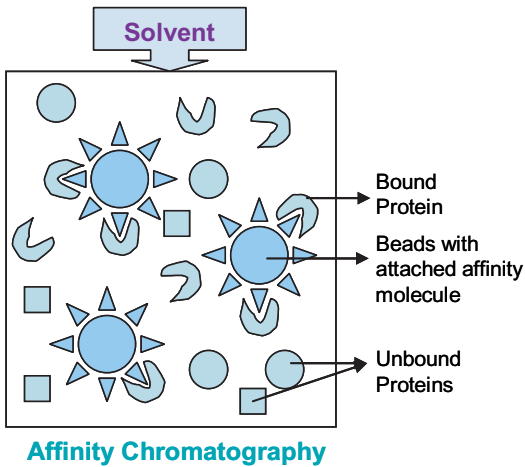


Fig. A.4 Affinity Chromatography. The resins have a head group which has a high binding affinity towards the protein of interest.

These molecules could either be co-factors, modified substrates, inhibitors or carbohydrates. This strategy of purification is used mostly in the later stages where the protein is relatively pure, and more specific approaches are required for additional purification. The affinity moiety or molecule is coupled to the matrix and used as a bait to fish the protein of interest (Fig. A.4). The protein could either be eluted with high salt in some cases or with increased amount of the affinity molecule itself.

A.2.4 Purification of Recombinant Proteins

This is the easiest method available for the purification of a protein, albeit it is a recombinantly expressed protein rather than an endogenous protein. The gene encoding a protein of interest is cloned into an expression vector (often with a tag such as GST or His) which is then introduced into the producer cell in order to express the protein as a fusion protein. The protein is then 'over-expressed' in higher than usual levels in a bacterial (e.g. BL21), yeast (e.g. *S. cerevisiae*), insect (e.g. sf9) or mammalian (e.g. CHO) cell system. The tag on the protein serves as a pull down, and thus separate and purify the protein from the cell lysate. The tag is usually a 6X His or Glutathione Transferase (GST). Thus, the column material is either Ni-NTA (Nitrilotriacetic acid) which binds tightly to 6His, or Glutathione

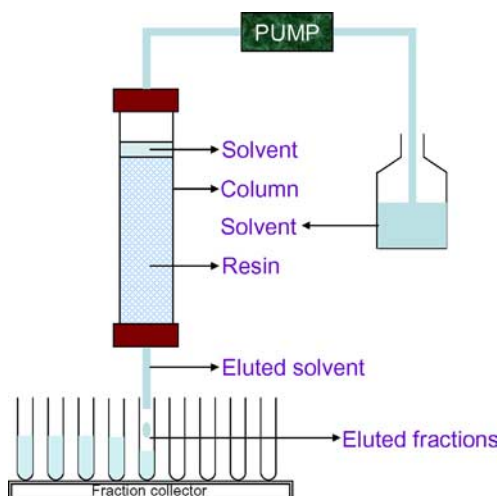


Fig. A.5 Flow Chart for column Chromatography. The Central part is the column from the sample and/or solvent is loaded at a controlled flow rate with a pump. The eluates from the column are collected in tubes of a fraction collector.

sepharose which binds to GST. Since these columns are very specific, the fusion protein is purified to near homogeneity. In order to attain complete purity, the protein then could be purified by other conventional chromatographic methods.

Protocol 2: (i) Column Preparation

- (1) Make a slurry of the respective resin or beads in the equilibration buffer.
- (2) Fill the glass column with the equilibration buffer with the nozzle of the column closed.
- (3) Open the nozzle with a slow flow rate.
- (4) Using a pipette, load the resin suspension onto the column.
- (5) Allow the material to settle till the required level.
- (6) Wash the column thoroughly with 2 to 3 column volumes of equilibration buffer before loading the sample onto the column.

(ii) Column Run: (Fig. A.5)

- (1) The sample is loaded at a slow rate onto the column from the top. The eluate from the column is collected as a flow through. In the case of size exclusion, the concentrated sample is layered on the top of the column bed.
- (2) The equilibration buffer or wash buffer is applied on the column at a monitored flow rate. The eluate is collected as the wash. For size exclusion, the eluates are collected in fractions.
- (3) The protein level can be monitored by scanning the eluates at O.D. 280 nm.
- (4) The bound proteins are eluted with increasing concentrations of salt or other elution buffers, depending on the column and enzyme. The elution can be carried out as step elution or gradient.
- (5) The eluates are collected as fractions.
- (6) The fractions can then be analysed for enzyme activity and run on SDS-PAGE for purity.

A.2.5 Commercially Pre-packed Column Kits

The columns are here designed specifically for a defined purpose. These columns are easier to use, faster and they require much less resources. Some of the columns include the NAP-25 or PD 10 desalting columns (from Amersham Biosciences), His Tag columns such as Ni-NTA spin column from Qiagen, His Bind from Novagen or His GraviTrap from GE Healthcare.

Desalting columns

The NAP-25 /PD-10 column contains Sephadex G-25 and is used for a rapid desalting or buffer exchange of nucleic acids, proteins and oligonucleotides.

Protocol 3:

- (1) Remove the top cap and pour off the excess liquid.
- (2) Cut the end of the column tip.

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- (3) Support the column over a suitable receptacle and equilibrate the gel with approximately 25 ml of the required buffer.
- (4) Allow the equilibration buffer to completely enter the gel bed.
- (5) Add the sample to the column in a maximum volume of 2.5 ml. If the sample volume is less than 2.5 ml, do not adjust it at this time. Allow the sample to enter the gel bed completely.
- (6) For sample volumes less than 2.5 ml, add equilibration buffer so that the combined volume of sample added in Step 5 and buffer added in Step 6 equals 2.5 ml. Allow the equilibration buffer to enter the gel bed completely.
- (7) Place a test tube for sample collection under the column.
- (8) Elute the purified sample with 3.5 ml buffer.

Purification of His-Tag Proteins

These columns are used for purification of recombinant fusion proteins tagged to 6XHis. The commercial columns contain the precharged Ni coupled to a tetradentate chelating absorbent such as the NTA (nitrilotriacetic acid), bound to a matrix which could be Sepharose or Cellulose.

Protocol 4:

- (1) Lyse the cells in the presence of protease inhibitors either by enzymatic lysis (0.2 mg/ml lysozyme, 20 µg/ml DNase, 1 mM mgCl₂) or by mechanical lysis (Sonication, homogenization, repeated freeze/thaw) in 20 mM sodium phosphate, 500 mM NaCl. Adjust the pH of the lysate to pH 7.4 using a dilute acid or base.
- (2) Centrifuge the lysate at 10000 rpm for 30 min at 4°C.
- (3) Collect the supernatant for purification step.
- (4) Cut off the bottom tip, remove the top cap, pour off excess liquid and place the column in the Workmate column stand.

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- (5) Equilibrate the column with 10 ml of 20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, pH 7.4
- (6) Load the sample.
- (7) Wash with 10 ml 20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, pH 7.4.
- (8) Apply 3 ml elution buffer (20 mM sodium phosphate, 500 mM NaCl, 100 mM imidazole, pH 7.4) and collect the eluate containing the purified protein.