Protein purification and analysis

Isolation of protein

- The first step in the isolation of a protein or other biological molecule is to get it out of the cell and into solution.
- Many cells require some sort of mechanical disruption to release their contents.
- Most of the procedures for lysing cells use some variation of crushing or grinding followed by filtration or centrifugation to remove large, insoluble particles.
- If the target protein is tightly associated with a lipid membrane, a detergent or organic solvent may be used to solubilize the lipids and recover the protein.

pH, Temperature, and Other Conditions Must Be Controlled to Keep Proteins Stable.

- Once a protein has been removed from its natural environment, it becomes exposed to many agents that can irreversibly damage it.
- These influences must be carefully controlled at all stages of a purification process.

Purification Is a Stepwise Process.

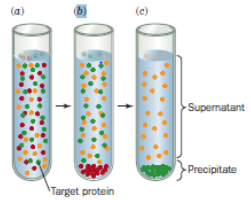
- Proteins are purified by **fractionation procedures.**
- In a series of independent steps, the various physicochemical properties of the protein of interest are used to separate it progressively from other substances.
- The idea is not necessarily to minimize the loss of the desired protein, but to *eliminate selectively the other components of the mixture so that only the required substance remains.*
- Protein purification is considered as much an art as a science, with many options available at each step. While a trial-and-error approach can work, knowing something about the target protein (or the proteins it is to be separated from) simplifies the selection of fractionation procedures.

Salting Out Separates Proteins by Their Solubility

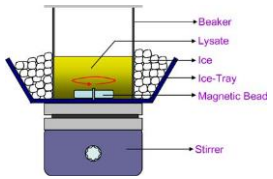
- Because a protein contains multiple charged groups, its solubility depends on the concentrations of dissolved salts, the polarity of the solvent, the pH, and the temperature. Some or all of these variables can be manipulated to selectively precipitate certain proteins while others remain soluble.
- The solubility of a protein at low ion concentrations increases as salt is added, a phenomenon called **salting in.** The additional ions shield the protein's multiple ionic charges, thereby weakening the attractive forces between individual protein molecules (such forces can lead to aggregation and precipitation).
- However, as more salt is added, particularly with sulfate salts, the solubility of the protein again decreases. This **salting out** effect is primarily a result of the competition between the added salt ions and the other dissolved solutes for molecules of solvent. At very high salt concentrations, so many of the added ions are solvated that there is significantly less bulk solvent available to dissolve other substances, including proteins.

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



Fractionation by salting out. (*a*) The salt of choice, usually ammonium sulfate, is added to a solution of macromolecules to a concentration just below the precipitation point of the protein of interest. (*b*) After centrifugation, the unwanted precipitated proteins (*red spheres*) are discarded and more salt is added to the supernatant to a concentration sufficient to salt out the desired protein (*green spheres*). (*c*) After a second centrifugation, the protein is recovered as a precipitate, and the supernatant is discarded.



Protein Precipitation using ammonium sulfate.

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- discarded.

Column Chromatography

- This method involves passing the protein through a column filled with resins of unique characteristics.

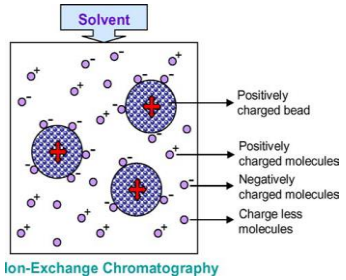
Depending on the type of the resin or beads, purification can be achieved through

(i) Ion Exchange, (ii) Size Exclusion or (iii) Affinity Chromatography

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



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

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.

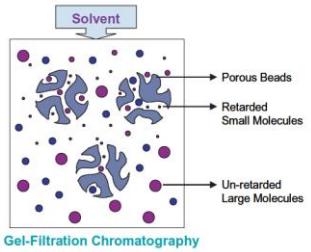
Hence, proteins would bind to positively charged molecules of the resin. Change in the buffer pH condition could make the protein relatively positive, thereby allowing it to bind to a negatively charged resin material.

Resins used

- 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.

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.



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

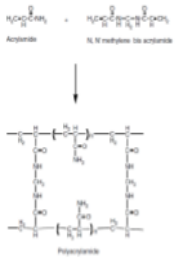
Affinity Chromatography

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

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. The protein could either be eluted with high salt in some cases or with increased amount of the affinity molecule itself.

Electrophoresis



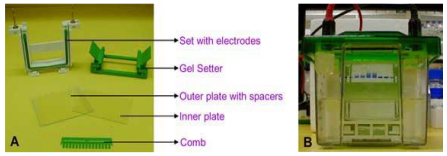
Proteins could be separated using electrophoresis.

Conventional electrophoresis uses a single electrical field to cause biomolecules to migrate through a matrix according to its mass-to-charge ratio

Most frequently used method is Poliacrylamide gel electrophoresis (PAGE).

How it works?

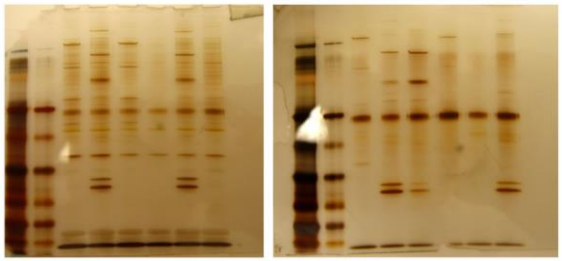
- Under denaturating condition sodium dodesyl sulfate cover proteins with a negative charge and there fore proteins move only according to their size.
- Two-dimensional gel electrophoresis (2-D electrophoresis) is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples.
- This technique separate proteins in two steps, according to two independent properties: the first-dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI); the second-dimension is SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weights (MW).
- In this way, complex mixtures consisted of thousands of different proteins can be resolved and the relative amount of each protein can be determined.



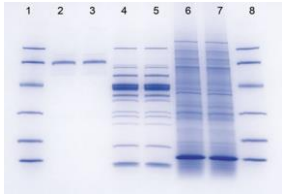
SDS-PAGE. (A) Different parts of the electrophoretic apparatus.
(B) Running the SDS-PAGE.

Gel Staining

- For visualisation of proteins after separation on gel, one could use different stains such as Coomassie blue stain or more sensitive silver staining.
- The Coomassie blue staining is relatively less sensitive than silver staining, but is highly convenient to use.
- **Coomassie Blue Staining**
- The dye Coomassie Brilliant Blue R250 nonspecifically binds to all the protein. The gel is soaked in the dye for it to seep in and bind to the proteins. The gel is then destained to remove the unbound dye. The dye binds to the protein and not the gel, and hence the protein bands can be visually seen.
- The binding of the dye to the protein is approximately in stoichiometry, so the relative amounts of protein can be determined by densitometry.
- For most SDS and native gels, separated proteins can be simultaneously fixed and stained in the same solution.



Silver staining



Coomassie blue staining