

Selective Precipitation of Proteins

Selective precipitation of proteins (Rothstein, 1994) can be used as a bulk method to recover the majority of proteins from a crude **lysate**, as a selective method to fractionate a subset of proteins from a protein solution, or as a very specific method to recover a single protein of interest from a purification step. Except for antibody-mediated precipitation (see *UNIT 10.8*), selective precipitation methods are usually not protein-specific; the process depends on the physical **and/or** chemical interaction of the precipitating agent with one or more proteins that possess certain characteristics. It may be necessary to use a combination of selective precipitation techniques to isolate the desired protein. The success of precipitation is monitored by measuring total protein content (*UNIT 3.4*), by identifying proteins in the fractions using SDS-PAGE (*UNIT 10.1*), and by performing a suitable bioassay to **identify** the fraction containing the desired protein.

Developing a selective precipitation technique for a particular protein requires identification of the appropriate precipitating agent and optimization of the precipitation procedure using that agent. Often, more than one reagent **will successfully** precipitate a particular protein, so selecting a reagent is a matter of identifying the one that provides the desired protein in the most optimal state. Some reagents cause denaturation or adversely affect bioactivity, and some **form** complexes that bind the protein tightly. Still others lack the ability to selectively enrich for the desired protein. This unit describes a number of methods suitable for selective precipitation (see Table 4.5.1). In each of the protocols that are outlined, the physical or chemical basis of the precipitation process, the parameters that can be varied for optimization, and the basic steps for developing an optimized precipitation are described.

Table 4.5.1 Protein Precipitative Techniques

Technique	Protocol
Salting out	Basic Protocol 1, Alternate Protocol 1
Isoionic precipitation	Basic Protocol 2, Alternate Protocol 2
Two-carbon (C ₂) organic cosolvent precipitation of proteins	Basic Protocol 3
C ₄ and C ₅ organic cosolvent precipitation, phase partitioning, and extraction of proteins	Basic Protocol 4
Protein exclusion and crowding agents (neutral polymers) and <i>osmolytes</i>	Basic Protocol 5
Synthetic and semisynthetic polyelectrolyte precipitation	Basic Protocol 6
Metallic and polyphenolic heteropolyanion precipitation	Basic Protocol 7
Hydrophobic ion pairing (HIP) entanglement ligands	Basic Protocol 8
Matrix-stacking-ligand coprecipitation	Basic Protocol 9
Di- and trivalent metal cation precipitation	Basic Protocol 10

Protein precipitation and coprecipitation frequently are quite cooperative (in the equilibrium sense of the term) like other types of phase **changes**—i.e., protein precipitations may tend to be all-or-none processes. Therefore **good** control sometimes can be obtained only within small ranges of conditions, such as in variation of precipitating agents. However, the neighborhood or range of parameters where that can be achieved may best be located by the first, wide-ranging set of experiments.

Pilot experiments should make it possible to decide whether the process shall be one of first precipitating out desired proteins, as opposed to first taking out unwanted proteins and perhaps other materials and then bringing down desired proteins in a second stage.

Investigation of optimum temperatures is usually useful and even mandatory. Pilot samples may be kept in refrigerators, at room temperature, and at still higher (heat-shock) temperatures, to find the best temperatures for **both** protecting and precipitating proteins. Protein precipitation, even when optimized, is sometimes nearly as dependent on rates (kinetics) as on equilibria (thermodynamics). In practice, therefore, attention must be paid to controlling the time of incubation of pilot samples under the various conditions. Strong precipitating agents (synthetic polyelectrolytes are leading examples) may form **coprecipitates** within a few seconds, but be irreversible. Intermediate and even quite weaker precipitants may take hours to develop protein **coprecipitates** but afford more controllability and **reversibility**.

Choice of Procedure

Table 4.5.1 lists ten possible techniques for protein precipitation. Choice of which to use likely depends on how easy or difficult it may be to reverse precipitation to recover the **protein(s)** and discard excess precipitating agent. For most **uses** to which precipitated proteins are **put**—e.g., nutrition, therapy, or simply for assay—the proteins must be freed of precipitating agents. The quite powerful agents, such as synthetic polyelectrolytes, can be difficult or slow (or both) to remove and release from desired proteins. Consequently, synthetic polyelectrolyte **coprecipitation** may best be used for first bringing down unwanted proteins (i.e., where those precipitates are to be discarded). Remaining proteins in the supernatant may then be captured using a less aggressive but more controllable, reversible precipitating agent from the list in Table 4.5.1 (e.g., Basic Protocols 1 to 5 or 8 to 10).

Removal of Nucleic Acids

Crude proteins from cell cytosols, especially from bacteria and yeasts, may be laden with nucleic acids (RNA and DNA). Nucleic acids may interfere with protein isolation and contribute to haze and colloidal character. It is useful to remove nucleic acids in the early stages of **crude workup**. Techniques for **removal** of nucleic acids also help remove some kinds of particulates when the particulates have much anionic **character**—e.g., many cellular and subcellular membranes. There are two general methods for removing nucleic acids—i.e., precipitation using **polyamines** (*UNIT 1.3*) and using positively charged **anion-exchanger** resins (*UNIT 6.1*). Nucleic acids are **polyanions** that are charged negatively and rather densely with phosphate groups. Naturally occurring "protamines" (Felix, 1960; Budavari, 1989), which are proteins themselves, but very **cationic** proteins, thoroughly coprecipitate most nucleic acids and ribosomes. However, overshooting the **endpoint**—i.e., adding more than enough protamine proteins to remove the nucleic acids—may leave excess protamines in samples, contaminating the sought-for proteins and perhaps **interfering** with their isolation downstream. Accordingly, it may be necessary to determine the endpoint for stopping protamine addition using a suitable spectroscopic technique for nucleic acid content (see *UNIT 7.2*). Synthetic polyethyleneimine (PEI; Sigma) and syn-

thetic polyamino butane compounds (spermine; Sigma) also precipitate nucleic acids, and are useful for large samples (milliliter to liter in volume). A detailed review of PEI (also called Polymin by some vendors) for nucleic acid precipitation is found in Jendrisak (1987). Between 20 and 80 μl of 5% PEI, added per ml of crudes containing 4% (dry weight) nucleic acids, nearly completely removes nucleic acids. This procedure should be carried out at a pH of -7.5 to 8, where PEI amine groups are fully cationic and nucleic acids are fully anionic. However, PEI is a very able precipitant for proteins with isoionic points below pH 7. If the sought-for protein coprecipitates with both PEI and nucleic acids, as some enzymes do, pH variation and salt extraction (e.g., with NaCl; Jendrisak, 1987) then becomes necessary to free such enzymes from the PEI agglomerate.

Anion exchangers may also be used to remove nucleic acids and have an advantage and a disadvantage compared to polyamines used in solution. The advantage is that an excess of solid exchanger may adsorb less of the sought-for proteins than an excess of such reagents as PEI in solution. The disadvantage is that—because solid resins can only adsorb very large macromolecules like nucleic acids at the resin surface (not in its interior)—rather large quantities of resin may be required. The Amberlite IRA-400 series of resins (Sigma) and Dowex-1 anion exchangers (Sigma) are adsorbants for nucleic acids in neutral pH ranges. The DEAE-celluloses (Sigma), which are quaternized amines and therefore cationic, also are capable of adsorbing nucleic acids to remove them from crudes preliminary to isolating the proteins from the crudes. See Basic Protocol 2 for directions on washing and conditioning such resins prior to use.

SELECTIVE PRECIPITATION BY SALTING OUT

Ammonium sulfate is the best, first-choice salt for initial development of a salting-out program to precipitate sought-for proteins (see Fig. 4.5.2). Other salts may be chosen, and frequently they yield successful results. However because of sulfate's kosmotropic and protein-molecule exclusionary powers (see Commentary), ammonium sulfate is most preferred and best understood. Sulfate salting out may be both thermodynamically limited and rate-limited. If precipitation of proteins does not occur quickly after addition of estimated amounts of sulfate salt, it may be best to wait for a period, perhaps a few hours, to allow time for precipitates to form. This becomes necessary for dilute protein solutions—less than -1% in overall concentration. For several mammalian enzymes, the threshold for protein precipitation and solubility—i.e., the visibly seen development of precipitation versus proteins remaining dissolved (apparent equilibrium)—occurs at -2 to 5 mg/ml protein in 2 to 3 M ammonium sulfate, at neutral pH (Scopes, 1987).

The pH and temperature also are likely to be important. If the isoelectric pH of the sought-for protein is known, it may be used as the center point around which to vary pH, in increments of -0.5 to 1 pH unit, in initial searches for optimum conditions. A number of proteins, peptide antibiotics, and drugs are best salted out as coprecipitates with SO_4^{2-} as a bound counterion. Hence their optimal pH for salting out may be acidic with respect to their isoelectric point. Figure 4.5.2 illustrates the main steps in salting out, starting with mixtures of proteins in solution without particulate matter.

In both this protocol and Alternate Protocol 1, the sample is first carried through all steps on a small scale for a pilot experiment to determine optimal conditions. The procedure is then scaled up to purify the protein sample, using those optimal conditions. Figure 4.5.3 illustrates the procedures for such a pilot experiment.

BASIC PROTOCOL 1

Extraction, Stabilization, and Concentration

4.5.5

Materials

Crude protein solution of interest, **particle-free** (see discussion of Clarification in Strategic Planning)

Appropriate pH buffer

Ammonium sulfate

Additional reagents and equipment for dialysis (*UNIT 4.4 & APPENDIX 3B*)

1. Dialyze protein samples against a pH buffer or a pH buffer/ammonium sulfate mixture having a sulfate concentration below that needed to start precipitation (see *UNIT 4.4 & APPENDIX 3B*).

By carrying our dialysis, three objectives may be achieved at the expense of an added step. First, it is possible to dialyze out unwanted lower-molecular-weight materials—e.g.,

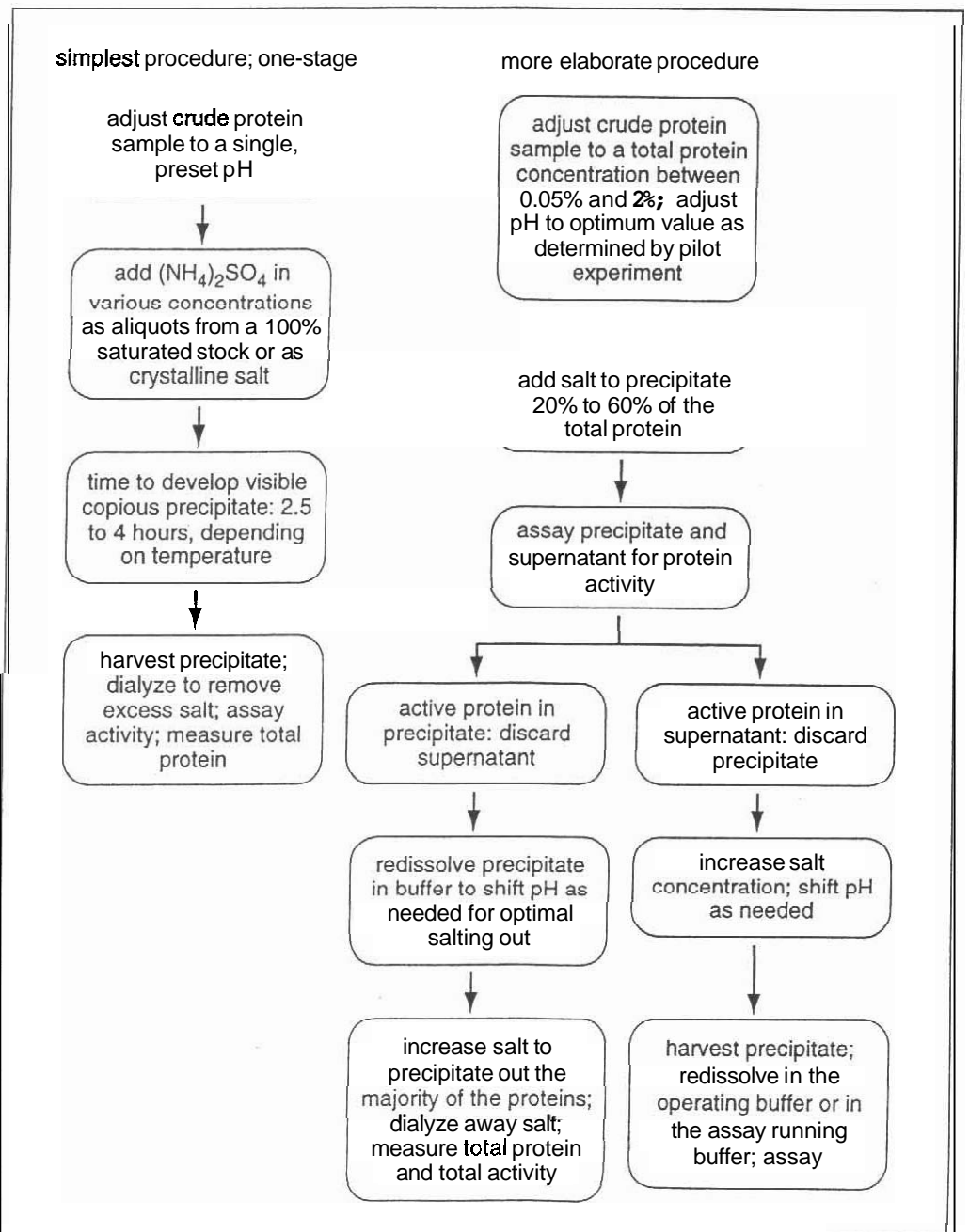


Figure 4.5.2 Two-salting out protocols, single-stage and two-stage, starting from a particle-free aqueous crude preparation (e.g., cell or tissue extract or centrifuged fermentation broth).

sugars, amino acids, and components of fermentation broth or cell extract. Second, dialysis makes it possible to bring the protein sample smoothly to the prescribed pH. Finally, dialysis allows the investigator to adjust the protein sample to a known beginning salt concentration—that of the dialyzing buffer. Such dialysis may be carried out in dialysis bags (cellulose acetate tubing), or by pressure membrane concentrators with membranes having nominal molecular-weight cutoff points below that of the sought-for protein.

2. Set up a pilot experiment (see discussion of Pilot Experiments in Strategic Planning) to determine the optimal protein concentration, pH, salt concentration, temperature, and incubation time.

See Critical Parameters and Troubleshooting for guidelines on how to vary these parameters. See Figure 4.5.3 for illustration of a small-scale pilot experiment.

pH adjustments generally are performed by addition of acid (HCl), base (NaOH, KOH, or ammonia), or a buffer. At the same time, it is usually necessary to avoid adding too much overall volume, so the buffering capacity of the sample itself must be taken into account. If feasible, it is generally best to adjust pH of proteins with 0.1 or 0.2 M HCl, or comparable concentrations of aqueous ammonia. Trying to move the pH appreciably with buffers, if dilute buffers must be used, increases overall volumes and decreases salt concentration. That, in turn, may require the use of considerably more salt to reach a specified concentration, in contrast to operations where pH has been adjusted by use of HCl or NaOH.

CAUTION: The protein solution should be stirred well during addition of strong acid or strong base. Beware of changes in temperature.

3. Add ammonium sulfate to the optimal concentration (see Critical Parameters and Troubleshooting). Incubate (for the optimal period of time) until a precipitate forms.

The salt may generally be added as a saturated solution. However, there is an advantage to adding the salt in solid form, as increases in volume are thereby lessened. There are few indications that addition of solid ammonium sulfate does harm to proteins already in solution.

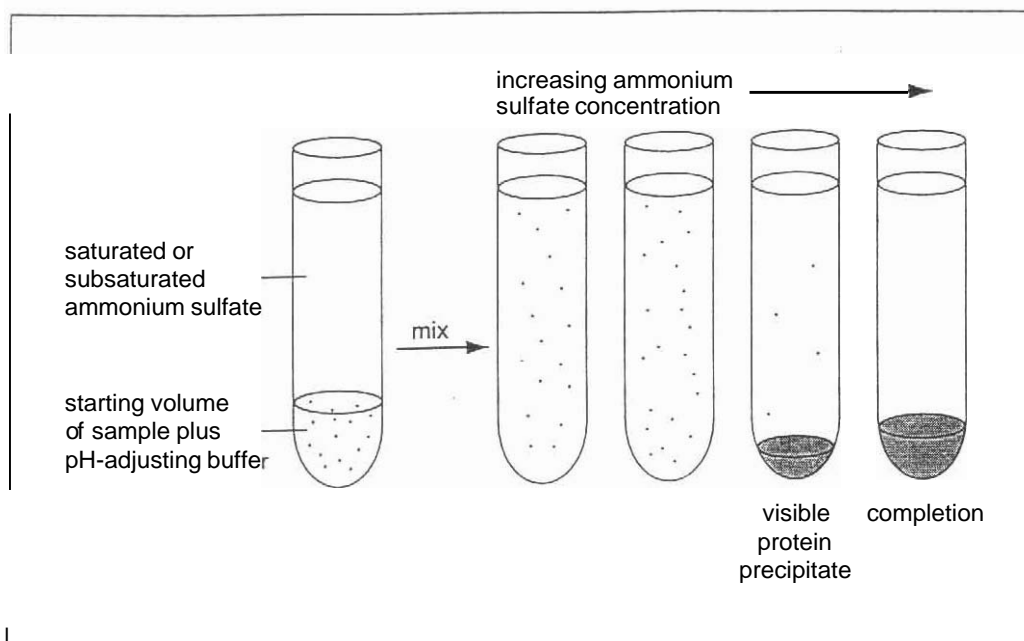


Figure 4.5.3 Salting out—small-scale investigation. Maximum ammonium sulfate concentration 4 M. Development of precipitate depends on the protein and unwanted materials which sometimes precipitate out in samples or fractions, apart from the sought-for protein. Other adjustable parameters are pH, temperature, and concentration of starting sample.

ALTERNATE PROTOCOL I

4. Collect the precipitate by simple decantation (pouring off the supernatant).

*Ammonium sulfate precipitates are usually **dense** and **settle** out readily. However, if **simple settling and decantation** will not work, a **slow-speed centrifugation** (10 to 100 x g) should easily **suffice**.*

5. Redissolve the precipitate in a buffer suitable for the next step (e.g., running buffer for electrophoresis or assay buffer).

*High **concentrations** of K^+ and other salts are **not** compatible with SDS-PAGE and **isoelectric** focusing. K^+ salts of SDS are poorly soluble. **Large** amounts of ammonium ion grossly **interfere** with some **total protein** assays (UNIT 3.4). Therefore, excess salt should be dialyzed away by simple cellulose-tubing dialysis (UNIT 4.4 & APPENDIX 3B) or by pressure ultrafiltration (UNIT 4.4).*

6. Perform a bioassay for the protein of interest.

*If the sample is to be diluted considerably **for** the bioassay, the redissolved ammonium sulfate may not need to be dialyzed beforehand. However, if **appreciable** salt interferes (as in SDS-PAGE and total protein assays), the assay may require a dialyzed sample input*

SELECTIVE PRECIPITATION BY STEPWISE SALTING OUT

A **stepwise** salting-out procedure is used to fractionate a **crude** mixture into two portions, only one of which retains the desired protein. The result is a partial purification of the desired protein from a crude mixture of proteins; this may improve the extent of recovery in subsequent purifications.

In this protocol, as in Basic Protocol 1, the sample is first **carried** through all steps on a small scale for a pilot experiment to determine optimal conditions. The procedure is then scaled up to purify the protein sample, using those optimal conditions. Figure 4.5.3 illustrates the procedures for such a pilot experiment.

1. Adjust the concentration and pH of the crude protein solution to the optimal values as determined by a pilot experiment (see discussion of Pilot Experiments in Strategic Planning).

*See Critical Parameters and Troubleshooting for guidelines on how to vary **these parameters**. See Figure 4.5.3 for illustration of a small-scale pilot experiment.*

2. Add ammonium sulfate at an appropriate concentration. Incubate until precipitate forms.
3. Collect the precipitate and supernatant and assay for the appropriate bioactivity in each.
- 4a. *If the active fraction is the precipitate:* Redissolve the material in a buffer suitable for assay or the next general procedure.

If the next steps are not to be performed immediately, the precipitate need not be redissolved. It should be stored with its salt because salts, especially ammonium sulfate, are protective and stabilizing.

- 4b. *If the active fraction (or part of it) **remains** in the supernatant:* Increase salt concentrations by 5% to 10% and perhaps change pH by -0.5 to 1 unit, in renewed pilot experiments and in larger-scale precipitation.
5. Collect the precipitate and supernatant and assay for the appropriate bioactivity in each.
6. Repeat steps 4b and 5 until the protein of interest is obtained in as clean a preparation as possible.

**SELECTIVE PRECIPITATION BY ISOIONIC PRECIPITATION:
COLUMN METHOD**

Proteins frequently are least soluble and most precipitable when they are isoionic. In the isoionic, salt-free state, protein molecules are in their most compact, least hydrated conformation — a phenomenon that is closely related to the condition of proteins at their isoelectric point. The distinction between isoionic and isoelectric properties is drawn in detail by Tanford (1961). Deionization using a column (this protocol) or dialysis (see Alternate Protocol 2) aim at rendering proteins isoionic to precipitate them. Two important parameters determine solubility of many proteins: solution pH with respect to each protein's isoionic point (pI) and the low salt concentration (zero to 0.1 to 0.2 M salt). A number of proteins—e.g., β -lactoglobulin—are sharply dependent on these parameters. Accordingly, if these parameters are well controlled and carefully adjusted, the solubility/precipitability behavior of a protein will be a practical basis for scaleable, selective isolation of the desired protein. The column method used in this protocol is appropriate only for proteins that remain soluble at their isoionic point.

In addition to adjusting proteins to their isoionic pH, the general method described here, using mixed-bed resin deionization, is able to strip away all salts from proteins. Salts, even in small concentrations (<0.05 M in many cases) often have large effects on protein solubilities and therefore on precipitability. Inorganic salts tend to "salt in" many proteins, thus enhancing their solubilities. Accordingly, sharp control and thorough removal of salts as desired is an important part of the general technique of isoionic precipitation.

Figure 4.5.4 shows a Dintzis deionization column with a lower, main mixed bed of anion-cation exchange resin beads in their respective OH⁻ and H⁺ form. Two categories

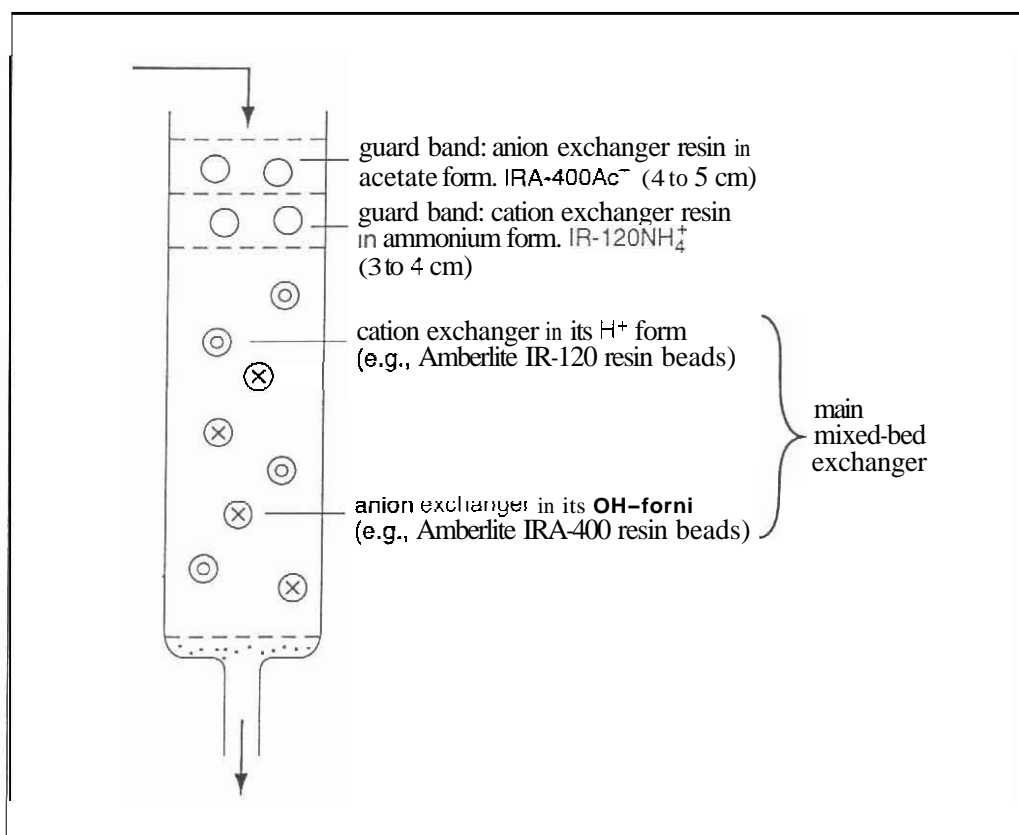


Figure 4.5.4 Deionization column. Dintzis design, described in Edsall and Wyman (1958). Passage through this column renders the protein salt-free and automatically adjusted to the protein's isoionic pH. This column is used for proteins that remain soluble at their isoionic point.

of salt ions are removed by the mixed bed: (1) the supporting **electrolyte**—i.e., the free salt in the solution; and (2) salt ions that are counterions, which are in effect bound to the protein if the protein is not isoionic. If the pH of the starting solution is below the isoionic point, the protein is a cation with anion counterions, commonly Cl^- . If the pH is greater than the isoionic point, the protein is a net macroanion with **cationic** counterions, commonly K^+ or Na^+ . In the mixed bed, **cationic** and anionic salt ions are exchanged for H^+ and OH^- , respectively, going on to simply form water. Protein molecules deprived of all mobile counterions are quantitatively forced to the single pH at which they can exist without mobile counterions or protein-bound ions—i.e., the isoionic pH. Therefore the effluent of the column is isoionic protein at the isoionic pH, in plain water. The net salt exchange and entrapment processes are similar to conventional desalting by resin ion exchangers, except the effluent is buffered by the isoionic protein and adjusted to its pH.

In the original Dintzis column, illustrated in Figure 4.5.4, guard exchanger bands may be positioned at the top of the column above the main bed. They exchange incoming salt cations and anions (or protein counterions) for NH_4^+ ion and acetate ion from the resins. This is intended to protect incoming proteins from direct mixing with the equivalent of strong acid and strong base, respectively, from the mixed-bed strong exchange resins in their respective H^+ and OH^- form. The NH_4^+ and acetate ions are exchanged and removed in the main bed, and the isoionic protein sample flows through.

Materials

Cation exchanger: Amberlite IR-120H⁺ resin (Sigma or Aldrich)

Anion exchanger: Amberlite IRA-400Cl⁻ resin (Sigma or Aldrich)

–0.5 and 1 M NaOH or KOH

0.5 to 1 M HCl

0.2 M acetic acid

0.2 M ammonium hydroxide

Crude protein solution of interest, particle-free (see discussion of Clarification in Strategic Planning)

Plastic beakers

Large Buchner funnel

Whatman no. 1 filter paper

Chromatography column of appropriate size

1. Determine exchange capacity required.

Necessary capacity for deionizing and trapping salts depends on the number of counterions and therefore on pH of the protein solution, concentration of the supporting electrolyte or buffer, and overall volume. The following is an example of the calculation method used. Assume that 50 ml of 5% serum albumin in 0.2 M acetate buffer, pH 4, is to be deionized. On the basis of the titration curve (with a pI of 5.0 for albumin: see UNIT 8.2 for discussion of titration curves), –35 counterions (e.g., Cl⁻) are present per protein molecule (mol.wt. 67,000), amounting to –1.3 meq of ions. The buffer contains –10 meq of total acetate, along with 10 meq of Na⁺, which were counterions for the buffer's acetate. Hence, there are a total of 22 meq of ions to be exchanged. The mixed bed is calculated to hold 1 meq/ml of settled mixed-bed resin, so –12 ml of resin, minimally, is required. Thus a 2 x 30-cm bed including 80 to 90 cm³ of mixed-bed resin is in good excess (by a factor of ~4) of the minimum required to strip out all inorganic ions, buffer, and protein counterions. See Critical Parameters for additional discussion.

2. Place IR-120H⁺ cation-exchange resin in a plastic beaker, cover with an excess of –0.5 M NaOH or KOH, and incubate –20 min to convert resin to the Na⁺ or K⁺ form. Transfer to a large Buchner funnel containing Whatman no. 1 filter paper, wash with water, then transfer to a beaker and cover with an excess of 0.5 to 1 M HCl. Incubate

20 min, then transfer to a Buchner funnel containing filter paper and wash with water again. Repeat this exchange cycle at least one more time before mixing the exchange resins.

*This full-cycle treatment removes undesirable low-molecular-weight hydrolysis fragments from resin polymers, which **otherwise** would bind strongly to many proteins and elute with them when proteins **are passed** through the column. Each step requires **-20 min**, to allow **penetration, diffusion**, and exchange of ions with the interiors of the resin beads. More detailed specifications are given in **full** treatises describing exchangers, such as Kressman (1957) and in the manufacturers' literature.*

3. Place IRA-400Cl⁻ resin in a plastic beaker, cover with an excess of **-1 M NaOH** or **KOH**, and incubate **-20 min** to convert resin to the OH⁻ form. Transfer to a large Buchner funnel containing Whatman no. 1 filter paper and wash with water to remove all excess OH⁻ ion.

*The resin is now IRA-400OH⁻. It should be kept **cold** and should not be **exposed** to air during long periods of storage because the CO₂ in air slowly neutralizes the IRA-400OH⁻ to a bicarbonate form. The resin should be taken through **this full exchange** cycle within a few days prior to use.*

*Anion exchangers, both old and new, which are made of quaternary amine polymers, self-hydrolyze and slough off organic amines, especially when stored in their **hydroxyl form** (e.g., IRA-400OH⁻). Accordingly, IRA-400 in whatever starting form (Cl⁻ or OH⁻), needs to be taken through **a full** exchange cycle. Each step requires **-20 min**, to allow penetration, diffusion, and exchange of ions with the interiors of the resin beads. More detailed specifications are given in **full** treatises describing exchangers, such as Kressman (1957) and in the manufacturers' literature.*

4. Prepare resin for preexchange guard bands as follows. React IRA-400OH⁻ (from step 3) with 0.2 M acetic acid, then wash with water to prepare anion-exchanger IRA-400Ac⁻. React IR-120H⁺ (from step 2) with 0.2 M ammonium hydroxide, then wash with water to prepare IR-120NH₄⁺.

5. Mix and load ion-exchange resins into columns (also see Fig. 4.5.4)

*See **Critical Parameters** for guidelines on preparing the mixed-bed resin.*

*Some precautions need to be taken to prevent **unmixing**—i.e., formation of separated bands of cation and anion exchangers in their respective H⁺ and OH⁻ forms—when filling the column. The density of IR-120H⁺ is appreciably larger than that of IRA-400OH⁻. The resins used for the guard bands have a similar disparity in density. If large volumes of premixed resins are dropped through several centimeters of water; they unmix on the way down. Hence, mixed-bed resins should be dropped in **small** increments, with **forward flow** of water, to prevent **unmixing**. The same general **considerations** pertaining to procedures such as packing and loading **chromatographic** columns, dead-volume minimization, and prevention of channeling that are described in UNIT 8.4 apply to this column technique.*

6. Determine flow rate required.

If there is adequate capacity in the mixed bed to trap all salts, a 2 × 30-cm column deionizes 50 to 100 ml of a 1% to 5% protein solution in ~3 hr. Suitable flow rates average 0.5 to 1 ml/min.

7. Perform column separation on crude protein solution

The first cut, ~20 to 30 ml of eluate, will be quite dilute as the protein solution displaces water in the column, and may be set aside.

8. Perform bioassay for the protein of interest

9. Recycle used resins.

Mixed-bed resins previously used for deionization—e.g., of serum proteins—are good general binding agents and can be reused. After being used for deionizing a protein, the

resin should be flushed with 0.2 M HCl to remove any protein and to prevent bacterial growth. Mixed-bed resin pairs of the IRA-400 and IR-120 series, as well many of the Dowex resin pairs, separate well from one another in saturated KCl. Crystalline KCl can be added to a slurry of used mixed-bed resin in water: As KCl saturation approaches, the anion exchanger that is least dense—i.e., the Cl^- form—floats upward. The cation exchanger in K^+ form sinks. The anion-exchange resin slurry is decanted off the top, away from the cation exchanger; isolating both resins. These may then be individually recycled back to their respective O^- and H^+ forms, whereupon they may again be mixed as needed (see steps 2 and 3).

ALTERNATE PROTOCOL 2

SELECTIVE PRECIPITATION BY ISOIONIC PRECIPITATION: DIALYSIS METHOD

One of the older means of rendering proteins salt-free or nearly salt-free (i.e., isoionic) is dialysis (also see UNIT 4.4 & APPENDIX 3B). However, two problems frequently arise with conventional dialysis: (1) when appreciable amounts of protein are present, osmotic effects result in swelling of dialysis bags as salt diffuses outward; and (2) often it is quite uncertain where the isoionic point is, even if it is feasible to deionize by dialysis against a buffer. The resin deionization method (see Basic Protocol 2), sometimes called Dintzis' method (Edsall and Wyman, 1958), automatically adjusts a protein precisely to its isoionic pH without prior knowledge of it.

Proteins that precipitate immediately when deionized are troublesome in the column method described in Basic Protocol 2 and Figure 4.5.4. Precipitates plug the column and pose the problem of separating precipitated protein from resin beads. β -lactoglobulin and a number of other proteins behave in this way; they have solubilities very sensitive to low salt conditions near the isoionic pH. This protocol (also see Fig. 4.5.5) describes a means of overcoming this problem. Proteins to be deionized and precipitated in isoionic form are confined in dialysis tubing with a molecular weight cutoff that will retain the protein of interest and the mixed-bed resin is placed outside as a slurry of 40 to 60 g of mixed-bed resin (50 to 80 ml of wet resin; see Basic Protocol 2) per 200 to 400 ml of dialyzing solvent. Salt ions and protein counterions exchange through the membrane and are trapped outside in the exchanger resins. After exchange is completed, precipitated protein in the dialysis tubing is recovered by centrifugation of the bag's contents. This general technique requires several hours. Salting out and diffusion slow as the protein concentration inside the dialysis bag decreases; hence this method is slower than the flow-through column method (see Basic Protocol 2).

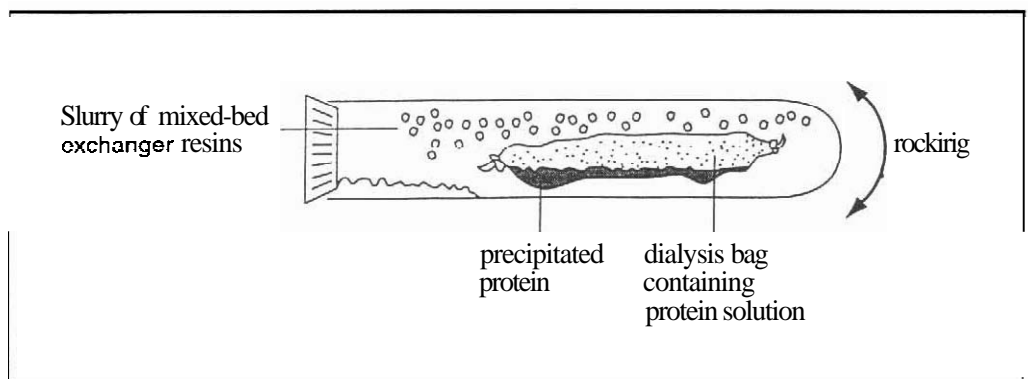


Figure 4.5.5 Deionization by dialysis. When proteins are insoluble and precipitate at their isoionic point, deionization is accomplished by placing dialysis tubing containing the protein sample in a slurry of mixed-bed resin exchangers (i.e., a mixture of IRA-400OH⁻ and IR-120H⁺) and incubating with rocking. Proteins insoluble at their isoionic point precipitate inside the bag. The mixed-bed exchange resins remove free salts, forcing the protein to its isoionic pH. This method is considerably slower than the column method.

SELECTIVE PRECIPITATION USING A TWO-CARBON (C₂) ORGANIC COSOLVENT

BASIC PROTOCOL 3

Protein precipitation using very cold ethanol or acetone is a method that **was** developed over a century ago. Both of these cosolvents are still frequently used. Ethanol surpassed acetone for precipitating proteins at the time E. J. Cohn, and co-workers at **Harvard** perfected the Cohn fractionation (Cohn et al., 1946) for large-scale preparation of plasma proteins—albumin, fibrinogen, prothrombin, and γ -globulins. The Cohn procedures are **still** employed, although modified. One of the advantages ethanol has over acetone pertains to fire hazard. Ethanol is less volatile than acetone and generates less flammable vapor under otherwise similar conditions. Both cosolvents, when handled in in >0.1-liter amounts, should be used in ventilated rooms.

Materials

Crude protein solution of interest, particle-free (see discussion of Clarification in Strategic Planning)
Buffering system
Ethanol
Acetone
Centrifuge

1. Adjust the **crude** protein solution of interest to the selected pH.

If the operating buffer (used as the solvent in the separation) or pH-calibration buffers (used to check pH-meter calibration) are to be selected for low-temperature operation, the tables by Alner et al. (1967) and Perrin and Dempsey (1974) give formulations for several buffers together with their pH values at temperatures in the 0°C range.

2. Arrange for good stirring and good metering to control mixing of organic cosolvents with the aqueous proteins.

It is necessary to suppress local high concentrations of C₂ cosolvents. It is also necessary to suppress the heat generated by mixing of cosolvents, especially of ethanol with water; to avoid undue denaturation and temperature increases greater than -2°C above the set temperature. Dilution of organic cosolvents with water or with aqueous running buffer before they are added to the protein solution may repress generation of heat during mixing and may also repress formation of large local concentrations of cosolvents at the point at which they are mixed with aqueous protein. However excessive dilution of organic cosolvents may add to the final volumes necessary to reach precipitation thresholds. A 3% to 8% (v/v) volume of water in organic cosolvent is a suitable dilution for achieving protein precipitation before overall volumes become excessive.

3. Cool the two components—i.e., protein in aqueous buffer and cosolvent system to be used—to the running temperature. Do not allow temperatures to rise more than 2°C above the running temperature.

Lower temperatures can be obtained by using an auxiliary refrigeration unit pumping coolant through an exchanger coil or by incubating C₂ cosolvents in the deepfreeze. Salt/ice baths are capable of reaching approximately -10°C.

4. Slowly mix the the cosolvent system into the aqueous protein solution while monitoring the mixture with a thermometer.

If the temperature rises much during cosolvent (or cosolvent/buffer) addition, slow the rate of solvent addition down to let the cooling system remove excess heat. Predilution of organic cosolvents (see annotation to step 2) may be helpful here.

5. Collect precipitate by centrifugation or allow precipitate to settle out. Remove supernatant and redissolve precipitate by adding water or suitable aqueous buffer.

Extraction,
Stabilization, and
Concentration

4.5.13

Organic cosolvents admixed with aqueous systems usually decrease the density of the solvent mixture, which may aid settling or centrifugation of precipitated protein. However, the centrifuge should also be cooled down to the precipitation running temperature. Some centrifuges become surprisingly warm, depending on the vintage and specific features. A test of the actual centrifugation temperature is advisable; this is done simply by immersion of a thermometer in centrifuge vessels after a run.

6. Perform a bioassay for the protein of interest.

SELECTIVE PRECIPITATION USING C₄ AND C₅ ORGANIC COSOLVENTS

Figure 4.5.6 outlines the technique for three-phase partitioning (TPP) using *t*-butanol in conjunction with ammonium sulfate (Lovrien, et al., 1987). Some C₅ and C₆ cosolvents — e.g., alcohols such as pentanol — may be useful in some cases. However C₅ and larger cosolvents tend to be so insoluble in water as to be of limited use. The cosolvent used here, *t*-butanol, is infinitely soluble in water alone, but in the presence of large concentrations of ammonium sulfate it forms a second phase. Simultaneously, proteins are precipitated, forming a third phase intermediate between the lower (aqueous) and upper (organic) liquid phases (see Fig. 4.5.6). The various phases are mutually saturated with respect to one another. The protein precipitate is usually well developed and easily retrieved by low-speed centrifugation.

TPP behavior depends on protein molecular charge (precipitated products are often sulfate salts of protein). Therefore, the pH of the aqueous phase is an important parameter for modulating protein precipitation, partitioning, and extraction of unwanted compounds. When proteins have been extracted from their original source using detergents, TPP or related methods such as Morton's *n*-butanol extraction technique (Morton, 1950) are frequently effective in removing detergents, lipids, and pigments from the sought-for protein.

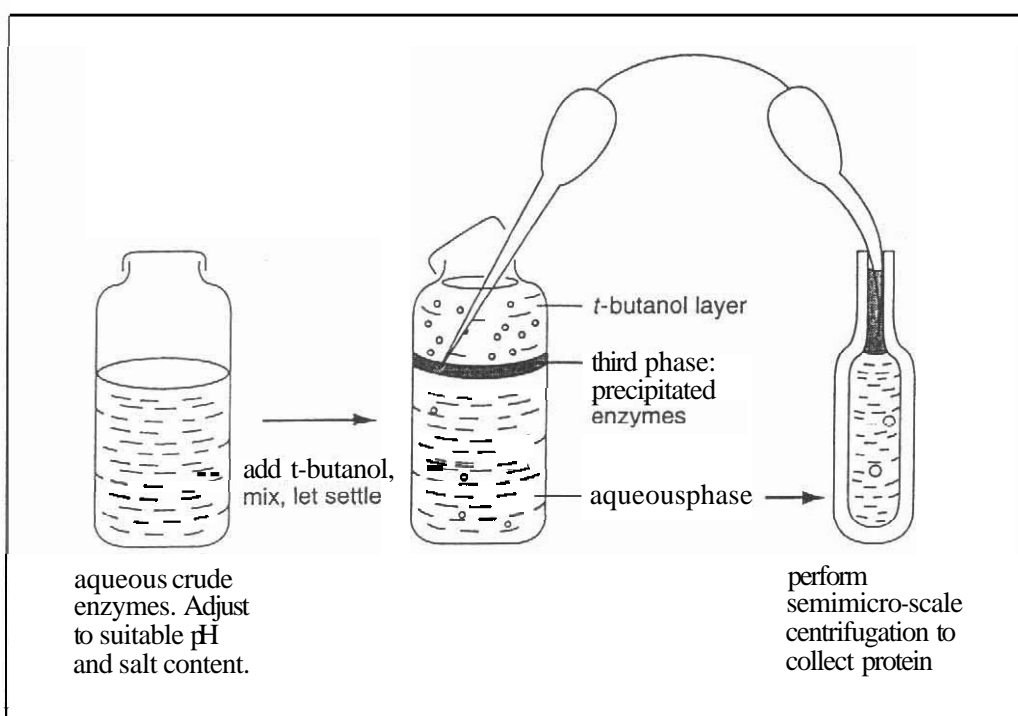


Figure 4.5.6 Procedure for precipitation of proteins from aqueous solutions using *t*-butanol. This figure applies to both large-scale precipitation and small-scale pilot experiments. The *t*-butanol layer develops when adequate salt is present in the aqueous solution. Both the *t*-butanol layer and the aqueous lower phase act as extraction solvents as well as partitioning systems.

Materials

- Ammonium sulfate
- Buffering system
- Crude protein solution of interest, particle-free (see discussion of Clarification in Strategic Planning)
- C, organic cosolvent (e.g., t-butanol, analytical grade)

1. Add ammonium sulfate and buffer to adjust the salt concentration and pH of the crude protein solution of interest appropriately.

The amount of ammonium sulfate used is determined by pilot experiments and is in general less than that used for simple salting out (20% to 100% saturation); i.e., the amount added is less than sufficient to induce precipitation.

2. Add –0.5 to 1 ml C, organic cosolvent per ml crude protein solution, mix, and incubate until three phases form.

If only technical grade t-butanol is available it may be either recrystallized (the melting point of pure t-butanol = 25.5°C) at room temperature, or distilled (the boiling point = 82°C).

3. Transfer the middle phase containing the precipitated protein to a centrifuge tube of volume approximately twice that of the protein precipitate. Centrifuge at 10 x g, 4° to 25°C.

See Critical Parameters for discussion of the separation of phases.

4. Redissolve the precipitate in an appropriate buffer and perform suitable bioassay

SELECTIVE PRECIPITATION USING PROTEIN EXCLUSION AND CROWDING AGENTS AND OSMOLYTES

Protein exclusion and crowding agents and osmolytes are neutral, very water-soluble compounds — such as sugars, organic diols, and polymers — used in concentrations of 2% to 20% to precipitate proteins. They operate primarily by entropically driven "crowding" (Herzfeld, 1996), by preferential hydration (Timasheff, 1992), or a combination of both mechanisms. Such agents push proteins out of solutions in the mechanical/physical sense and in the thermodynamic sense. At concentrations greater than ~5% by weight, exclusion agents and osmolytes occupy so much volume on the molecular scale that they leave less room in solution available for protein molecules.

Crowding and molecular-exclusion polymers that have been used for protein precipitation include polyethylene glycol (PEG), polypropylene glycol (PPG), polyvinyl alcohol (PVA), methylcellulose, dextran, and hydroxypropyl dextran. Osmolytes and zwitterions used for protein precipitation include sucrose, 2-methyl-2,4-pentanediol (MPD), raffinose, maltose, sarcosine, and betaine. These compounds are readily available from suppliers such as Sigma and are able to protect and precipitate a number of proteins. Development of each protein-precipitation program requires attention to pH, temperature, and other factors that modulate parameters governing protein solubility and precipitability. The literature is scant concerning rate limitation of protein precipitation by these parameters. It is likely that in lower concentration ranges of proteins, neutral polymers, and osmolytes (<1% to 2%), precipitation rates may be slowed. Accordingly the general method should be treated as a rates (kinetics) problem when precipitates are not quickly formed.

The sample is first carried through all steps on a small scale for a pilot experiment to determine optimal conditions. The procedure is then scaled up to purify the protein sample, using those optimal conditions. Figure 4.5.3 illustrates the setup for such a pilot experiment.

BASIC PROTOCOL 5

Extraction, Stabilization, and Concentration

4.5.15

Materials

Crude protein solution of interest, particle-free (see discussion of Clarification in Strategic Planning)

Appropriate pH buffer

Precipitating agent: neutral polymer or osmolyte

1. Set up a pilot experiment (see discussion of **Pilot Experiments** in Strategic Planning) to determine the optimal concentrations of protein and precipitating agent as well as the optimal pH, temperature, and incubation time.

*In preliminary searches, use a series of test tubes as in Figure 4.5.3. On addition of components to each other, mixing or **strong vortexing** may be needed because some neutral polymers are highly viscous.*

2. Prepare protein solution and solution of precipitating agent.

*Neutral polymers should be dissolved in water before adding **them** to aqueous proteins. Moderate heating may speed dissolving. It is poor practice to attempt **addition** of dry polymers directly to dissolved proteins as the polymers tend to form gummy masses that dissolve very slowly.*

*Mechanisms here depend on molecular crowding; hence concentrations of proteins and dissolved polymers to be added **must** be maximized. These components dilute each other when mixed. If protein samples need pH adjustment before the mixing step, use buffers in fairly concentrated form—or even 0.2 to 1 M acid or base if feasible—to keep volumes minimized and component concentrations **maximized**.*

4. Mix protein solution and solution of precipitating agent. Incubate (for the optimal period of time) until a precipitate forms.

*If mixtures of proteins are very crude and only one or two proteins are sought after, it may be preferable not to drive the precipitation too hard. In such cases it may be best to accept more slowly developed precipitates to **maximize** the amount of **unwanted protein** and excess precipitant left behind in the **supernatant** and the amount of sought-after protein captured in the precipitate.*

5. Collect the precipitate.

*Trapped and coprecipitated neutral polymers in precipitates may need be "reversed" or split away later to release **proteins free** of polymers. How **this** can be done depends on the chemistry of the systems, and perhaps also on the chromatographic properties of the precipitated proteins. In the case of **dextrans**, modest amounts can be degraded by dextranase enzyme. Coprecipitated proteins may be captured on ion-exchange matrices, whereupon neutral polymer may be eluted and the **protein** displaced from the exchanger using a **pH shift and/or salt** (see UNIT 8.2).*

6. Perform bioassay for the protein of interest.

BASIC PROTOCOL 6

SELECTIVE PRECIPITATION USING SYNTHETIC AND SEMISYNTHETIC POLYELECTROLYTES

Four categories of polyelectrolytes can precipitate proteins. First, there are the synthetic polyelectrolytes—e.g., vinyl polymers such as polyacrylate (PAA) and polymethacrylate (PMA); acid salts (polyanions); and polyethyleneimine (PEI, a polycation). Next, there are the semisynthetic polyelectrolytes—carboxymethylcellulose (CMC), sulfated cellulose, and sulfated dextrans. The third category includes the protamines (very basic proteins), and the fourth includes naturally occurring sulfate polysaccharides—e.g., heparin and chondroitin sulfate. Heparin and related biochemical polyelectrolytes are not generally used on a large scale because of their cost; they are used in research for precipitating narrow classes of proteins such as β -lipoproteins. For a recent, short review

Table 4.5.2 Synthetic Polyelectrolyte Coprecipitating Agents for Proteins

Polyelectrolyte	Proteins precipitated	References
Polyacrylate	Lysozyme, hemoglobin	Sternberg and Hershberger (1974)
Polyacrylate	Eight industrial enzymes	Sternberg (1976)
Polyacrylate	Ferrihemoglobin, catalase, bovine serum albumin	Berdick and Morawetz (1954)
Polymethacrylate	Cellulase enzymes	Whitaker (1953)
Polymethacrylate	Serum albumin, hemoglobin	Morawetz and Hughes (1952)
Polyethyleneimine	Recombinant (RecA) protein	Shibata et al. (1981)
Polyethyleneimine	RNA polymerase II	Jendrisak and Burgess (1975)
Polyethyleneimine	β -galactosidase fusion protein	Niederauer et al. (1994)
Polyethyleneimine	Restriction endonucleases	Bickle et al. (1977)
Carboxymethylcellulose (CMC)	β -lactoglobulin, α -lactalbumin	Hidalgo and Hansen (1971)
Polylysine	Fibrinogen	Sela and Katchalski (1959)
Copolymer Eudragit	Protein A	Mattiasson and Kaul (1994)
Tri-block polyampholytes	Trypsin, RNase A, lysozyme	Patrickios et al. (1994)
Polydimethyldiallylammonium chloride	Serum albumin	Dubin et al. (1987)

of polyelectrolytes for protein precipitation see Singh (1995). Table 4.5.2 lists a number of polyelectrolytes in each category and cites references regarding their use. Figure 4.5.7 outlines the main steps for polyelectrolyte-mediated protein precipitation.

Frequently, the main practical problem with this technique is in reversing coprecipitation—i.e., dissolving the coprecipitate in a way that releases proteins and splits away the polyelectrolyte. In the case of polyacrylate and polymethacrylate, these polymers bind calcium and barium ions rather strongly. Thus protein/polyacrylate coprecipitates may first be shifted upward in pH, which renders proteins and polyelectrolytes negatively charged and helps them redissolve. After this, Ca^{2+} or Ba^{2+} is introduced to precipitate away the polyelectrolyte and release the protein. Several papers describing examples of the use of this technique are referred to in the Commentary (see Critical Parameters and Table 4.5.2).

The general range of starting protein concentrations from which proteins may be coprecipitated in fair to good yield (at optimum pH) is -0.05% to 3% (w/v) protein. The range of quantities of polyelectrolyte required, given a suitable kind of polyelectrolyte, tends to be less than the amount of protein. By weight, -2% to 20% polyelectrolyte with respect to the protein is generally required. The variation between systems is large, partly because polyelectrolytes have large variations in charge density—e.g., completely ionized polyacrylic acid carries one anionic charge per 56 g polymer; carboxymethylcellulose, substituted on each glucose unit, carries one anion per 240 g polymer if completely ionized.

Materials

- Water-soluble polyelectrolytes (Aldrich or Sigma)
- Crude protein solution of interest
- Test tubes
- Glass or plastic cuvettes
- Spectrophotometer

**Extraction,
Stabilization, and
Concentration**

4.5.17

1. Choose a polyelectrolyte.

For proteins not previously investigated, first experiment with from two to four easily available, water-soluble polyelectrolytes. Polyacids (e.g., polyacrylic acids), and polybases (e.g., polyethyleneimine), can be titrated with NaOH, or HCl respectively, to two or three pH units, respectively, above or below the target protein's isoelectric point (if known). Such polyelectrolytes are buffers themselves, as are proteins, over the pH range of acid/base titration. Hence, it is unnecessary to use additional conventional inorganic buffers to control pH. If polyelectrolytes and proteins are mismatched in pH before mixing, the mixture may shift in pH; the pH should be measured, if necessary, to determine H⁺ transfer on mixing the main components.

2. Prepare stock polyelectrolyte solutions.

Initial stock polyelectrolyte solutions should be prepared in water in concentrations three to ten times the final coprecipitating concentrations. Dilutions will be performed in the succeeding steps.

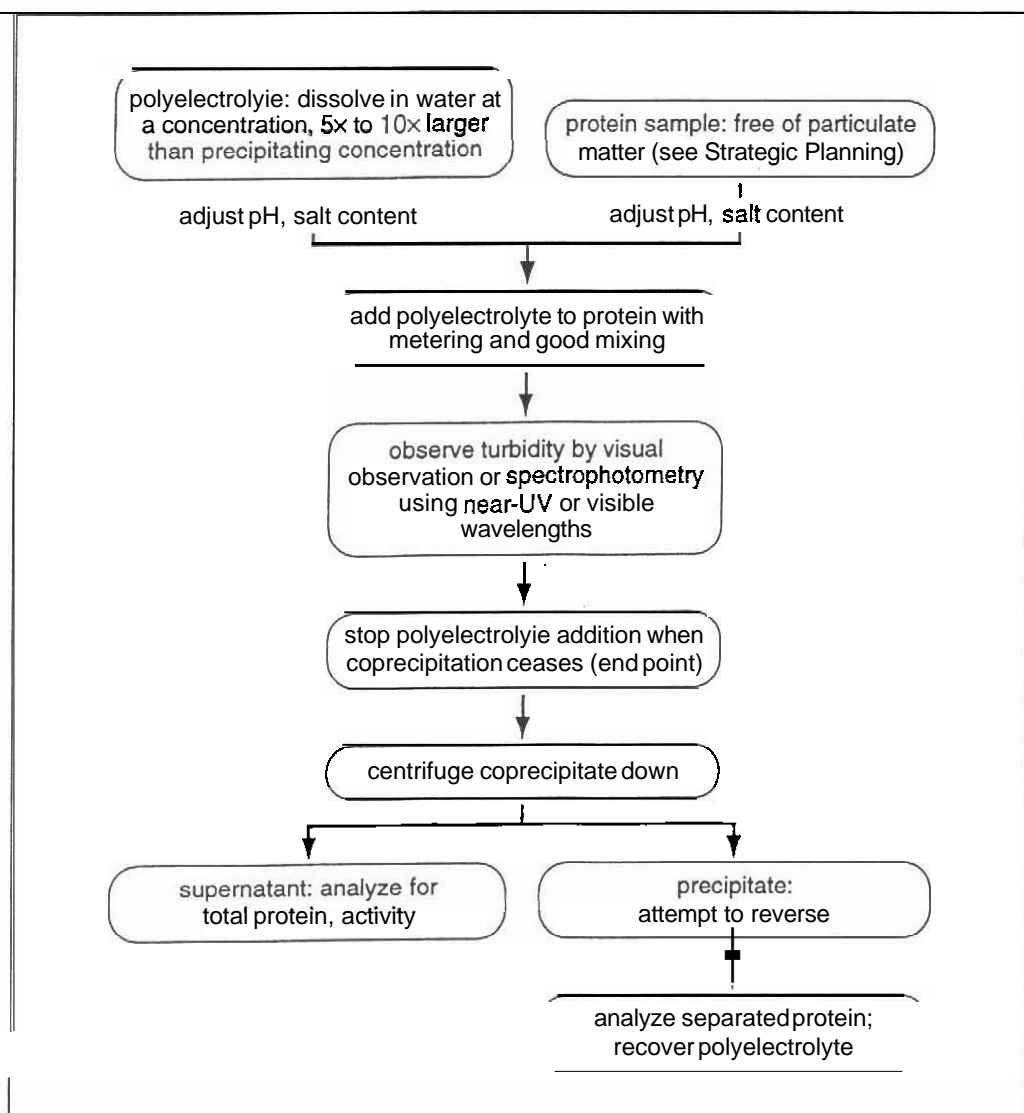


Figure 4.57 Principal steps in protein coprecipitation with polyelectrolytes. "Metered" addition is slow addition (with good stirring) of aliquots of polyelectrolyte stock to the protein sample near the threshold of precipitate formation as seen by eye or spectrophotometric turbidity determination.

3. Render protein sample clear and nonparticulate (see discussion of Clarification in Strategic Planning).
4. Set up several test tubes on a small scale, adding ~ 2 ml sample and 0.05 to 1 ml of each polyelectrolyte stock solution at three or four different pH values to construct a matrix of **polyelectrolyte/protein** mixtures. Observe the point at which turbidity appears.

The intent is to roughly locate (by visual comparison to detect turbidity) the coprecipitation starting point, the coprecipitation endpoint, the relative concentrations of polyelectrolyte and protein to be used, and the optimum pH. This enables construction of a rough phase diagram (i.e., a plot of turbidity versus variables such as relative amount of polyelectrolyte).

Also see discussion of Pilot Experiments in Strategic Planning.

5. Set up a more finely grained pilot experiment as in the previous step, but carry out turbidity measurements in glass or plastic **cuvettes**. For scale-up, use controlled rates of polymer addition (i.e., adding polymer to the protein sample slowly in reproducible aliquots of stock solution near the threshold of precipitate formation), temperature control (water bath), and mixing using a magnetic stir-bar. Measure **spectrophotometric** absorbance in the 400- to 500-nm range and determine where overt coprecipitation begins.

Sharply climbing absorbances mark the ranges where turbidity forms as a result of particulate coprecipitates. Coprecipitation is in large part a rate process as well as an equilibrium process. If the aim is to achieve selection of particular proteins, rather than precipitation of all proteins, the best strategy may be to bring down some protein(s) in a first-stage separation of the coprecipitate, followed by a second stage in which polyelectrolyte is again added.

6. Carry out full-scale separation. Centrifuge to collect coprecipitate; retain the supernatant and analyze for total protein activity.
7. Reverse coprecipitation to free captured protein from synthetic polymers.

This step should start with resuspension of particles in water or in buffers to shift pH to a position where polyelectrolyte and protein have similar charges—e.g., both anionic or both cationic. Separating our polyelectrolytes is often achieved by addition of metal ions (Ca^{2+} or Ba^{2+} in the form of chlorides) to carboxylate polyelectrolytes. An attempt may also be made to adsorb polyelectrolytes on bead resins of opposite charge—e.g., on DEAE cellulose for carboxylated polyelectrolytes.

In developing a procedure for polyelectrolyte removal and release of sought-for proteins, measurement of supernatant protein concentration and/or activity if the protein is an enzyme should facilitate monitoring of protein release. Most of the polyelectrolytes in Table 4.5.2 have low A_{280} absorbances (and small molar extinction coefficients). Hence simple measurement of A_{280} should locate the conditions at which proteins are released into solution, provided there are no particulates present. However, such particulates cause very strong scattering and turbidity in the UV range, far beyond the absorbance of equivalent amounts of truly dissolved protein.

8. Perform bioassay for protein of interest and recover polyelectrolyte.

SELECTIVE PRECIPITATION USING METALLIC AND POLYPHENOLIC HETEROPOLYANIONS

Under strongly acid conditions, inorganic and some organic strong anions—known as heteropolyanions—ably precipitate proteins. Inorganic anions used under acid conditions for this purpose include **perchlorate**, tungstate, phosphotungstate, molybdate, **phosphomolybdate**, **tungstosilicate**, and ferrocyanide. Inorganic ions (used under acid to neutral conditions) include **sulfosalicylate**, **picrate**, and diverse plant **polyphenols** and **tannates** (also see Critical Parameters).

Prior to overt precipitation, protein molecules under strongly acidic conditions are acid-expanded and remain soluble. When the heteropolyanions are introduced and **allowed** to bind, the protein molecules are forced back to a compact, poorly hydrated conformation. Molecular expansion and contraction of the protein in solution, which leads to precipitate formation, may be followed by biophysical tools (Fink, 1995). Driven further with additional heteropolyanions, such as perchlorate and tungstate in -0.2% to **2%** or **3%** concentration, proteins coprecipitate with these anions in dense aggregates that easily settle or centrifuge down.

In some procedures for precipitating proteins, perchloric acid (**HClO₄**) is the precipitating agent of choice because it is ultraviolet-transparent above 250 nm. Perchloric acid precipitates whole protein molecules, but not their low-molecular-weight **fragments**—amino acids and oligopeptides. This is the basis for analysis with many proteolytic enzymes—at the end of the proteolytic cleavage assay, whole or intact proteins are precipitated out by a few percent **HClO₄**. Proteolytic fragments are left in the supernatant for subsequent **A₂₈₀** measurement, giving a rather direct measure of the amount of hydrolysis that occurred before adding the **HClO₄**.

SELECTIVE PRECIPITATION USING HMROPHOBIC ION PAIRING (HIP) ENTANGLEMENT LIGANDS

Hydrophobic ion pairing (HIP) is **coprecipitation** of proteins using flexible hydrocarbon "tails" of alkane anions (detergents) that have their anion head groups bound in the target proteins. Figure 4.5.8 illustrates the composition of such a coprecipitate. Organic anions, especially sulfonates and sulfates, often bind strongly to proteins bearing **cationic** sites.

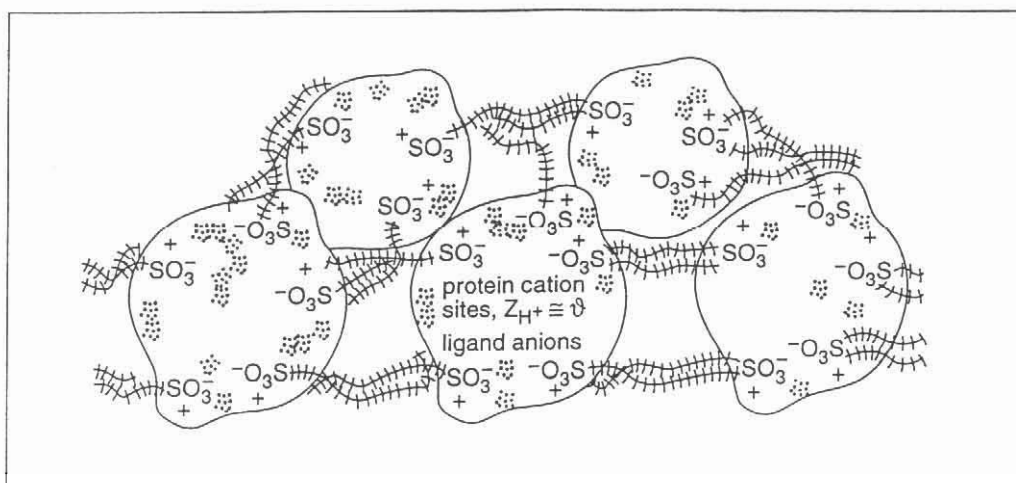


Figure 4.5.8 HIP coprecipitate. Strong anions (sulfate or sulfonate-bearing C_{10} – C_{14} alkane tails, e.g., dodecyl sulfate) bind to protein **cationic** sites forming ligand-protein complexes. Complexes draw together via tail-tail hydrophobic entanglement, aggregate, and coprecipitate. At maximum coprecipitating efficiency with -10^{-7} M protein, the stoichiometric ratio of bound ligand anions (ν) is to cation side chains (Z_{H^+}) in the protein molecule is close to 1:1.

The organic alkane groups entangle and bind to each other in a manner similar to **tail/tail** association in micelles. Entanglement draws **protein/ligand** complexes together as shown in Figure 4.5.8. Such complexes go on to aggregate, then coprecipitate. Ordinary detergents such as dodecyl sulfate. (DDS^-) in correct **protein/detergent** ratios are able protein precipitants. Such ligands, especially detergent anions, need to be carefully regulated with regard to concentration. Suitable overall concentrations of precipitating detergents are usually 10^{-4} to 10^{-6} M when used with overall protein concentrations ranging from 0.01% to 0.001% (w/w) or 10^{-4} to 10^{-6} M protein. If suitable ligand concentrations are exceeded, the ligands go on to form **micelles** and **solubilize** proteins rather than precipitate them. Inside their optimum coprecipitation range with respect to total protein concentration, these ligands are rather generally effective towards many proteins.

Protein coprecipitation by HIP is frequently easy to reverse. The precipitate is redissolved in solvent with a pH -2 or 3 units more alkaline than the coprecipitation pH. Detergent anions may be quantitatively stripped out with strong ion exchange resins, usually in the chloride form—e.g., Amberlite IRA-400Cl $^-$ or Dowex-1Cl $^-$, or equivalent resins. Released proteins—e.g., enzymes in the supernatant—are then ready for assay.

SELECTIVE PRECIPITATION BY MATRIX-STACKING LIGAND COPRECIPITATION

Matrix-stacking ligands function (see Fig. 4.5.9) to draw protein molecules together in dense complexes that aggregate and coprecipitate protein. **Ligand/ligand** association forms a matrix host; protein molecules trapped in the matrix are guests. Occasionally, these ligands **cocrystallize** proteins and **peptides** (Conroy and Lovrien, 1992). Ligand organic tails bear rather rigid coplanar groups that stack and bind to one another π -face- π -face fashion, in a manner similar to **stacking** of aromatic **bases** in nucleic acid double helices. Tail-to-tail matrix association is reinforced by short alkane groups on the periphery of the **tails**—i.e., methyl, ethyl, and *t*-butyl substituents. The **alkyl** substituents lend hydrophobicity and promote water displacement, which helps drive tail-to-tail association. The structures of a few anionic ligands, are shown in Figure 4.5.10.

As with flexible-entanglement ligands (see Basic Protocol 8), the more rigid matrix-stacking ligands achieve maximal coprecipitation efficiency when v ligands are bound per protein molecule with close to a 1:1 ratio of v to Z_{H^+} —i.e., $v \sim Z_{\text{H}^+}$, where Z_{H^+} = protein net cationic charge from H^+ titration of side chains. Hence coprecipitation depends on pH and how pH governs the protein charge, Z_{H^+} . Stacking and entanglement ligands in their most useful overall concentrations of 10^{-4} to 10^{-5} M often protect proteins as well as precipitate them. Such ligands tighten protein conformation, sometimes profoundly (Matulis and Lovrien, 1996). Protection often starts in solution before overt coprecipitation. The capacity to protect proteins against several kinds of degradation is an advantage of matrix-stacking ligand coprecipitation.

Two variables around which to design early protein coprecipitate experiments are illustrated in Figure 4.5.11. The following steps may be used for developing a matrix-ligand coprecipitation protocol.

Materials

- Crude protein solution of interest, particle-free (see discussion of Clarification in Strategic Planning)
- Potential ligands (Fig. 4.5.10; also see Aldrich catalog)
- Appropriate buffers
- Dowex-1Cl $^-$ resin
- Small test tubes or 2- to 3-ml plastic microcentrifuge tubes

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Extraction,
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4.5.21

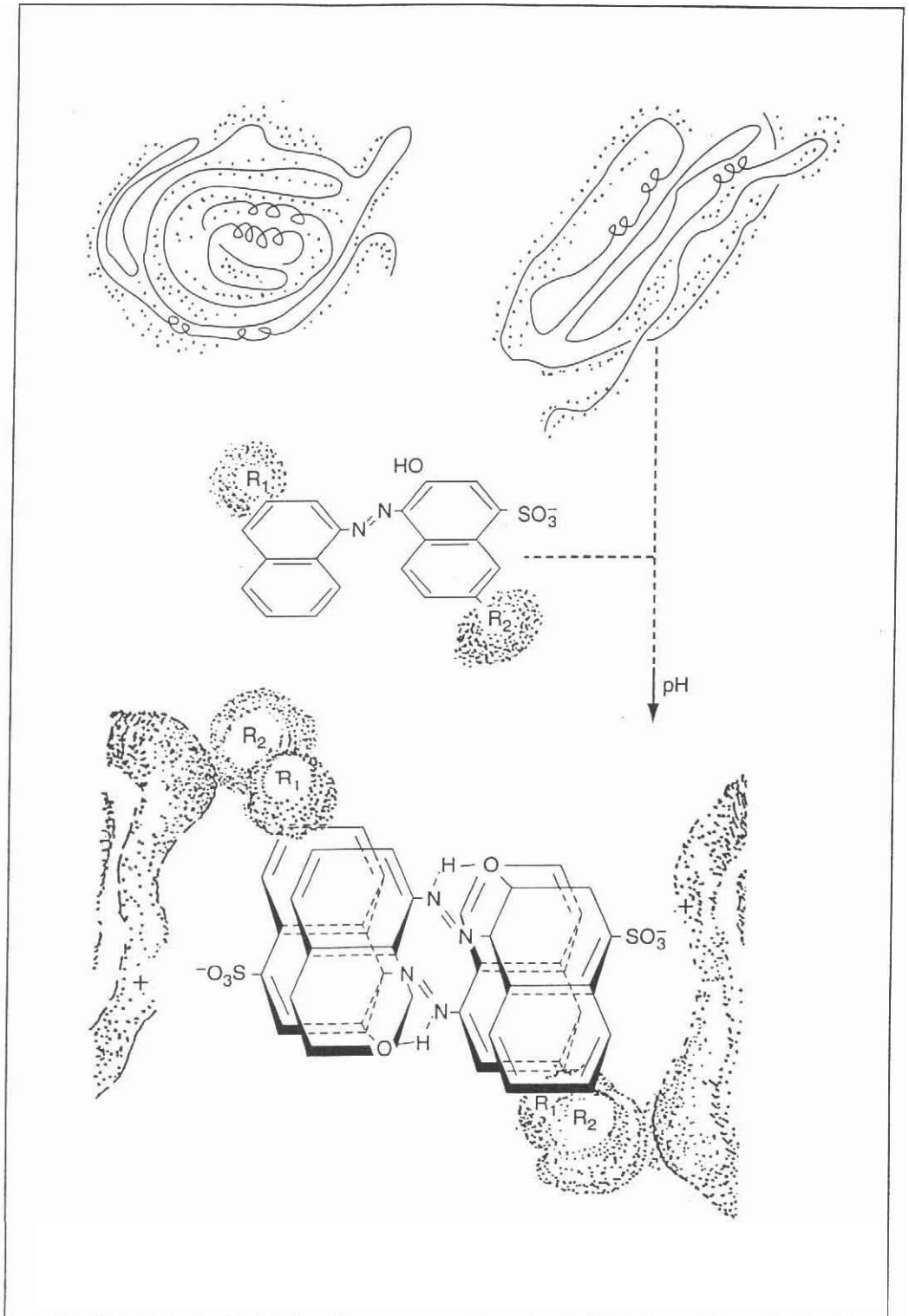


Figure 4.5.9 Molecular structure-function basis for matrix-stacking ligand coprecipitation of proteins. Protein molecules initially soluble in water and bearing a positive net charge attract strong anion (sulfonate) ligands. Such ligands associate via organic tail/organic tail stacking interaction, reinforced by alkane R-groups. Ligand/protein complexes are thus drawn together and coprecipitate out of solution.

1. Set up grids of small test tubes or 2- to 3-ml plastic **microcentrifuge** tubes as in Figure 4.5.11. In each grid, vary two parameters versus one another to optimize coprecipitation—e.g., pH versus ligand concentration (with a fixed total protein concentration), pH versus kind of ligand, and other pairs of variables as described in Figure 4.5.11.

2. If possible, determine protein concentrations in samples to be precipitated (see UNIT 3.4).

Usually if stacking and entanglement ligands are to function, proteins need be adjusted in pH in such a way that proteins of 20- to 60-kDa molecular weight carry a Z_{H^+} of -15 to 40 net cationic charges. If protein molecular weights are unknown, assume -40,000 Da, or assume the molecular weights typical of the kinds of proteins being worked with (e.g., common microbial and digestive proteases range from 20 to 35 kDa, and coagulation proteases from 50 to 75 kDa).

3. Determine isoionic point for the protein and optimal pH for precipitation.

Expect to adjust the pH of some samples to between 2.5 and 5 if information from titration curves or amino acid analysis is not available for predicting Z_{H^+} . Preferably this is done

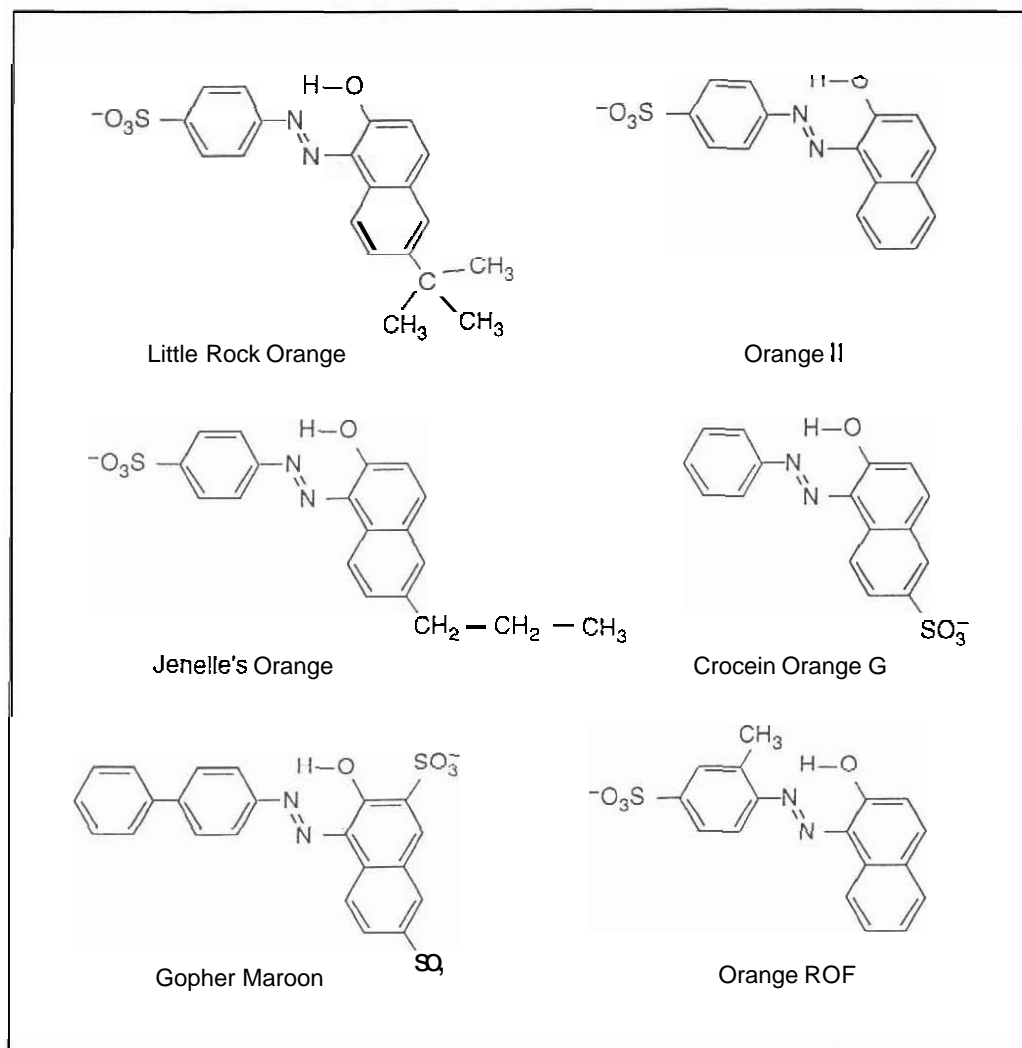


Figure 4.5.10 Anionic ligands synthesized with reinforcing alkane groups (Little Rock Orange and Jenelle's Orange) are very strong protein coprecipitants. Commercially available dye anions—Orange II, Crocein Orange G, and Orange ROF (available from Aldrich)—frequently are also efficient coprecipitating ligands for cationic proteins in pH ranges 2 to 4 units below the isoionic pH of the protein.

by direct **acidification** with **0.01 M HCl** accompanied by **pH** monitoring. **Alternatively, buffers—e.g., glycine or formate buffers—are recommended. Do not adjust protein pH into acid regions before adding the ligands unless there is a need for protection by ligands in control experiments. If sought-for proteins are fragile at acid pH, ligands should be added before titrating the proteins to the lowered pH. Control (contrast) experiments may use the opposite sequence—i.e., acidify first and add ligand second. If there turns out to be much difference in the results obtained via the two sequences, protection of proteins from acid-pH denaturation is indicated.**

- Experiment with two or three ligands from Figure 4.5.10 or choose other potential ligands from among the **azoaromatic** sulfonate dyes listed in the **Aldrich** catalog. Prepare ligand stock solutions.

Ligands are preferably dissolved in water or in dilute buffer at the running pH. Stay below the limit of ligand solubility, which can vary considerably, in making stock solutions. Upper solubility limits for a number of such ligands are 0.01 to 1 mM.

- Add ligands from ligand stock solutions to protein sample in various volume ratios—e.g., **1 vol** stock solution to **2 vol** protein solution and vice versa. Briefly vortex the mixtures.

Extreme ratios—e.g., 10 vol of ligand solution to 1 vol protein samples—should be avoided as these will dilute the protein too much. If possible, arrange for modest mutual dilutions of ligand and proteins on mixing.

- Optimize temperature, carrying out initial investigations at room temperature and proceeding to higher or lower temperatures as needed.

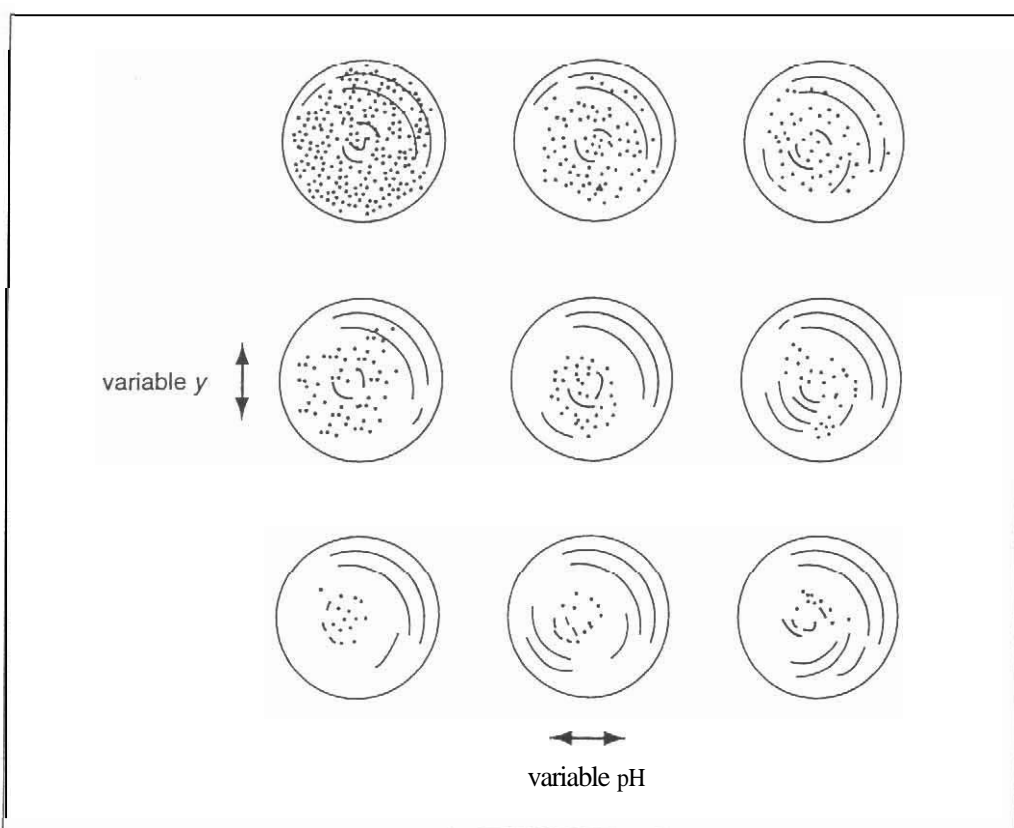


Figure 4.5.11 Sets of small test tubes or plastic microcentrifuge tubes, 2 to 3 ml in volume, may be used to explore optimum conditions for matrix-ligand/protein mutual coprecipitation by varying pairs of important parameters. Five principal variables determining coprecipitation are: kind of ligand; ligand-protein ratio (y ; often between 2 and 20); initial pH; temperature; and concentrations of auxiliary coprecipitating agents such as Zn^{2+} (10^{-3} to 10^{-4} M).

7. Determine rate of precipitation giving the optimal yield.

Matrix-ligand coprecipitation is sometimes rate-limited when protein concentrations start from <0.1% (w/v). If this is the case, incubate ligand/protein mixtures for a few hours. Rather often, coprecipitation occurs rapidly; however, to avoid including unwanted compounds in coprecipitates, do not drive the coprecipitation reaction too rapidly (if possible).

8. Collect coprecipitates by centrifugation at 5 to 50 × g. Perform bioassay of the coprecipitates and, if necessary, of the supernatant for the protein of interest.

In some systems, e.g., pemxidase isolations from crudes, myriad unwanted proteins precipitate out first and the pemxidase enzyme is left in the supernatant.

9. Redissolve coprecipitates by shifting pH upward with 0.1 M acetate, imidazole, or phosphate buffers, pH 5 to 7. Add ~100 mg of Dowex-1Cl⁻ resin and incubate 5 to 15 min with gentle shaking to trap the anionic ligands. Monitor the completeness of trapping via the color of the matrix ligand.

Approximately 100 mg of resin per milliliter of the higher-pH redissolving buffer provides a good excess of trapping capacity. See Basic Protocol 2 for discussion of the capacities of anion exchange resins. Dower-1 resins have ~1 meq of capacity per ml (wet volume) of resin beads. Complete trapping is easily followed from matrix ligand's color. Released proteins in solution should be colorless.

10. Measure total protein (and total released activity if an enzymatic or other bioassay is available) to determine specific activity, total recovered activity, and recovered protein for comparison to samples before coprecipitation.

SELECTIVE PRECIPITATION USING DI- AND TRIVALENT METAL CATION PRECIPITANTS

Di- and trivalent metal cations—e.g., Zn²⁺, Mn²⁺, Ca²⁺, and Al³⁺ (general symbol M²⁺ or M³⁺)—provide two means for precipitating proteins: (1) they may act directly, or (2) frequently they are useful as auxiliary agents for other precipitation methods such as the crowding method using neutral polymers (see Basic Protocol 5) or matrix-stacking ligand coprecipitation (see Basic Protocol 9). This technique is reviewed by Rothstein (1994) and Gurd and Wilcox (1956). Zinc ions are the ions most commonly used for direct precipitation and as auxiliary agents, especially when working with plasma and coagulation proteins (Cohn et al., 1950). Zinc ions are required for insulin crystallization, including large-scale industrial production of this polypeptide.

The discussion below represents a small sector of the landscape of chemical behavior in regard to metal cations. Metal cations provide a diverse, largely undeveloped means of protein precipitation. The intrinsic solution behavior of metal ions (without proteins), especially their chelation-coordination reactions, must be taken into account for application of such ions to the development of protein precipitation methods.

Materials

Analytical Reagent (AR)-grade metal salts (generally as chloride or nitrate)
Crude protein solution of interest, particle-free (see discussion of Clarification in Strategic Planning)
Appropriate buffer(s)

1. Determine optimal buffering system.

The precipitation system may be buffered in two respects: (1) toward H⁺ ions and pH in the conventional way and (2) also with respect to metal ions, using metal ion buffers which are coordinaring and chelaring agents (Perrin and Dempsey, 1974). A number of com-

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pounds—e.g., EDTA, citrate, and several amino acids—can serve both buffering purposes. Evidence that **compounds serve** as one or both of these types of buffers is that **pH dependency and/or metal ion concentration dependency** of protein precipitation is rather sharply dependent on the kind of buffering compound. **Zwitterionic** buffers such as HEPES **may** be useful for controlling pH because they have much less tendency to bind or coordinate with metal ions than conventional Sorenson buffers (e.g., phosphate). Because proteins are also buffers, their self-buffering action may suffice to control pH if present in concentrations 20.3%.

2. Select an appropriate metal cation and precipitate protein.

Di- and trivalent metal cations (M^{2+} and M^{3+}) are vigorously precipitated by salts and inorganic buffer anions, which represent in protein extracts and samples at various stages of isolation. Sulfate (from salting out; see Basic Protocol 1 and Alternate Protocol) powerfully precipitates Ca^{2+} as $CaSO_4$. Solubilities of <100 mg/ml or even <10 mg/100 ml are seen with $Zn(NH_4)PO_4$, $Mn_3(PO_4)_2 \cdot 7H_2O$, $MnCO_3$, $Mg(NH_4)PO_4 \cdot 6H_2O$, $MgCO_3$, $CaCO_3$, and a number of calcium phosphates. Sulfates and carbonates of Ba^{2+} are extremely insoluble (Hogness and Johnson, 1954). Before committing a metal ion as a precipitating agent: (1) review general behavior with respect to **precipitability** by buffer and salt anions in the protein solvent; (2) carry out control experiments with protein-free samples to find if the solvent(s) are likely to produce massive precipitates of simple inorganic compounds with the metal ion; and (3) arrange to remove **interfering** anions if the anions are in concentrations ≥ 0.05 M or if concentrations of ions such as SO_4^{2-} and CO_3^{2-} are <0.05 M.

Trivalent metal ions such as Al^{3+} and Fe^{3+} form a complicated series of **oxo-**, hydrate, and **hydroxylated** species in water: They go on to form insoluble hydroxides (solubility products 10^{-30} to 10^{-40} ; Hogness and Johnson, 1954), at neutral and even somewhat acidic pH. Metal cations of this kind are interesting protein precipitants (Gurd and Wilcox, 1956). However; instead of **complexing** with protein molecules, they may form very insoluble **hydroxyl gels and flocculate proteins** by adsorption on the gels at $pH \geq 5$. Hence development of protein precipitation with such cations may need be confined to rather acidic pH regions.

It is not necessarily a disadvantage if **low-molecular-weight** salts or other solvent components precipitate M^{2+} first, before proteins are precipitated. Inorganic precipitates of **sulfate**, phosphate, and carbonate **may form** first. These can then be centrifuged away, whereupon more metal ions are added to bring down the sought-for protein, in a second stage of precipitation. Total protein measurements (UNIT 3.4) are used to locate the starting point for protein precipitation by the metal ions.

3. Analyze precipitate.

Analysis of metal content of coprecipitates and their supernatants is useful for characterizing products and following how variations in procedures determine product composition. The most generally accessible **methods are spectrophotometric, using color-developing reagents** capable of quantitating metal cations at 10^{-4} to 10^{-5} M concentrations (Sandell and Onishi, 1978). Figure 4.5.12 shows a calibration curve for Zn^{2+} analysis using pyridylazoresorcinol (PAR; Aldrich). The optimal pH for use of PAR is ~ 7 (Cheng et al., 1992) in HEPES or other nonchelating buffer. 0.1 mM PAR is prepared in 0.05 M HEPES buffer; pH 7.0, then dilutions of Zn^{2+} (as zinc acetate) are prepared in the same buffer at various concentrations ranging from 5 to 30 μ M. The PAR reagent may be made up as a 1 mM stock solution and diluted 10-fold to provide the working reagent at 0.1 mM. Spectrophotometric samples are made by mixing 1.0 ml of 0.1 mM PAR with 1.0 ml of each of the Zn^{2+} dilutions. A_{495} is then measured versus a reference of equal PAR concentration without zinc. The slope of the plot in figure 4.5.12 is the molar extinction coefficient, $\epsilon_{495} = 4.5 \times 10^4$ M $^{-1}$ cm $^{-1}$. An example of Zn^{2+} release from a zinc enzyme monitored by PAR color development is given in Hunt et al. (1985). Zinc analysis is a criterion for the identification and purity of insulin.

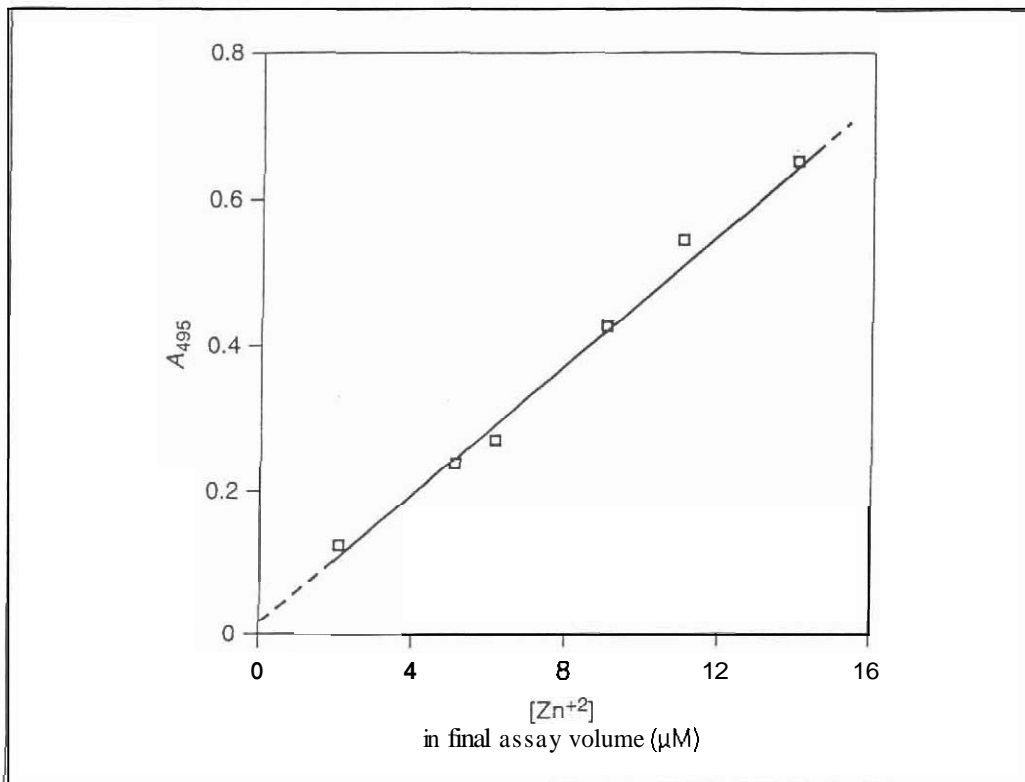


Figure 4.5.12 Calibration plot for zinc ion spectrophotometric analysis using pyridylazoresorcinol reagent.

4. Remove metal ions from precipitate.

Metal ions may be removed from protein precipitates via cation-exchange resin trapping, or by treating precipitates with a buffer at a pH at which it is expected to dissolve. It is preferable to mix the precipitate with a release buffer first to see if in fact it does dissolve, then test the resin-bead exchanger afterwards. Trapping resins in the Na⁺ or K⁺ form may be tested; conventional cation resins such as Amberlite IR-120Na⁺ usually give adequate results. A specialty chelating resin—Chelex 100 exchange beads (Bio-Rad)—may also be used. The exchange capacities of such resins are 1.8 and 0.4 meq (milliequivalents) per milliliter of wet resin. The resins should not be used in great excess (i.e., >5× resin equivalents per estimated equivalent of metal to be removed) because such resins may also adsorb proteins. Exchange of metal ions out of precipitates may be accelerated by conventional soluble chelating agents (e.g., 10⁻⁴ M EDTA) or by amino acids such as aspartic acid in the case of Zn²⁺ (Fiabane and Williams, 1977).

5. Perform bioassay for protein of interest.

COMMENTARY

Background Information

In practice, most protein-precipitating agents and methods yield a variety of different products depending upon conditions such as pH, metal ions present in the solution, and/or presence of cosolvents. Many "precipitates" are actually coprecipitates in which molecules of the precipitating agent remain bound to the proteins. How much precipitating agent or ligand becomes bound and thus coprecipitated usually remains unclear until the coprecipitate is analyzed. Precipitation and coprecipitation

are most important in "upstream" stages of protein isolation—for crude, dilute, solutions in large volumes up to several liters. However, precipitation is sometimes useful downstream also, as it reduces volume—e.g., excess solvent and buffers may be removed from chromatographic eluates via this technique. Sometimes it is an efficient strategy to precipitate unwanted proteins in the first stages of purification and then capture the sought-for protein in a second precipitation step later.

Some precipitation methods are old, and

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some have been developed more recently. Some of **the old** methods, particularly salting out with ammonium sulfate (which is more than 130 years old), remain as useful as ever. Ammonium sulfate offers the ability to protect sensitive proteins and may be scaled up at a reasonable cost. Some precipitating and **coprecipitating** agents yield precipitates that readily reverse. Reversibly acting agents can be **stripped** away **later**—e.g., ammonium sulfate salting out is reversed by dialysis, pressure filtration, or resin exchange to remove sulfate. Other agents, despite their great powers in precipitating, may be difficult to remove completely, or may catalyze damage to proteins (e.g., **sulfhydryl** oxidation). Consequently, precipitating agents must be evaluated in relation to later steps that may have to be used to compensate for the disadvantages that some strong precipitating agents carry with them. Sometimes the optimal precipitating agent is a weaker rather than a very strong agent, if the weaker agent is more easily dissociated, trapped out, or dialyzed away to release the sought-for protein.

Besides the agents and individual methods listed in Table 4.5.1, combinations of methods are frequently advantageous. For example, sometimes it is helpful to add modest concentrations of Zn^{2+} to proteins that one is **trying** to precipitate by another method. **Another precipitation** technique, TPP (see Basic Protocol 4), is also a "hybrid"—a **combination** of organic cosolvent precipitation and sulfate **kosmotropic** precipitation. Precipitation and **coprecipitation** are often quite protective of **proteins**, especially in salting-out techniques. However, whether or not protection is achieved frequently depends on the sequence of steps used in each protocol. For example, if salting out or coprecipitation with a particular ligand needs to be **carried** out at a pH very different from the starting point, a choice must be made between shifting pH first and then adding salt, or alternatively adding salt or ligand first and then readjusting pH. The latter is usually best choice in terms of protecting the protein.

Critical Parameters and Troubleshooting

Salting out

In the simplest version of salting out, desired proteins are precipitated relatively early and the supernatant is set aside or discarded. In this case, unwanted proteins are often left in the supernatant. The supernatant may, however, be readjusted in pH, increased in salt concentra-

tion, or both, to bring down yet other proteins, perhaps the sought-for protein, in a second stage or "second cut" of salting out. Capture of **sought-for protein** activities in second **and even** third stages or cuts, with intervening pH shifts, are discussed in various sources (e.g., Scopes, 1987).

In each step, or cut, the sequence of salt addition, pH **shifting**, and changes in other **parameters** (such as temperature) must be considered. The salt (ammonium sulfate) may be added before shifting other parameters, especially the pH. Alternatively, the opposite sequence may be used: adjust the pH first to **some** desired set point then add the salt. These two sequence options can have quite different effects for several reasons, stemming from the fact that **salts**, particularly ammonium sulfate in concentrations greater than -0.5 M, often serve as protein-protective agents. The **protein-protective** effects of ammonium sulfate include conformation tightening, which guards against pH-induced unfolding. Sometimes it is advantageous to impose quite acidic or alkaline pH extremes or a thermal shock, to first precipitate unwanted, denatured proteins, and leave sought-for proteins in the supernatant. However, the amount of salt and when to add it are **variables**; these need attention in weaving the optimum path for salting out.

Several treatises and textbooks present tables, formulas, and nomograms for relating percent saturation, **molar concentration**, weight of ammonium sulfate to be added, and dilution factors that are useful for increasing or **decreasing** amounts of ammonium sulfate so as to arrive at prescribed concentrations of the salt. The numerical relationships are mostly cited for 0° to $30^\circ C$, although the solubility of ammonium sulfate in water changes little. Saturated ammonium sulfate in water is close to 4.1 M. Two relationships cited by Scopes (1987) give the number of grams (g) to be added to a liter of solution at $20^\circ C$ for shifting molarity (M) and percent saturation (S). The first of these equations is for shifting to a molarity M_2 starting at molarity M_1 :

$$g = \frac{553(M_2 - M_1)}{4.05 - 0.3M_2}$$

The second equation is for shifting to a percent saturation S_2 starting at percent saturation S_1 .

$$g = \frac{553(S_2 - S_1)}{100 - 0.3S_2}$$

These relationships pertain to solutions of

ammonium sulfate in water. Proteins that are already in diverse buffers and that contain other salts, in unknown concentrations, throw off the accuracy of **nomograms** and formulas for shifting saturation percentages and **molarities**. Nevertheless, these are useful for estimating added salt concentrations to be used and **for reporting**.

Neutral salts other than ammonium sulfate, such as sodium or potassium chloride, may be tested in attempting to precipitate the **sought-for** protein (and later in attempting to split excess salt **away**—i.e., reverse the process). However, ammonium sulfate is usually the best primary choice, not because of tradition but because the sulfate anion, SO_4^{-2} , is one of the strongest yet most benign **Hofmeister kosmotropes** (*UNIT 8.4*) over nearly the whole "biological" pH range, approximately from pH 2 to 10 (Collins and Washabaugh, 1985). The orthophosphate dianion, HPO_4^{-2} is also a strong, inexpensive kosmotrope and salting-out agent. However the **pK** of H_2PO_4^- is 7.2; accordingly, below pH ~ 6.5 the H_2PO_4^- monoanion predominates even though it is a considerably weaker kosmotrope. Other anions such as fluoride, F^- , are good **kosmotropes** but are very toxic. For a combination of desirable properties, including least **expense**, the **sulfate** systems remain the most convenient, reliable, and most tested. One disadvantage of sulfate arises in the case of **proteins** requiring calcium ion, Ca^{2+} . Because calcium sulfate and calcium phosphate are extremely insoluble, both SO_4^{-2} and HPO_4^{-2} precipitate Ca^{2+} . Hence, for calcium-requiring proteins, salting out may have to be developed with salts other than **sulfates**—e.g., acetates are a possibility.

Isoionic precipitation

Amberlite resins **IR-120** (a cation exchanger) and **IRA-400** (an anion exchanger), in the form of 6% or 8% cross-linked beads, are the basis of the original **Dintzis** mixed-bed **exchangers**. The equivalent **Dowex** exchangers, Dowex 1 and Dowex 50 series, in the form of 6% to 8% cross-linked resins, serve equally well but are not superior.

Anion and cation exchangers have differing exchange capacities per settled milliliter of wet resin. **IR-120H⁺** has -1.8 meq/ml of capacity, and **IRA-400OH⁻** has -1.2 meq/ml. Accordingly each **100 ml** of mixed exchanger may be made by mixing **40 ml** of the cation exchanger in the **H⁺** form with **60 ml** of anion exchanger in the **OH⁻** form, a volume ratio inversely proportional to their intrinsic exchange capacity ratio. Mixed-bed exchangers may be stored in

the cold (do not freeze them) for long periods without self-neutralization.

Mixed-bed **exchangers**, such as the MB-I Amberlite series, may be purchased premixed. However it is recommended that the exchanger be mixed in the laboratory to ensure that resins have been "exercised" (see Basic Protocol 2, steps 2 and 3) and leached of any polymer hydrolysis products. Anion exchangers based on various quaternary **amines** are especially susceptible to self-hydrolysis (detectable by an **amine** odor) upon storage in the **OH⁻** form. Commercial ready-mixed bed resins usually incorporate dye chromogens (acid-base indicator molecules), which show (by a blue to amber color change) when their capacity has been **titrated**. Such resins may be usable with some protein molecules, but if there is any leaching out of resin materials, the eluants will be strongly colored, indicating contamination of proteins.

Two-carbon organic cosolvent precipitation

In Basic Protocol 3, no general criterion can be put forth for choosing between acetone and ethanol, for deciding how pH should be adjusted versus the isoionic point of the protein, or for **determining the exact** salt concentration to be used. **There exists a large** body of literature describing empirical, experimental means for **C₂** cosolvent-based isolation of dozens of proteins. A few guidelines may be useful. Ethanol was the **C₂** cosolvent used for the isolation of most blood-plasma proteins. Many respiratory enzymes have been isolated with a step involving an acetone cosolvent. In systems where lipids and triglycerides are troublesome, many workers have used acetone cosolvents, sometimes at nearly 100% acetone, because acetone and acetone-water mixtures extract lipid materials. Acetone in such applications functions as an extractant as well as a protein precipitating agent. Proteins and whole-tissue agglomerates that **have been rendered nearly lipid-free** by acetone, then **lyophilized**, are referred to as "acetone powders."

A review with many references was recently written by Rothstein (1994), which includes discussion of the seminal papers by Cohn and coworkers. With a number of proteins, a complex relationship exists between salts, the **C₂** organic cosolvents, the proteins, (which bind salts to some extent), and the pH. These parameters operate to make organic cosolvent precipitation quite empirical.

Methanol and the two-carbon **organic cosolvents** appear to be able to penetrate protein

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molecules and compete with water for sites inside. However C_4 and larger cosolvents are largely excluded from the inside of proteins if the proteins are kept compact and folded. Accordingly, it is unlikely that the larger cosolvent molecules, such as C_4 alcohols, behave congruently with the C_1 and C_2 cosolvents for most proteins.

C₄ and C₅ organic cosolvent precipitation

Some C_4 organic cosolvents, particularly *t*-butanol and *n*-butanol, are able precipitating and extracting agents for proteins. They are useful in "upstream" stages of isolation, starting with crude solutions. They are variously used in combination with osmolytes and salt-ing-out agents such as ammonium sulfate. Conditions are adjusted in such a way that separated layers of cosolvent are obtained, along with an aqueous layer and a layer of precipitated protein. The separated liquid layers act as extraction and partitioning systems as well as protein precipitation systems. Precipitated proteins usually appear at the interface between liquid layers. Because some of the C_4 and C_5 cosolvents act either as kosmotropes or in parallel to kosmotropes like the sulfate anion, these cosolvents frequently are protective of aqueous proteins even at normal temperatures, 20° to 30°C. They induce protein-molecule conformation tightening, from which the protein-molecule protection partly originates. The similarity between sulfate interaction with proteins and C_4/C_5 cosolvent interaction with proteins does not, however, have the same origin. Sulfate exerts electrostatic forces toward many proteins (Chakrabarti, 1993), whereas *t*-butanol exerts much less electrostatic-based interaction, perhaps none at all. A rough model of the effect of the *t*-butanol cosolvent is that it behaves like a neutral osmolyte in water.

It is not always strictly necessary to choose *t*-butanol as the cosolvent. However, *t*-butanol has some advantages. First, it is relatively inexpensive. Only -0.3 to 0.5 ml of *t*-butanol is required per milliliter of starting aqueous protein solution, after the optimal amount of salt has been added. When *t*-butanol is used, smaller amounts of ammonium sulfate are usually required for a given precipitation, relative to the amounts needed in conventional salting out. Second, *t*-butanol is much less volatile than C_2 cosolvents, especially in water-dominated systems. Hence it poses less of a fire hazard and less hazard from inhalation. Third, *t*-butanol is, in general, far less denaturing at 20° to 30°C temperatures than C_2 cosolvents. Fourth, *t*-bu-

tanol behaves similarly to kosmotropes and osmolytes, protecting proteins. Finally, the extractive and partitioning capacities of three-phase partitioning (TPP) using C_4 and C_5 organic cosolvents (see Fig. 4.5.6) tend to remove pigments, lipids, and amino acids from proteins.

C_4 and C_5 organic cosolvents in the aqueous phase produced by techniques like TPP exert themselves as osmolytes and crowding agents, up to a point, when they reach concentrations of several percent. However, the main differences between the C_4/C_5 organic compounds (of the types discussed here—e.g., butanols and pentanols) and water-soluble osmolytes such as sugars are: (1) osmolytes like sugars and amino acids tend not to form a separate organic phase that goes on to act as an extraction phase for lipids, pigments, and other nonprotein material, whereas the butanols and pentanols do; and (2) hydrocarbonaceous C_4 and C_5 compounds bind to some extent to proteins that have a high degree of nonpolar, hydrophobic character. This latter property causes such proteins to position themselves as illustrated in Figure 4.5.6, floating above the aqueous layer. When the protein precipitate (i.e., coprecipitate) is redissolved in buffer with low salt, then shifted away from the precipitative pH (usually upward -2 to 3 pH units), in most instances the C_4/C_5 compounds dissociate. In contrast, conventional osmolytes, especially sugar, tend not to bind to proteins.

In some versions of TPP, the system is operated in two stages. In the first stage, the precipitate containing unwanted proteins that forms at the initial pH is discarded. The aqueous phase is then shifted in pH, and/or more salt is added, giving a second precipitate containing the sought-for protein. In the example described in Lovrien et al. (1987), the enzyme α -amylase is isolated in this way. Many sorts of variations can be made in pH, salt concentration, and other parameters.

Protein-exclusion and crowding agents

Exclusion agents and osmolytes have high affinities for water and compete with proteins in solution for water. The crowding action, aided by any degree of affinity of protein molecules for one another (protein-to-protein association) promotes protein precipitation. Thermodynamic pushing occurs because hostile environments imposed on proteins in osmolytes and in crowded solution raises protein chemical potential in a positive direction. This is then relieved by protein precipitation, which

enables protein molecules to exist with lowered chemical potential.

Crowding and exclusion agents frequently act upon conformationally motile (loose), water-penetrated proteins before overt precipitation. Conformationally loose protein molecules are also "squeezed" on by these agents, promoting protein molecule tightening and sometimes promoting an ordered protein conformation. Proteins thus "squeezed on" often become protected, sometimes dramatically. Moreover if their conformation is forced into good order, protein molecules may crystallize instead of simply being precipitated in amorphous form. Thus, the osmolyte MPD (2-methyl-2,4-pentanediol) is often used with success for protein crystallization. MPD crowds, excludes, and squeezes to push proteins into a narrow or even single conformation necessary for arriving at the crystalline state.

As a general rule, osmolytes and crowding agents do not bind strongly to proteins. Hence, ideally, precipitated proteins are free of such agents. However in fact, some agents bind rather weakly, and appear in the precipitate (which is in fact a coprecipitate). Accordingly, selection of the agent may depend on the ease or difficulty of removing trapped agent from protein precipitates if in fact the desired redissolved protein needs to be stripped of precipitating agent.

Neutral polymers such as polyethylene glycol (PEG) and dextran, which do not affect electric charge, might be expected to act toward proteins independently of electrostatic parameters such as protein net charge and salt concentration. However, the opposite often occurs: i.e., buffer, salt, and pH-dependent protein charge frequently sharpen and either greatly enhance or sometimes depress protein precipitation via PEG. Salts in concentrations quite below normal kosmotropic concentrations—i.e., 0.01 to 0.5 M salts—have a large influence, decreasing chymotrypsin solubility and increasing its precipitation by PEG (Mieška and Ingham, 1978). In other examples, chaotropic anions and "Hofmeister-neutral" anions (e.g., chloride) increase protein solubility and depress precipitation. Readers may profit by consulting some of the papers referred to here, particularly to Mieška and Ingham (1978) to review how large and variable these "electrostatic" effects may be.

Neutral polymers, acting as steric-exclusion precipitants, and the monomers of such polymers, acting as osmolytes, are far from equivalent in protein-precipitating power when used

in equal concentrations. The polymers appear to be considerably most effective. For example, Laurent (1963) investigated dextran polymer and its monomer glucose as precipitants for albumin and fibrinogen. Dextran polymer considerably outstripped glucose in precipitation power when each reagent was used at a concentration of 0% to 10% (w/v). On the other hand, concentrations of proteins for achieving protein precipitation using neutral polymers usually must be from 1% to 5% (w/v). Hence the neutral polymers are considerably less sensitive and less effective at low protein concentrations than some of the other methods discussed in this unit—e.g., the matrix-stacked ligand technique (see Basic Protocol 9 and discussion below).

Neutral polymers are equivalent to neutral polymers used in aqueous two-phase partitioning (Walter and Johansson, 1994) and as "precipitants" in protein crystallization (McPherson, 1982). PEG is the dominant polymer used in two-phase partitioning and as a precipitant for protein crystallization. PEG polymers in several molecular-weight ranges—low, medium, and high—are available from Sigma and other suppliers. Choice of PEG molecular weights must be balanced between two considerations. Higher-molecular-weight polymers tend to be more effective precipitants for some proteins (i.e., able to precipitate albumins out of solution in 5% to 15% PEG concentration ranges: Atha and Ingham, 1981). Lower-molecular-weight PEG polymers of molecular weight <3000 to 4000 generally must be used in 20% to 50% concentrations to effectively precipitate a number of proteins that have been studied. However, the practical problem of ridding precipitated proteins of trapped PEG polymers is eased with low-molecular-weight PEGs because they dialyze away relatively easily. Proteins generally are more sensitive to precipitation by high-molecular-weight PEGs than low molecular-weight-PEGs, frequently precipitating, if indeed they do so, in 3% to 20% concentrations of large PEGs (molecular weight greater than 4000; Atha and Ingham, 1981). The downside of high-molecular-weight PEGs is that, if trapped, these PEGs become difficult to separate from coprecipitated proteins by simple dialysis. Also, high-molecular-weight PEG solutions tend to be quite viscous, whereas oligomeric low-molecular-weight PEGs are easier to handle and mix.

Alkane ether polymers (trade name Ucon; Union Carbide: see SUPPLIERS APPENDIX), which are copolymers of ethylene and propylene ox-

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ide related to PEGs, go into and out of solution with marked temperature dependency (i.e., they "phase-separate"; Albertsson and Tjerneld, 1994). Accordingly, with the use of such polymers, manipulation of temperatures in initial precipitation and in redissolving protein precipitates may afford means for balancing these two problems.

Synthetic and semisynthetic polyelectrolytes

Polyelectrolytes have been synthesized as copolymers with nonionic groups to help alleviate problems that arise with homopolymers such as polyacrylic and polymethacrylic acid. The latter homopolyelectrolytes, when ionized, are so strongly coprecipitating that they are often poorly selective. Hence, copolymers of acrylic acid and methacrylic acid with a nonionic methylmethacrylate ester side function as agents with softened coprecipitating power but increased selectivity. A series of copolymers of this type, with varying ratios of ionic and nonionic groups, are named "Eudragit" agents, and are available commercially from Rohm GmbH (see SUPPLIERS APPENDIX and Mattiasson and Kaul, 1994). Copolymerization of two and even three separate vinyl monomers provide polyampholytes of myriad kinds, differing in ratios of vinyl monomers. Synthetic polyampholytes take on net positive, net negative, or zero charge, depending on the pH (like proteins, which are also polyampholytes). The advantage of such compounds is that after coprecipitating proteins, the synthetic polyampholyte can be split away from the protein by adjusting the pH of the mixture to the isoionic pH of the polyampholyte, which will precipitate it separately (Patrickios et al., 1994). Many of the polymers that have been discussed so far—e.g., carboxymethylcellulose and polymethacrylic acid anion—are negatively charged above the pK of their side-chain carboxyl groups, -pH 5. Such polymers should primarily be used for precipitating proteins that are cationic—i.e., in pH ranges below the target protein's isoelectric point. However, it may be necessary to experiment with synthetic polyelectrolytes which are cations and which are therefore primarily aimed at precipitating anionic proteins in pH ranges impractical for synthetic polyanions. The most common, readily available neutral-cationic synthetic polyelectrolyte of this type is polyethyleneimine (PEI). The H⁺-titratable nitrogen groups of PEI are integral parts of its backbone, not relegated to side chains. PEI is very basic and exists as a polycation over a very wide range of pH. PEI is also a powerful copre-

cipitating agent for proteins. Table 4.5.2 lists a small number of polyelectrolytes, several of which are sold by Aldrich and Sigma. References in Table 4.5.2 should enable the reader to survey conditions used for coprecipitation of various enzymes.

Metallic and polyphenolic heteropolyanions

There is considerable variability but lack of much quantitative information concerning recovery and bioactivity measurements of most proteins that have been precipitated with heteropolyanions, in cases where their activities must be recovered. The great acidity and also the oxidizing power of some of these agents—e.g., HClO₄—tends to seriously denature many proteins. Deamidation of proteins sometimes follows strong acidification, as well as unwanted hydrolysis and conformational unfolding. However, weaker acids such as tannic acid (a mixture of several phenolic products) are less denaturing, and yield intact active enzymes and other proteins after tannate precipitation at neutral pH. Accordingly, choosing among heteropolyanion precipitation agents depends on the use to which the protein precipitates are to be put. For very rapid, complete precipitation of unwanted proteins down to -50 µg/ml (Bensadoun and Weinstein, 1976), in cases where destruction of proteins is acceptable, HClO₄ in 1% to 5% concentration is used. Picric acid is almost equally effective, but it is highly colored and hence interferes in analytic procedures requiring spectrophotometric measurement. As with polyelectrolyte precipitation, which agent to use may depend on the ease versus difficulty of removing such agents from proteins after coprecipitation.

Examples of precipitation of diverse proteins by heteropolyanions at mildly acidic pH (i.e., pH 4 to 6) are described in two papers. Sternberg (1970) coprecipitated ovalbumin, hemoglobin, pepsin, and amyloglucosidase from protein crudes using four heteropolyanions. Sternberg removed the inorganic anions by dialysis, ultrafiltration, and ion exchange, recovering fair to good activities in some cases. Astrup et al. (1954) worked with six heteropolyanions in staged purification of serum proteins including α- and γ-globulins.

Tannic acids are polyphenolic compounds (sometimes conjugated with one or more saccharides) from plant extracts. They are quite diverse in structure, depending on the plant source. Sigma sells a crude tannic acid that is convenient to start with. An advantage to using tannates as precipitants is that they function in

neutral pH regions, pH -4 to 8. Hence, severe acid and hydrolytic conditions, often necessary with the metallic heteropolyanions, may be avoided with tannates, which are all-organic anions. Little detail is known concerning how tannate precipitation works. **Tannates are sometimes effective in precipitating proteins on a broad front, and inclusively, but in some systems fail to precipitate proteins to which they are bound (Hagerman and Butler, 1991). Amounts of tannic acid and of tannate needed for precipitation of diverse proteins range from approximately half as much by weight as the sought-for protein, down to quite smaller amounts of tannin; these quantities are also quite dependent on pH. Hagerman and Butler (1978, 1991) survey principal parameters for controlling precipitation of individual proteins such as albumin and lysozyme. They recommend quebracho tannin and commercial tannic acids as initial agents for attempting precipitation of mixtures of proteins. Although there are gaps in the precipitating power of tannate compounds for proteins, they provide alternatives for scaleable, reversible procedures under conditions where the metallic heteropolyanions (strong acid) are too severe. The strong spectrophotometric absorption spectra of tannins when reacted with Folin phenol reagent or with ferric chloride, both of which generate characteristic colors for phenols, afford convenient means for quantitating tannates in coprecipitates and in soluble remnants (Hagennan and Butler, 1978).**

Hydrophobic ion-pairing entanglement ions

It may seem surprising that ligands such as those used in Basic Protocol 8—especially detergents—are efficient precipitating agents; these reagents are normally used in solubilizing and denaturing proteins. Detergent anions (i.e., dodecyl sulfate) acting as protein coprecipitants were first studied at length by Putnam and Neurath (1944) for seven proteins. Since then, the detergent/protein and subsequent complex/complex association reactions have sometimes been called hydrophobic ion-pairing reactions. A rather strict detergent-anion to protein-cation association occurs in 1:1 stoichiometry to form ion pairs between charged groups; simultaneously an alkane/alkane association occurs between the hydrophobic tails. The two sets of forces strongly pull the complexes together and out of the solution to agglomerate and form the visible coprecipitate. Protein-molecule to protein-molecule association may also occur, but there are rather few

detailed studies of this. The ion/ion association, which is the electrostatic component of HIP, is dependent on and adjusted by the pH, because the pH and the number of protein H⁺-titratable side chains determine the number of net cationic charges on the global protein molecule—i.e., Z_{H⁺}. This, in turn, determines the number of ligand anions expected to bind when coprecipitates are formed. Because strong anions from detergents usually bind strongly, there is seldom a need to add extra ligand (detergent) to provide for large amounts of free (unbound) ligand. Between 60% and 95% of the total detergent anions added under the aforementioned conditions are likely to be bound for 10-carbon, 12-carbon, and larger detergent sulfates. However, short-chain detergent anions—e.g., hexyl and octyl sulfates—are usually poor HIP precipitants.

Matrix-slacking ligand coprecipitation

In organic anion-matrix ligands, the rigid azoaromatic sulfonates are able to stack their tail groups to force association and coprecipitation. These reagents have several similarities to flexible detergent coprecipitating anions (HIP agents; see discussion above). As in the case of the flexible detergents, matrix-ligand coprecipitation is sharply dependent on protein electrostatic charge, thence on pH, on protein side-chain composition, and on the isoionic point. In contrast to the detergent anions, whose coprecipitating powers are mainly dependent on alkane-chain length, matrix-stacking ligands are dependent on organic structural detail and placement of groups, not simply on size. The placement of short alkane "reinforcement" substituents and the location of sulfonate anions on aromatic rings, as well as the location of tautomerizing hydroxyl groups, make stacking-ligand structural character more complicated and unpredictable than that of flexible-detergent ligands. In practice, one may need to experiment with a variety of matrix ligands to find an optimal kind for selectively coprecipitating each protein.

It appears that matrix-stacking ligands need their hydrophobic tail groups to extend some distance away (greater than -10 to 15 Å) from the sulfonated head group (Matulis and Lovrien, 1996). This requirement presumably maximizes tail-tail stacking and hydrophobic association, distancing them so as not to interfere with sulfonate group-protein cation association. In any case, functions of matrix ligands are sensitive to placement of substituents such as alkane groups on the azoaromatic rings, as

well as the number of such substituents. In practice, one expects to initially experiment with -3 to 5 such ligands to find a ligand suitable for investigation in more detail. **Because matrix ligands generally are quite protective of proteins with which they coprecipitate, it may not be necessary to always develop the coprecipitation stage of protein isolation at cold temperatures. Indeed, the method often interfaces well with a heat-shock step to clear out unwanted proteins susceptible to heat shocking. Pilot experiments therefore can generally be carried out at ordinary room temperatures.**

Matrix ligands strongly coprecipitate proteins. However, overall isolation processes for most proteins usually need an exit. The protein and ligand should be released from one another by resin-exchange trapping as described in Basic Protocol 2. Hence, it may occur that somewhat more weakly coprecipitating, but more easily released ligands may be optimal in the procedures, so as to allow proteins to dissolve, ready for assay or the next isolation steps..

Di- and trivalent metal cation precipitation

Metal cations exert themselves in three ways to precipitate proteins. First, their charges add to protein molecule **net charge, enhancing** binding of coprecipitating ligand ions such as matrix or detergent sulfate anions. Second, metal ions draw in protein side chains and **lysine** amines, as well as carboxylate and **histidyl** side chains, to fill metal ion orbitals with their electron pairs. Protein molecules thus act as large chelating agents in a manner analogous to the behavior of EDTA and other multidentate chelators and become tightened and sometimes protected because of the chelation to **M²⁺**. Finally, **M²⁺ and M³⁺ ions can become complexed with buffer ions and other electrolytes chosen by the researcher. When that happens, actual net charge and remaining metal ion ligand positions available to the protein may be adjusted to modulate protein precipitation. Metal ion complexes of M²⁺ and M³⁺ may actually be made neutral, or even anionic, depending on presence of chloride, acetate, or citrate, and their concentrations (in the 10⁻³ to 10⁻¹ M range); this phenomenon is also dependent on pH. Cotton and Wilkinson (1988) cite Zn(H₂O)₆⁺², ZnCl(H₂O)₅⁺¹, ZnCl₂(H₂O)₄, ZnCl₄⁻², and ZnCl₄(H₂O)₄⁻² as examples. Cobalt and a number of other transition metal cations complex with ammonia and water molecules to make a series of coordinate cations of considerably larger size than the parent Co²⁺ but which still carry the central metal ion's**

charge. On the other hand, addition of CN⁻ or SCN⁻ generates a series of complexes such as Fe(CN)₆⁻³ which are strong, very stable anions. A number of metal **cations—e.g., Ni⁺² and Zn⁺²—are** capable of switching configuration from square planar to tetrahedral to octahedral, depending on coordinate ligands, chelating agent, and solvent conditions. Some metal cations useful in protein precipitation are amphoteric, switching water molecules and **OH⁻ ligands** into and out of metal orbital positions depending on pH. Metal cations such as **Al³⁺** strongly bind **oxo-ligands**, including water molecules and hydroxyl (OH⁻) to form metallic gels that settle or centrifuge down and capture proteins by adsorption (Collingwood et al., 1988). This mode of precipitation is often called flocculation. Flocculated products tend to have uncertain composition, but have the advantage of being dense and separable by low-speed **centrifugation** or simply by settling.

If the choice of metal ion is otherwise not obvious, the first choice as protein **coprecipitant** is the zinc ion. Zn²⁺ is of intermediate strength in chelation-coordination reactions with respect to the kinds of ligands offered by mine, carboxylate, and imidazole side chains. Therefore it can be **"reversed"**—i.e., removed from coprecipitates. Zn²⁺ rather rapidly exchanges ligands that coordinate with it (Berg and Shi, 1996). More importantly, zinc ions do not promote electron transfer and oxidation-reduction reactions, whereas many of the transition **metals—e.g., iron, copper, and nickel ions—indeed do so. Hence Zn²⁺ is fairly safe to use for precipitating proteins with sensitive sulfhydryl (-SH) groups. Other metal ions—e.g., Cu²⁺ and Ni²⁺ promote oxidation, usually irreversibly, and therefore can be rather destructive. Zn²⁺ in the form, for example, of zinc chloride, is also the probable first choice to use as an "assist" agent in conjunction with other precipitants. Atha and Ingham (1981) show excellent examples of the ability of ZnCl₂ to decisively decrease the concentration of PEG crowding agent necessary to precipitate out human serum albumin from a 0.5% solution. Zn²⁺ appears to tighten the albumin molecule's conformation, easing PEG's task in precipitating the protein.**

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