Protein Extraction and Purification Methods-4

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Reference: Protein Purification techniques Second edition
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Purification and concentration by precipItation

- Many of the early protein purification procedures used only precipItation methods as a means of separating one protein (or class of proteins) from another. For example, the core histones (H2a, H2b, H3, and H4) were purified by ethanol and/or acetone precipItation. Differences in solubility have also been used to purify albumins and globulins from serum (the globulins are precipItated by diluting serum with water, whilst the albumins remain soluble).

- The solubility of a protein molecule in an aqueous solvent is determined by the distribution of charged hydrophilic and hydrophobic groups on its surface.

- The charged groups on the surface will interact with ionic groups in the solution (Figure 10). Protein precipItates are formed by aggregation of the protein molecules, induced by changing pH or ionic strength, or by addition of organic miscible solvents or other inert solutes or polymers.

- Temperature will also affect the degree of aggregation achieved. PrecipItates can be recovered by filtration or centrifugation, washed, and redissolved in an appropriate buffer, if required. To remove final traces of the precipItating agent, which might interfere with subsequent purification steps, the redissolved precipItate should be dialysed, diafiltered, or desalted on a gel filtration column

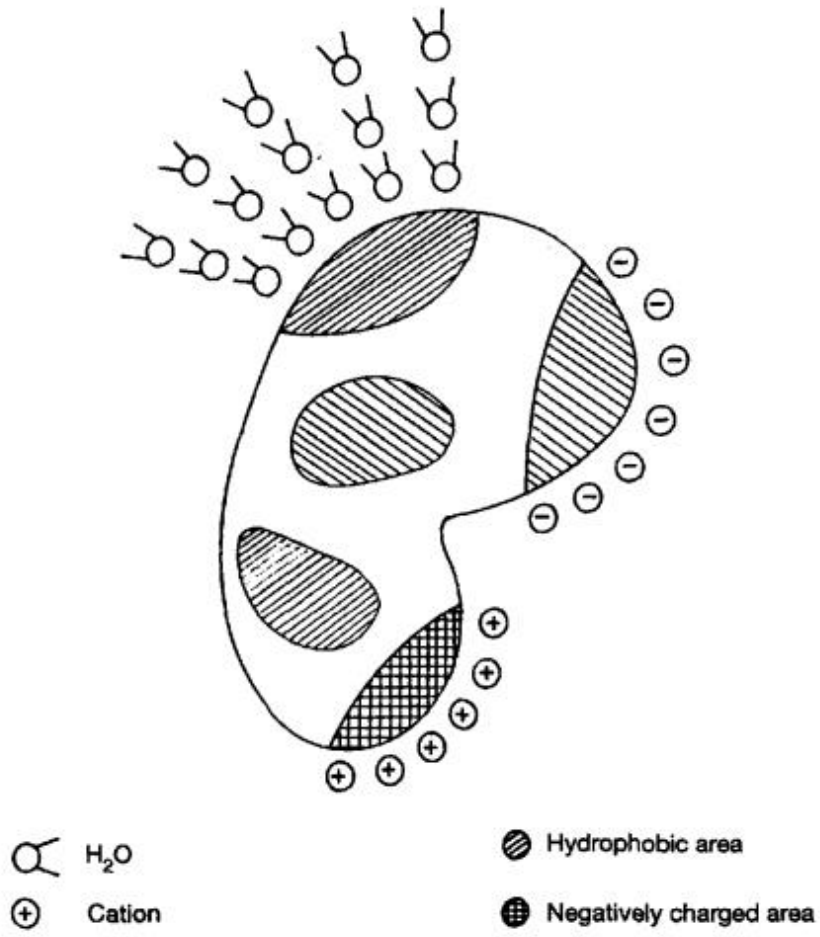


Figure 10 Schematic representation of a protein showing negatively and positively charged areas on the protein interacting with ions in the solution. The hydrophobic areas on the protein interact with water molecules causing an ordered matrix of water molecules to form over these areas.

1. PrecipItation by alteration of the pH

- One of the easiest methods of precipItating a protein and achieving a degree of purification is by adjusting the pH of the solution to close or equal to the pI of the protein (termed isoelectric precipItation).

- The surface of protein molecules is covered by both negatively and positively charged groups.

- Above the pI the surface is predominantly negatively charged, and therefore like-charged molecules will be repelled from one another; conversely below the pI the overall charge will be positive and again like-charged molecules will repel one another.

- However, at the pI of" the protein the negative and positive charges on the surface of a molecule cancel one another out, electrostatic repulsion between individual molecules no longer occurs, and electrostatic attraction between molecules may occur, resulting in formation of a precipitate.

2. Precipitation by decreasing the ionic strength (salting in)

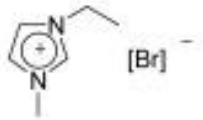
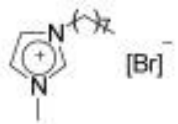
- Some proteins can be precipitated by lowering the ionic strength. This can rarely be achieved with crude extracts, since the ionic strength can only be lowered by addition of water, which will also lead to a decrease in the concentration, and hence an increased solubility (a notable exception is the serum globulins).

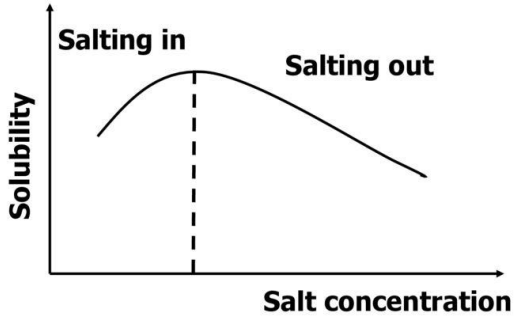
- However, this form of precipitation can often occur at later stages of a purification, for example, when removing salts by diafiltration, dialysis, or gel filtration. This may not always be a welcome occurrence, for example, when using gel filtration a precipitated protein will be trapped and may block the matrix.

- Precipitation at low ionic strength is more likely to occur at or close to the pI of the protein, since the causes of precipitation are similar and therefore additive.

3.Precipitation by increasing the ionic strength (salting-out)

- Precipitation by addition of neutral salts is probably the most commonly used method for fractionating proteins by precipitation. The precipitated protein is usually not denatured and activity is recovered upon redissolving the pellet.
- In addition these salts can stabilize proteins against denaturation, proteolysis, or bacterial contamination. Thus, a salting-out step is an ideal step at which to store an extract overnight, either before or after centrifugation.
- The cause of precipitation is different from that for isoelectric precipitation, and therefore the two are often used sequentially to obtain differential purification. Salting-out is dependent on the hydrophobic nature of the surface of the protein.
- Hydrophobic groups predominate in the interior of the protein, but some are located at the surface, often in patches. Water is forced into contact with these groups, and in so doing becomes ordered

Salting-out		Salting-in	
Inorganic	**Organic**	**Inorganic**	**Organic**
LiCl, NaCl, NaBr, $CaCl_2$, MgCl2, Na_2SO_4, and KCl	 [EMIM][Br], β-cyclodextrin	Urea, guanidium hydrochloride, tetrabutyl ammonium bromide and $LiClO_4$	 [OMIM][Br]



- When salts are added to the system, water solvates the salt ions and as the salt concentration increases water is removed from around the protein, eventually exposing the hydrophobic patches.
- Hydrophobic patches on one protein molecule can interact with those on another, resulting in aggregation. Thus, proteins with larger or more hydrophobic patches will aggregate and precipitate before those with smaller or fewer patches, resulting in fractionation.
- The aggregates formed are a mixture of several proteins, and like isoelectric precipitation the nature of the extract will affect the concentration of salt required to precipitate the protein of interest.
- In contrast to isoelectric precipitation, increasing the temperature increases the amount of precipitation; however, salting-out is usually performed at 4°C to decrease the risk of inactivation

- The effectiveness of the salt is mainly determined by the nature of the anion, multi-charged anions being the most effective; the order of effectiveness is phosphate > sulfate > acetate > chloride > (and follows the Hofmeister series).

- Although phosphate is more effective than sulfate, in practice phosphate consists of mainly HPO_4^{2-} and $H_2PO_4^{"}$ ions at neutral pH, rather than the more effective $P0_4^{3-}$.

- Monovalent cations are most effective, with $NH_4^+ > K^+ > Na^+$. The salt must be relatively cheap with few impurities present.

- The solubility is also an important consideration, since concentrations of several molar are required; thus, many potassium salts are not suitable. Because of the risk of possible denaturation, or changes in solubility, there should be little increase in heat caused by the salt dissolving.

In practice ammonium sulfate is the most commonly used salt (other salts which have been used in particular applications are ammonium acetate, sodium sulfate, and sodium citrate). Ammonium sulfate is cheap, and sufficiently soluble; a saturated ammonium sulfate solution in pure water is approximately 4 M. The density of a saturated solution is 1.235 g/ml, compared to 1.29 g/ml for a protein aggregate in this solution. In practice the density of a 75-100% saturated solution may be higher than 1.235 g/ml, due to the presence of other salts and compounds in the extract, therefore making recovery of a protein aggregate by centrifugation difficult.

- Rather than quote the pore size of the ultrafiltration membranes it is more common to quote a nominal molecular weight cut-off (NMWC) for the membrane. The NMWC is defined as the minimum molecular weight globular molecule which will not pass through the membrane.
- Concentration by ultrafiltration offers several advantages over alternative methods.
- Ammonium sulfate will slightly acidify the extract, therefore a buffer of about 50 mM should be used to maintain a pH between 6.0-7.5. If a higher pH is preferred then sodium citrate should be used instead of ammonium sulfate.
- Although ammonium sulfate is sufficiently pure for most applications, if the enzyme of interest is sensitive to heavy metals, EDTA should be included in the buffer

Table 2 The amount of solid ammonium sulfate to be added to a solution to give the desired final saturation at 0°C

Initial concentration of ammonium sulfate	Final concentration of ammonium sulfate, % saturation at 0°C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	g solid ammonium sulfate to add to 100 ml of solution																
0	10.7	13.6	16.6	19.7	22.9	26.2	29.5	33.1	36.6	40.4	44.2	48.3	52.3	56.7	61.1	65.9	70.7
5	8.0	10.9	13.9	16.8	20.0	23.2	26.6	30.0	33.6	37.3	41.1	45.0	49.1	53.3	57.8	62.4	67.1
10	5.4	8.2	11.1	14.1	17.1	20.3	23.6	27.0	30.5	34.2	37.9	41.8	45.8	50.0	54.5	58.9	63.6
15	2.6	5.5	8.3	11.3	14.3	17.4	20.7	24.0	27.5	31.0	34.8	38.6	42.6	46.6	51.0	55.5	60.0
20	0	2.7	5.6	8.4	11.5	14.5	17.7	21.0	24.4	28.0	31.6	35.4	39.2	43.3	47.6	51.9	56.5
25		0	2.7	5.7	8.5	11.7	14.8	18.2	21.4	24.8	28.4	32.1	36.0	40.1	44.2	48.5	52.9
30			0	2.8	5.7	8.7	11.9	15.0	18.4	21.7	25.3	28.9	32.8	36.7	40.8	45.1	49.5
35				0	2.8	5.8	8.8	12.0	15.3	18.7	22.1	25.8	29.5	33.4	37.4	41.6	45.9
40					0	2.9	5.9	9.0	12.2	15.5	19.0	22.5	26.2	30.0	34.0	38.1	42.4
45						0	2.9	6.0	9.1	12.5	15.8	19.3	22.9	26.7	30.6	34.7	38.8
50							0	3.0	6.1	9.3	12.7	16.1	19.7	23.3	27.2	31.2	35.3
55								0	3.0	6.2	9.4	12.9	16.3	20.0	23.8	27.7	31.7
60									0	3.1	6.3	9.6	13.1	16.6	20.4	24.2	28.3
65										0	3.1	6.4	9.8	13.4	17.0	20.8	24.7
70											0	3.2	6.6	10.0	13.6	17.3	21.2
75												0	3.2	6.7	10.2	13.9	17.6
80													0	3.3	6.8	10.4	14.1
85														0	3.4	6.9	10.6
90															0	3.4	7.1
95																0	3.5
100																	0

The temperature at which the precipitation is carried out is also important; the higher the temperature the lower the solubility of the protein. Slow addition of the ammonium sulfate and efficient stirring are important, particularly as the desired saturation is approached. Dissolved air may come out of solution and cause frothing; this is not deleterious, but frothing caused by over-vigorous stirring may cause denaturation of the protein. The precipitate is usually removed by centrifugation, though filtration can be used, particularly if the density of the protein aggregate is similar to or lower than that of the solution.

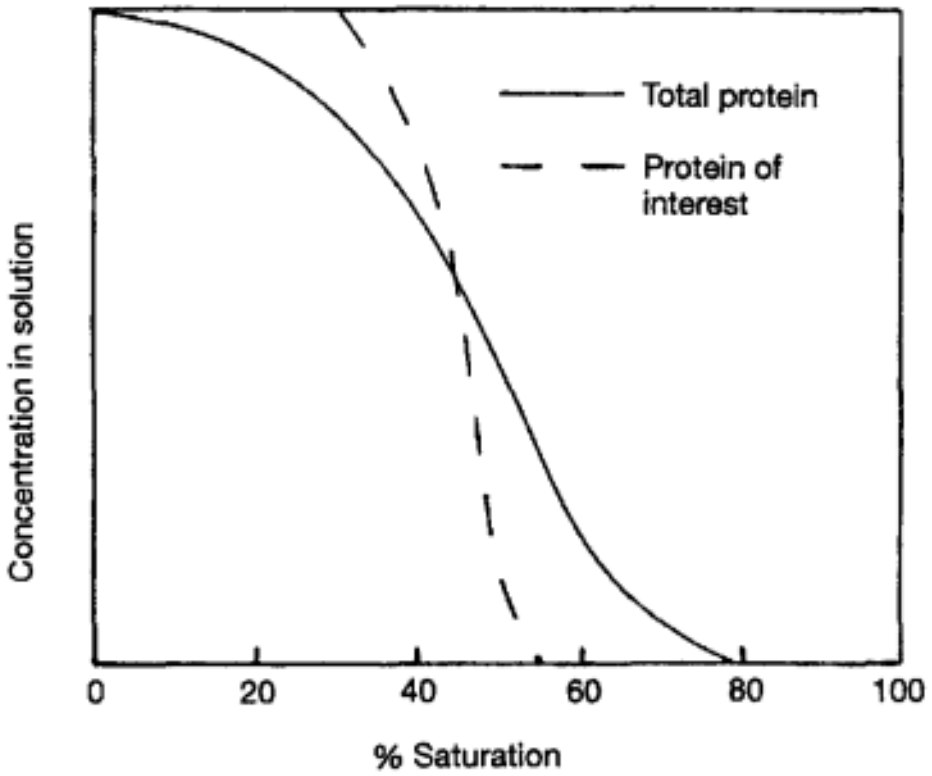


Figure 11 Typical profile for ammonium sulfate precipitation. The optimum % saturations for an ammonium sulfate cut would be 30% and 55% for purification of this protein.

4. Precipitation by organic solvents

- Many proteins can be precipitated by addition of water-miscible organic solvents, such as acetone and ethanol. The factors which influence the precipitation behaviour of a protein are similar to those involved in isoelectric precipitation and different from those involved in salting-out; thus, this method can be used as an alternative to isoelectric precipitation in a purification sequence, perhaps in conjunction with salting-out.
- Addition of the organic solvent lowers the dielectric constant of the solution, and hence its solvating power. Thus, the solubility of a protein is decreased and aggregation through electrostatic attraction can occur.
- Precipitation occurs more readily when the pH is close to the pI of the protein. The size of the protein also influences its precipitation behaviour; thus, a larger protein will precipitate in lower concentrations of organic solvent than a smaller protein with otherwise similar properties.
- However, some hydrophobic proteins, particularly those which are located in the cellular membranes are not precipitated by organic solvents, and in fact can be solubilized from the membranes by addition of organic solvents

- To minimize denaturation, precipitation with organic solvents should be carried out at or below 0°C. At higher temperatures the protein conformation will be rapidly changing, thus enabling molecules of the organic solvent to gain access to the interior of the protein, where they can disrupt the hydrophobic interactions and cause denaturation.

- Acetone and ethanol are the most commonly used solvents; others which have been used are methanol, propan-1-ol, and propan-2-ol. Safety aspects should be considered, particularly when working on a large scale; thus, the solvent should be relatively non-toxic and have a relatively high flashpoint, above 20 °C

5. Precipitation by organic polymers

- PEG is the most commonly used organic polymer. The mechanism of precipitation is similar to that of precipitation by organic solvents, however, lower concentrations are required, usually below 20%.
- Higher concentrations result in viscous solutions, making recovery of the precipitate difficult. The molecular weight of the polymer should be greater than 4000; the most commonly used molecular weights are 6000 and 20 000.
- PEG can be removed by ultrafiltration, provided its molecular weight differs significantly from that of the protein of interest. However, PEG does not interfere with many of the possible subsequent purification steps (e.g. ion exchange or affinity chromatography).