CHAPTER TWENTY

Protein Precipitation Techniques

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Contents

Abstract

After cell lysis, the most often used second step in a protein purification procedure is some sort of a rapid, bulk precipitation step. This is commonly accomplished by altering the solvent conditions and taking advantage of the changes in solubility of your protein of interest relative to those of many of the other proteins and macromolecules in a cell extract. This chapter will focus on the two most widely used precipitation methods: (1) ammonium sulfate precipitation and (2) polyethyleneimine (PEI) precipitation. These two methods work through entirely different principles, but each can achieve significant enrichment of target protein if optimized and applied carefully.

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1. Introduction

For both laboratory scale and larger scale protein fractionation, there is a need for a quick, bulk precipitation to remove much of cellular protein and other components. It is especially important to remove proteases as early in the procedure as possible to avoid protein degradation. This precipitation must be rapid, gentle, scalable, and relatively inexpensive. In addition to the fractionation, it can also achieve a significant concentration of the enriched protein. While many different precipitation methods have been used over the last hundred years, ammonium sulfate (AS) has remained the most widely used and polyethyleneimine (PEI) has increased in popularity, especially for acidic proteins. These two methods will be discussed in detail, followed briefly by several other precipitation methods and some general advice on handling precipitates and obtaining maximal purification from your precipitation step. An extensive and very useful general overview of various types of protein precipitation procedures can be found in Scopes (1994). An excellent review of AS and organic solvent (ethanol and acetone) precipitation can be found in Englard and Seifter (1990).

2. Ammonium Sulfate Precipitation

2.1. Principles

While several salts can be used as precipitants, AS has several properties that make it the most useful. It is very stabilizing to protein structure, very soluble, relatively inexpensive, pure material is readily available, and the density of a saturated solution (4.1 M) at 25 °C ($\rho = 1.235$ g/cm^3) is not as high as another salting-out agent, potassium phosphate (3 M, $\rho = 1.33$ g/cm^3).

Figure 20.1 shows a typical protein solubility curve where the log of the protein solubility is plotted as a function of AS concentration. The main features of this curve are a region at low salt where the solubility increases (called "salting in"), and then a region where the log solubility decreases linearly with increasing AS concentration (called "salting out"). The latter part of the curve can be described by the equation $\log_{10} S = \beta - K_s(\Gamma/2)$ where S in the solubility of the protein in mg/ml of solvent, $\Gamma/2$ is the ionic strength, and β and K_s are constants characteristic of the protein in question. K_s is a measure of the slope of the line and β is the log of the solubility if the salting-out curve is extrapolated to zero ionic strength. In general, most proteins have similar K_s values but vary considerably in their β value.

Suppose that the curve in Fig. 20.1 is valid for your protein and that the concentration of your protein in a cell extract is 1 mg/ml. The upper

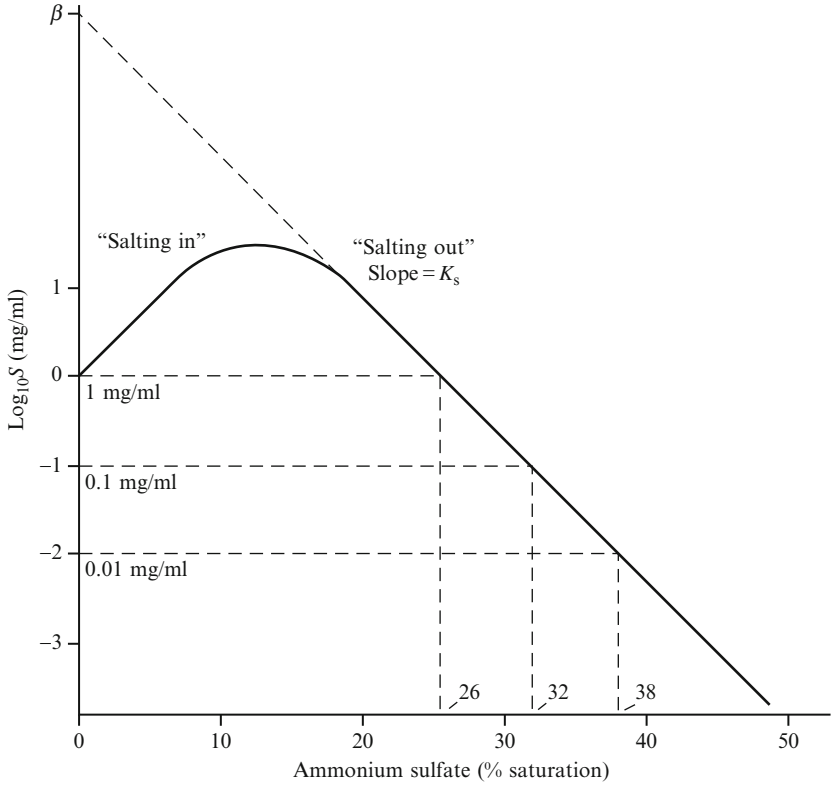

Figure 20.1 **Ammonium sulfate solubility curve for a hypothetical protein. This represents the log solubility of a hypothetical protein as a function of percent saturation of ammonium sulfate. The "salting-out" line follows the relationship $\log S = \beta - K_s(\Gamma/2)$ as described in the text, where $\Gamma/2$ is the ionic strength, which here is given as ammonium sulfate percent saturation.**

horizontal dotted line intercepts the solubility line at $\log S = 0$ ($S = 1$ mg/ml) and at an AS percent saturation of 26%. This means that if you add AS to 26% saturation, all of your protein would be soluble. Now if you increased the AS to 32% saturation (the middle horizontal dotted line), the $\log S$ would be -1 ($S = 0.1$ mg/ml) so 90% of your protein would become insoluble and precipitate out. For this extract, an excellent strategy would be to make a 26–32% saturated AS cut: add AS to 26%, spin out insoluble material, and then make the supernatant 32% saturated and collect what precipitates, which would contain 90% of your protein. You would remove those proteins and cell components that precipitate at 26% saturation and those that fail to precipitate at 32% saturation.

It is instructive to consider what would happen if you diluted the extract 10-fold with buffer. Now the initial concentration of your protein in the

extract is 0.1 mg/ml or $\log S = -1$. You can add AS to 32% saturation and your protein will not precipitate. To achieve 90% precipitation of your protein, you would have to increase the AS to about 38% saturation (bottom horizontal dotted line) or carry out a 32–38% saturated AS cut. You would end up having to use more than 10 times as much AS with the diluted extract to obtain your protein. This illustrates how important it is to specify the concentration of your extract.

You do not usually have a curve like that shown in Fig. 20.1 for your protein of interest so you have to determine the appropriate AS concentrations experimentally as described below.

2.2. Basic procedure

While there are numerous variations on AS precipitation, the most common ones are to add solid AS to a protein extract to give a certain percent saturation. Adding an amount of solid AS based on Table 20.1 is convenient, reproducible, and practical.

1. Generally one determines a lower percent saturation at which the protein of interest just does not precipitate and a higher percent saturation that gives $> 90\%$ precipitation as described in the section below.
2. Add solid AS to reach the lower value. Take care to add the AS slowly with rapid stirring so that the local concentration does not "overshoot" the target value. Some people carefully grind the solid AS with a mortar and pestle to a fine powder that dissolves rapidly. Once the AS is completely dissolved, allow the precipitation to continue for about 30 min. This is a compromise between waiting several hours as precipitation slowly approaches equilibrium and the desire to move along with the purification and not to introduce long delays in the procedure. Generally, one carries out all operations in an ice bucket or cold room.
3. Centrifuge at about $10{,}000 \times g$ for about 10 min in a precooled rotor to pellet the material that is insoluble.
4. Carefully pour off the supernatant and determine its volume. Determine the grams of AS from Table 20.1 to go from the lower desired percent saturation to the final higher percent saturation. Again add the AS slowly with rapid mixing to avoid high local concentrations and let the solution sit for 30 min to allow precipitation to occur.
5. Centrifuge as above. Let the pellet drain for about 1 min to remove as much as possible of the supernatant. If you have carried out the test precipitation carefully, the pellet will contain 90% or more of your target protein. This protein can be dissolved in an appropriate buffer and after either dialysis, desalting, or dilution used in the next step of the purification.

Table 20.1 Final concentration of ammonium sulfate: Percentage saturation at 0 °C[a]

Initial concentration of ammonium sulfate (percentage saturation at 0 °C)	Percentage saturation at 0 °C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (g) to be added to 1 l 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

[a] Reprinted from Englard and Seifter (1990), which was adapted from Dawson *et al.* (1969).

2.3. Doing an ammonium sulfate precipitation test

Generally one can precipitate 90% of a given protein with a 10% increase in AS saturation so one should restrict the range of the "AS cut" to no more than 10% (the proteins that are just soluble at 30% saturation but precipitate at 40% saturation are referred to as the 30–40% AS cut).

Figure 20.2 illustrates a method to determine the optimal AS precipitation conditions using only two centrifugation steps. Basically you place a volume of cell extract, for example, 10 ml in each of five tubes. You add with mixing amounts of solid AS to give 20%, 30%, 40%, 50%, and 60% saturation based on Table 20.1, let sit 30 min to allow precipitation, and then centrifuge to pellet the insoluble material. The pellets represent the 20%, 30%, 40%, 50%, and 60% saturated AS pellets. The volumes of the corresponding supernatants are determined and again solid AS is added to raise each to a 10% higher level of saturation. Again you mix, allow 30 min to precipitate, and then spin. The five pellets are the 20–30%, 30–40%, 40–50%, 50–60%, and 60–70% AS cuts. Each of these is dissolved in buffer and assayed for enzyme activity and total protein and perhaps subjected to SDS gel analysis. Most of the activity should be in one of the cuts, but if, for example, half is in 30–40% cut and half is in the 40–50% cut, then perhaps a 35–45% cut would be optimal. While this test may seem onerous, it is really

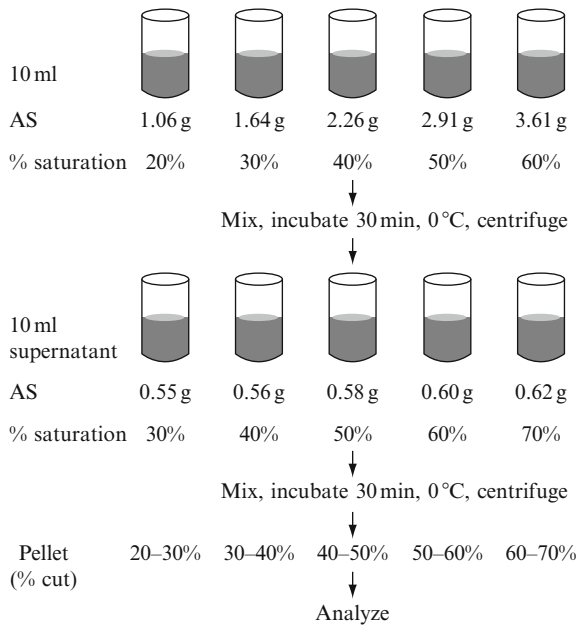

Figure 20.2 How to do an ammonium sulfate precipitation test. This test is carried out as described in the text and is self-explanatory.

quite an efficient way to determine the optimal conditions that will result in higher enrichment in this important step.

2.4. Comments/problems/solutions

1. Pellet is not solid. If the AS pellet is not firm after centrifugation it will be difficult to cleanly pour off the supernatant. One solution is simply to centrifuge 50% longer such that the precipitate that sedimented to the bottom of the tube has more time to compact. Another reason for a loose pellet is the presence of DNA that increases viscosity and slows sedimentation rates. If viscosity is a problem, it can be reduced by sonicating longer to break cells and shear DNA into shorter pieces. Another approach is to treat with the recombinant nuclease Benzonase (EMD/Novagen).
2. Pellet floats in high concentrations of AS. Since the density of very high concentrations of AS approach that of protein aggregates, the AS precipitate might float rather than sediment to the bottom of the tube during centrifugation. This can be a problem especially when your protein contains lipid or if there are nonionic detergents around that bind to the protein and decrease its density.
3. Published protocols are often hard to follow. Many published procedures fail to indicate the AS saturation convention (saturated at 0, 20, or 25 °C) or the protein concentration of the extract. As cautioned above, the amount of AS needed to achieve a given precipitation is dependent on the protein concentration.
4. You must interrupt an AS precipitation procedure. If you must stop the procedure, leave the protein as an AS precipitate. Proteins are remarkably stable in AS, either as a suspension of precipitated protein or as a pellet.

3. Polyethyleneimine Precipitation

3.1. Principles

PEI, whose trade name is Polymin P, is a basic cationic polymer made by BASF in large quantities for use in the textile and paper industry. PEI is a product of polymerization of ethyleneimine to yield a basic polymer with the structure: $CH_3CH_2N–(–CH_2CH_2–NH–)_n–CH_2CH_2NH_2$. Typically, n equals 700–2000 to give a molecular weight range of 30,000–90,000 Da. Since the pK_a value of the imino group is 10–11, PEI is a positively charged molecule in solutions of neutral pH. The use of PEI in protein fractionation originated at Boehringer Mannheim and was published by Zillig *et al.* (1970). More extensive examples of its application in protein purification

and several reviews have been published (Burgess, 1991; Burgess and Jendrisak, 1975; Jendrisak, 1987; Jendrisak and Burgess, 1975).

PEI can be thought of as similar to soluble DEAE cellulose. It binds to negatively charged macromolecules such as nucleic acid and acidic proteins and forms a network of PEI and bound acidic molecules that rapidly precipitates. The binding is stoichiometric. A heavy precipitate rapidly forms that can be pelleted by centrifugation for 5 min at 5000 rpm. Whether an acidic protein binds to PEI depends on the salt concentration. At low salt (0.1 *M* NaCl), a mildly acidic protein will bind and precipitate, but at intermediate salt (0.4 *M* NaCl), it will elute from the polymer and become soluble. A highly acidic protein will bind at low salt, not be solubilized at intermediate salt, and will be eluted at high salt (0.9 *M* NaCl). It should be noted that when a protein is eluted from a PEI pellet both the protein and the PEI become soluble. Thus it is necessary to remove the PEI from the protein before returning to low salt (see below).

3.2. Different modes of use of PEI

There are three different strategies for the use of PEI precipitation.

Strategy A: Precipitate with PEI at high salt (1 *M* NaCl). This precipitates the nucleic acids and leaves almost all protein in the supernatant.

Strategy B (for neutral or basic proteins): Precipitate with PEI at 0.1 *M* NaCl to remove nucleic acids and acidic proteins. This leaves protein of interest in the supernatant.

Strategy C (for acidic proteins such as E. coli RNA polymerase): This protocol will be presented in detail below and is based on Burgess and Jendrisak (1975) as refined by Burgess and Knuth (1996).

3.3. Basic procedure for Strategy C

1. Prepare a 10% (v/v) [5% (w/v)] PEI stock solution. PEI comes as viscous liquid that is 50% (w/v). (We use PEI from MP Biochemicals, $M_w = 50{,}000$–$100{,}000$, but one can also use material from other sources, for example, Sigma and Aldrich, $M_w = 750{,}000$). Ten milliliter is diluted to 70 ml with ddH_2O and concentrated HCl (3.8–4.0 ml) is added until the pH reaches 7.9. The volume is made up to a final volume of 100 ml with ddH_2O. This stock solution is stable at cold room or room temperature indefinitely. It should be noted that some companies sell PEI solutions that have been diluted one to one with ddH_2O to reduce viscosity and aid in dispensing. This material is only 25% (w/v).
2. Break *E. coli* cells (about 3 g wet weight cell pellet) by sonication in 30 ml buffer containing 50 m*M* Tris–HCl, pH 7.9, 5% glycerol, 0.1 m*M*

EDTA, 0.1 m*M* DTT, and 0.15 *M* NaCl. Centrifuge out cell debris at 15,000 rpm for 15 min. All operations are carried out on ice.

3. Based on a PEI precipitation test for your system (see below) add 10% (v/v) PEI, pH 7.9 to a final concentration of, for example, 0.3% (v/v) and mix well for 5 min to allow formation of a dense white precipitate.
4. Centrifuge at 5000 rpm for 5 min. *Note: Do not centrifuge too hard or the pellet will be harder to resuspend.* Decant the 0.3% PEI supernatant and save for later analysis. Let the pellet drain for 1–2 min to get rid of as much of the supernatant as possible.
5. Thoroughly resuspend the 0.3% PEI pellet in 30 ml of the above buffer containing 0.4 *M* NaCl. If available, the Tissue Tearor homogenizer (BioSpec Products, Inc. Cat # 985370-07) works very well to finely resuspend the pellet and effectively washes out proteins physically trapped in the pellet, and elutes out mildly acidic protein that are weakly bound to the PEI in the pellet. Let sit 5 min and then centrifuge at 5000 rpm for 5 min and decant the 0.4 *M* NaCl wash.
6. Resuspend thoroughly the 0.4 *M* NaCl pellet in 30 ml of buffer above but containing 0.9 *M* NaCl. This elutes more acidic proteins (like *E. coli* RNA polymerase), but leaves the nucleic acids in the pellet (it takes about 1.6 *M* NaCl to elute the nucleic acids). Let sit about 5 min, mix, and centrifuge at 15,000 rpm for 10 min.
7. To the 0.9 *M* NaCl eluate, add solid AS to 60% saturation (add 3.61 g per 10 ml of eluate). Mix well and let precipitation occur for at least 30 min. Centrifuge at 15,000 rpm for 10 min. Let pellet drain for 5 min. The pellet contains the AS precipitated protein, but almost all of the PEI remains in the supernatant. While traces of PEI are trapped in the pellet, they usually do not interfere with subsequent operations. If it is necessary to more completely remove these PEI traces, one can resuspend the pellet in buffer containing 60% saturated AS and recentrifuge.

This procedure typically gives a sixfold purification of RNA polymerase from other proteins, greater than 90% recovery, and a nearly complete removal of nucleic acids in 1–2 h.

3.3.1. Additional comments

1. It is important to emphasize that it is necessary to remove PEI from the 0.9 *M* NaCl eluate. If you merely dilute to low salt or dialyze to low salt, the proteins will again bind PEI and reprecipitate.
2. We have found that the PEI precipitation can occur even in the presence of 1% Triton X-100.
3. In contrast to AS precipitation, when you dilute an extract 10-fold with buffer, you use the same total amount of PEI (e.g., if you found that 0.3% PEI gave good precipitation of your protein with a normal extract,

you would only have to add 0.03% PEI to achieve the same precipitation with the 10-fold diluted extract, but of course there would be 10 times the volume of extract). This reflects the fact that PEI binds tightly to the acidic components and essentially titrates them.

3.4. Doing an PEI precipitation test

Since one cannot predict how much PEI to add to precipitate a given acidic protein nor how much salt it will take to elute the protein from the PEI pellet, one is advised to carry out simple PEI precipitation and elution tests (Burgess and Jendrisak, 1975; Burgess and Knuth, 1996). Basically this involves taking about six 200-μl samples into six microfuge tubes and adding 10% (v/v) PEI to final concentrations of 0%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% (v/v). Mix well and microfuge for 1 min at high speed. Analyze the supernatants by enzyme activity or by SDS polyacrylamide gel electrophoresis and, if necessary, by Western blot to determine the minimum amount of PEI needed to precipitate all of the target protein. Let us say that it takes 0.3%. Now prepare a set of six microfuge tubes which each contain a small 0.3% PEI pellet prepared as above and add 200 μl of buffer containing 0, 0.2, 0.4, 0.6, 0.8, and 1.0 *M* NaCl. Resuspend well. Let sit for 15 min, microcentrifuge, and analyze supernatant for your protein as above. The highest salt concentration that does not elute any of your protein is used as a wash and the salt concentration where all of your protein is eluted is used as the eluent.

3.5. An example of using PEI to precipitate a basic protein bound to DNA

Recently (Duellman and Burgess, 2008), in trying to purify the very basic protein Epstein–Barr virus nuclear antigen 1 (EBNA1) expressed in *E. coli*, we carried out a PEI precipitation test at 0.1 *M* NaCl to see if we could precipitate the nucleic acid and acidic proteins and leave the basic EBNA1 in the supernatant (this is Strategy B). To our surprise, the EBNA1 precipitated as we added some PEI (0.15%), but then failed to precipitate when we added more PEI (0.4%). It appears that the EBNA1 was bound to DNA and precipitated out with the DNA. However, at higher PEI concentrations the PEI preferentially bound the DNA and displaced the EBNA1. We found that precipitating with 0.15% PEI, washing with 0.3 *M* NaCl, and then eluting out of the PEI pellet with 0.8 *M* NaCl gave both a good enrichment for EBNA1 and rapid removal of nucleic acids.

4. Other Methods

This chapter has focused on AS and PEI precipitation. Other methods for protein precipitation mentioned very briefly below have been well described in numerous publications (see, Englard and Seifter, 1990; Ingham, 1990; Scopes, 1994).

4.1. Ethanol and acetone precipitation

Precipitation with organic solvents, such as ethanol and acetone, has been in use for well over a hundred years, but is probably best known for its use in fractionating human serum in the classic work of Cohen and Edsall. Care must be taken to carry out precipitations at very cold temperatures to avoid protein denaturation.

4.2. Isoelectric precipitation

Proteins are less soluble at their isoelectric point where they have zero net charge and can most easily approach each other with minimal charge repulsion. Since proteins are also less soluble at very low ionic strength, isoelectric precipitation is usually done at very low or no salt.

4.3. Thermal precipitation

In this method, cell extracts are heated to a temperature at which many proteins denature and precipitate, where the protein of interest is more stable and stays soluble. This approach is particularly useful in purifying enzymes from thermophiles expressed in *E. coli* where the extract is heated to a high enough temperature, often to denature and precipitate almost all *E. coli* protein, leaving the heat stable enzyme in solution.

4.4. Polyethylene glycol (nonionic polymer) precipitation

This subject has been reviewed extensively by Ingham (1990).

5. General Procedures When Fractionating Proteins by Precipitation

1. Thorough resuspension of precipitated protein pellets is important during washing or elution. While a pellet may seem quite solid, there is a very significant amount of supernatant trapped in a pellet and adhering

to the walls of the centrifuge tube. As mentioned earlier, it is wise to let the pellet drain well to remove as much of the supernatant as possible. If the pellet is large compared to the total amount of supernatant then it is recommended that one resuspend the pellet in 10 volumes of an appropriate buffer to remove supernatant proteins trapped in the pellet. For example, with a 40% saturated AS precipitate, one can wash by resuspending the pellet in 40% saturated AS and recentrifuging. For washing pellets of PEI precipitated material, washing is very useful and the use of a homogenizer like a Tissue Tearor is recommended to break up the precipitate into a very fine suspension. If resuspension is not thorough, then material that you want to wash out is not efficiently removed and the fractionation is less effective.

2. Try to avoid frothing when mixing. Whipping air into a protein solution can promote oxidation of proteins and also cause protein denaturation at the air–water interface.

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