Chapter 1

Preparation for Protein Isolation

I. Introduction

This book is devoted to laboratory techniques for the analysis and separation of proteins. Proteins are an extremely heterogeneous class of biological macromolecules. They are often unstable when not in their native environment, which in itself varies considerably among cell compartments and extracellular fluids. Of the many types of proteins, we can distinguish between those that are soluble or membrane-bound, those with catalytic or purely structural roles, and those with various post-translational modifications.

Each protein may have specific requirements once it is extracted from its normal biological milieu. If these requirements are not satisfied, the protein can rapidly lose its ability to carry out specific functions, and an already limited lifetime may be drastically reduced. Thus, determination of these requirements has often been a major hurdle in protein characterization. In some cases, the difficulty has been to stabilize the protein against external proteolysis, while in other cases the problem has been to maintain ligand-binding or enzymatic activity. Solutions to these problems are highly individual. Nonetheless, some fundamental parameters must be considered by anyone studying proteins. In this chapter, we discuss a number of these parameters and attempt to provide general guidelines or sources of information for laboratory work with proteins.

II. Buffers

A. Buffer Characteristics

- A buffer is defined as a mixture of an acid and its conjugate base which can reduce changes in solution pH when acid or alkali are added. The selection of an appropriate buffer is important in order to maintain a protein at the desired pH and to ensure reproducible experimental results. A rudimentary description of key concepts behind buffering, such as pH and pK_a, can be found in the Calbiochem "Buffers" booklet and in Stryer (1988, pp. 41-42).

- There are eight important characteristics to consider when selecting a buffer (adapted from Scopes, 1982):
  1. pK_a value (see Table 1.1)
  2. pK_a variation with temperature
  3. pK_a variation upon dilution
  4. Solubility
  5. Interaction with other components (such as metal ions and enzymes)
  6. Expense
  7. UV absorbance
  8. Permeability through biological membranes

- Some General Observations

  1. Ideally, different buffers with a similar pK_a should be tested to determine whether there are undesired interactions between a certain buffer and the protein under investigation (Blanchard, 1984).

  2. Once a buffer is chosen, it is best to work at the lowest reasonable concentration to avoid nonspecific ionic strength effects. A 50 mM buffer is a good starting point.

3. The useful buffering range diminishes significantly beyond 1 pH unit on either side of the pK_a. Note that many enzymes are irreversibly denatured at extreme pH values (Tipton and Dixon, 1979).

4. The physiological pH in most animal cells is 7.0 - 7.5 at 37°C. Due to the effect of temperature, this value rises to close to 8.0 near 0°C (Scopes, 1982).

5. The buffer of choice also depends on the methods employed:
   - For gel filtration chromatography, almost any buffer can be chosen that is compatible with the protein of interest.
   - For anion exchange chromatography, cationic buffers such as Tris are preferred.
   - For cation exchange or hydroxyapatite chromatography, anionic buffers such as phosphate are preferred (Blanchard, 1984).

6. 'Good's' buffers (for example, MES, PIPES, MOPS) were developed by Good and colleagues (1966) to be biologically inert, to have low UV absorbance, and to be minimally affected by temperature or ionic strength.

7. Buffer mixtures with wide buffering ranges at constant ionic strength are described by Ellis and Morrison (1982).

8. A description of buffers and cryosolvents for low temperature conditions is found in Fink and Geeves (1979).

9. All chemical products should be reagent grade or higher.

B. Preparation of Buffers

- In principle, the pH of a solution can be adjusted directly at the temperature at which the buffer is to be used. However, this requires that the pH electrode be standardized at the working temperature, often 4°C, while in practice, buffer is usually prepared at room temperature and the pH adjusted so that it will be correct after the solution is brought to the desired temperature. It should be noted that temperature effects on buffer pH may be large. A notable example is Tris, which has a pK_a that changes from 8.06 at 25°C to 8.85 at 0°C [Blanchard, 1984]). An experimental solution should be tested for its pH after all the components (e.g. EDTA, DTT, Mg^{2+}) have been added since the pH may change after their addition.

- Unless other instructions are given, assume that the pH of a buffer is adjusted down with HCl and up with either NaOH or KOH.

- The basicity of tetramethylammonium hydroxide is equivalent to NaOH or KOH. Tetramethylammonium hydroxide should be used in adjusting the pH of a buffer for a reaction which requires the complete absence of mono-, di-, or trivalent metal ions (Calbiochem "Buffers" booklet).

- By convention, the molarity of a buffer corresponds to the acid component. Thus, 1 M Tris-acetate, pH 4.8 means that 1 M acetic acid is titrated with about 0.5 M Tris base to give the final pH. However, in common usage, just the opposite is often intended: a solution of Tris base may be titrated with acetic acid. Or, even more confusingly, Tris-HCl (the "acid" form) may be titrated with acetic acid, resulting in a three-component system of Tris, chloride, and acetate. Be aware of this potential for ambiguity when trying to duplicate recipes from the literature.

- If both protonated and unprotonated forms of a buffer are readily available, solutions of the two forms at the same concentration can be mixed until the desired pH is obtained. It is preferable to use established tables or calculations for mixing components, although verification with a pH meter is always advisable as in the following example:

Preparation of a phosphate buffer between pH 5.8 and 7.8 (Calbiochem "Buffers" booklet):

Stock Solution A (0.2 M NaH_2PO_4):

Dissolve 27.6 g NaH_2PO_4 to make 1 liter in deionized water.

Stock Solution B (0.2 M Na_2HPO_4):

Dissolve 28.4 g Na_2HPO_4 to make 1 liter in deionized water.

pH	% A	% B	pH	% A	% B
5.8	92.0	8.0	6.9	45.0	55.0
5.9	90.0	10.0	**7.0**	**39.0**	**61.0**
6.0	**87.7**	**12.3**	7.1	33.0	67.0
6.1	85.0	15.0	7.2	28.0	72.0
6.2	81.5	19.5	7.3	23.0	77.0
6.3	77.5	22.5	7.4	19.0	81.0
6.4	73.5	26.5	**7.5**	**16.0**	**84.0**
6.5	**68.5**	**31.5**	7.6	13.0	87.0
6.6	62.5	37.5	7.7	10.5	89.5
6.7	56.5	43.5	7.8	8.5	91.5
6.8	51.0	49.0			

Note: pH values are approximate and will vary according to exact temperature and the accuracy at which components are mixed and measured. Therefore, they should be verified with a pH meter. However, pH electrodes themselves are subject to significant variability. Thus, for maximum reproducibility, it is best to adjust pH by adding known amounts of each component, rather than titrating each time with a pH meter.

Table 1.1
pK_a Values of Common Biological Buffers

Trivial Name	Buffer Name	pK_a*
Phosphate (pK_{a1})		2.15
Citrate (pK_{a1})		3.06
Formate		3.75
Succinate (pK_{a1})		4.21
Citrate (pK_{a2})		4.76
Acetate		4.76
Pyridine		5.23
Citrate (pK_{a3})		5.40
Succinate (pK_{a2})		5.64
MES	2-(N-Morpholino)ethanesulfonic acid	6.15
Cacodylate	Dimethylarsinic acid	6.27
Carbonate (pK_{a1})		6.35
BIS-Tris	[Bis-(2-hydroxyethyl)imino]tris(hydroxymethyl)methane	6.46
ADA	N-2-Acetamidoiminodiacetic acid	6.59
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)	6.76
BIS-Tris propane	1,3-Bis[tris(hydroxymethyl)methylamino]propane	6.80
ACES	N-2-Acetamido-2-aminoethanesulfonic acid	6.90
Imidazole		6.95
MOPS	3-(N-Morpholino)propanesulfonic acid	7.20
Phosphate (pK_{a2})		7.20
TES	2-[Tris(hydroxymethyl)methylamino]ethanesulfonic acid	7.50
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid	7.55
HEPPS (EPPS)	N-2-Hydroxyethylpiperazine-N'-3-propane-sulfonic acid	8.00
Tris	Tris(hydroxymethyl)aminomethane	8.06
Tricine	N-[Tris(hydroxymethyl)methyl]glycine	8.15
Glycylglycine		8.25
Bicine	N,N-Bis(2-hydroxyethyl)glycine	8.35
TAPS	3-{[Tris(hydroxymethyl)methyl]amino}propane-sulfonic acid	8.40
Borate		9.23
Ammonia		9.25
CHES	Cyclohexylaminoethanesulfonic acid	9.55
Glycine		9.78
Carbonate (pK_{a2})		10.33
CAPS	3-(cyclohexylamino)propanesulfonic acid	10.40
Phosphate (pK_{a3})		12.43

* Values from Calbiochem "Buffers" booklet or Blanchard (1984).

C. Concentration Effects of Buffer on pH

- It is useful to prepare buffers as 10x or 100x stocks. This permits smaller storage volumes and the addition of bactericidal agents such as 0.02% sodium azide which are diluted to insignificant levels before use (Scopes, 1982). Saturating solubilities of some buffers at 0°C (for full chemical names, see Table 1.1):

MES	0.65 M
PIPES	2.3 M
MOPS	3.0 M
TES	2.6 M
HEPES	2.3 M
Tris	2.4 M
Phosphate	2.5 M (as K^+ salt)

- Note that dilution of concentrated stock buffer solution may change the pH. For example, a buffer with 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 is pH 6.7. Tenfold dilution raises the pH to 6.9 while after one hundredfold dilution it is 7.0 (Tipton and Dixon, 1979).

- The pH of Tris decreases by 0.1 unit per tenfold dilution (Calbiochem "Buffers" booklet).

D. Limitations of Certain Buffers

Buffers are often present at the highest concentration of all components in a protein solution and may have significant effects on a protein or enzyme. Buffers composed of inorganic compounds (phosphate, borate, bicarbonate) may interact with enzymes (or their substrates), affecting their activities. Most seriously, some buffers form coordination complexes with di- and trivalent metal ions resulting in proton release, lower pH, chelation of the metal, and formation of insoluble complexes. Buffers with low metal binding constants such as PIPES, TES, HEPES, and CAPS are preferred for studying enzymes with metal requirements (Blanchard, 1984).

- Phosphate:
  1. is a feeble buffer in the pH range 8 - 11;
  2. precipitates or binds many polyvalent cations;
  3. inhibits a large variety of enzymes, including kinases, phosphatases, dehydrogenases, and other enzymes with phosphate esters as substrates (Blanchard, 1984);
  4. exhibits a dependence of pK on buffer dilution (see Section C).

- Citrate binds to some proteins and forms metal complexes (Scopes, 1982).

- Cacodylate is toxic (Scopes, 1982).

- Carbonate has limited solubility and, since it is in equilibrium with CO_2, studies must be carried out in a closed system (Blanchard, 1984).

- ADA absorbs light at wavelengths up to 260 nm and binds metal ions (Good et al., 1966).

- MOPS interferes with the Lowry protein assay, but not with either the Bradford or Bicinchoninic Acid assays (see Chapter 3).

- HEPES:
  1. interferes with the Lowry protein assay, but not with either the Bradford or Bicinchoninic Acid assays (see Chapter 3);
  2. as for all piperazine-based Good buffers (HEPES, EPPS, PIPES; see Good et al., 1966) forms radicals under various conditions and should be avoided in systems where redox processes are being studied (Grady et al., 1988).

- Tris:
  1. is a poor buffer below pH 7.0;
  2. possesses a potentially reactive primary amine;
  3. participates in various enzymatic reactions such as that catalyzed by alkaline phosphatase;
  4. passes through biological membranes (Calbiochem "Buffers" booklet);
  5. is affected by buffer concentration and temperature (see Section C above).

- Borate forms complexes with mono- and oligosaccharides, nucleic acids and pyridine nucleotides, and glycerol (Blanchard, 1984).

E. Preventing Buffer Contamination

- Phosphate-buffered solutions are highly susceptible to microbial contamination. However, 1 M phosphate stock solutions do not usually become contaminated with bacteria (Schleif and Wensink, 1981).

- Filtering the buffer through a sterile ultrafiltration device may be useful for preventing bacterial or fungal growth, especially at pH 6 - 8 (Blanchard, 1984).

- To prevent buffer contamination during storage, 0.02% (3 mM) sodium azide is often used. Sodium azide does not interact significantly with proteins at this concentration. Note: Sodium azide should be used with caution in the presence of heavy metal cations, as it can dry down into highly unstable salt crystals (Rozycki and Bartha, 1981).

- Refrigeration helps to reduce buffer contamination.

F. Water Purity

Water is the primary ingredient in almost every laboratory solution. Most contaminating substances are removed by distillation and deionization, but traces of some compounds sometimes remain and reliable measurements with protein solutions may be affected. A description of various treatment systems for high-level purification of water for laboratory research can be found in Ganzi (1984).

III. Salts, Metal Ions, and Chelators

A. Ionic Strength: 0.1 - 0.2 M KCl or NaCl simulates physiological conditions for many applications (O'Sullivan and Smithers, 1979).

B. Divalent Cations

If a complex is formed between the buffer and a divalent cation such as Ca^{2+} or Mg^{2+}, the capacity for buffering hydrogen ions is reduced. In addition, the availability of the metal ions to participate in an enzymatic reaction may be diminished. Thus, beware of buffers with affinities for metals.

1. Avoid Tris buffers when a metal cofactor is required for protein activity or stability. In 100 mM Tris with 2 mM Mn^{2+}, 29% of the metal is chelated (Morrison, 1979).

2. For purposes of reproducibility, if working with an ATP-binding enzyme, add Mg^{2+} in 1 mM excess over ATP to ensure that essentially all ATP is present as Mg·ATP (Watts, 1973).

C. Chelators

When it is necessary to limit metal effects, specific metal ion chelators should be used. Metal ion chelators also inactivate metalloproteases.

1. To eliminate trace amounts of heavy metals in buffers, 0.1 - 5 mM ethylene diamine tetraacetic acid (EDTA) is commonly used (Scopes, 1982).

2. The most commonly used chelating agents are EDTA and ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA). While EDTA displays strong and nonspecific affinity for a variety of metals, the affinity of EGTA for calcium is significantly higher than its affinity for magnesium, permitting the preferential sequestering of calcium in solutions with EGTA (Blanchard, 1984).

3. *o*-Phenanthroline chelates zinc, while *m*-phenanthroline does not (Todhunter, 1979).

IV. Reducing Agents

A. General Considerations

Within the cell, various reducing compounds, notably glutathione, prevent protein oxidation. Once the cell has been disrupted, care must be taken to counteract effects due to increased contact with oxygen and dilution of naturally occurring reducing agents. Many proteins lose activity when oxidized, although this activity may sometimes be restored by reduction of critical thiol groups. The presence of divalent cations may accelerate the formation of disulfide bonds (Scopes, 1982).

B. Specific Recommendations

- 2-Mercaptoethanol, which is easy to use since it may be stored as a solution at 4°C, must be used at a concentration of 5 - 20 mM. Within 24 hours of its introduction into the buffer, 2-mercaptoethanol becomes oxidized, after which it may accelerate protein inactivation (Scopes, 1982).

- Dithiothreitol (DTT or Cleland's reagent) is supplied as a powder and must be stored at -20°C as a stock solution. DTT may be used at 0.5 - 1.0 mM, and oxidation results in the formation of a stable intramolecular disulfide which does not endanger protein sulfhydryls.

- A good strategy is to use 2-mercaptoethanol at a 1:1000 dilution (about 12 mM) during a protein preparation, but 1 - 5 mM DTT for long term storage (Scopes, 1982).

- Note that certain enzymes are sensitive to reduction (Schleif and Wensinek, 1981).

V. Detergents

A. Introduction

Detergents are used most often for the extraction and purification of membrane proteins, which otherwise are usually insoluble in aqueous solution. A number of classes of detergents may be used and some general guidelines for the solubilization and stabilization of membrane proteins are presented in this section.

Detergents are amphiphilic molecules with substantial solubility in water. With the exception of bile salts, the hydrophobic portion of the molecule usually consists of a linear or branched hydrocarbon "tail" whereas the hydrophilic "head" may have one of a number of very different chemical structures. An important property of detergents is the formation of micelles, which are clusters of detergent molecules in which the hydrophilic head portions face outward. Solubilized membrane proteins form mixed micelles with detergent. The hydrophobic (or transmembrane) domain of the protein is shielded from contact with the aqueous buffer by detergent molecules. The critical micelle concentration (CMC) is defined as the lowest detergent concentration at which micelles form. A detergent with a high CMC (e.g., octyl glucoside) will return to the monomeric state upon dilution below this concentration, thus permitting rapid removal of the detergent by dialysis. In addition, the total molecular weight of the protein plus the micelle may be important for dialysis, gel filtration chromatography, and electrophoresis under non-denaturing conditions. Factors affecting the CMC include temperature, pH, ionic strength, presence of multivalent ions or organic solvents, and detergent purity.

Although the presence of high concentrations of detergent often results in protein denaturation, sometimes the subsequent removal of detergent allows the protein to renature. If protein denaturation is a problem, nonionic detergent concentrations below 0.1% are not usually harmful to proteins (Scopes, 1982).

B. Classes of Detergents

Consult Table 1.2 for specific properties of detergents.

- Ionic Detergents

Ionic detergents contain head groups with either positive charges (cationic detergents) or negative charges (anionic detergents). Ionic detergents have the disadvantage of being highly denaturing. However, they permit the separation of proteins into their monomeric forms, facilitating molecular weight determination.

1. Sodium or Lithium Dodecyl Sulfate (SDS, LiDS)

Of these two anionic detergents, lithium dodecyl sulfate (LiDS) has the advantage that it is soluble at 4°C while sodium dodecyl sulfate (SDS) is not. As much as 10 mg or more of LiDS may be necessary for complete solubilization of one mg of membrane protein (Boehringer Mannheim Catalog). Note that the use of these detergents in the presence of potassium buffers or ammonium sulfate may lead to their precipitation at room temperature. The critical micelle concentration is dramatically affected by the salt concentration; for SDS, the CMC drops from 8 mM in the absence of salt to 0.5 mM in 0.5 M NaCl (Helenius et al., 1979).

2. Sodium Cholate and Sodium Deoxycholate

Sodium cholate and sodium deoxycholate (DOC) are anionic detergents that are less denaturing than other ionic detergents (Harlow and Lane, 1988). Two classes of micelles, termed primary (containing up to 9 molecules) and secondary (9 - 60 molecules), occur above the critical micelle concentration. In contrast to other detergents, free cholate or deoxycholate monomers continue to accumulate above the CMC. The pK_a of these detergents is between 8 and 9, and precipitation of the detergent in its acid form is a problem below pH 7.5. Divalent cations cause DOC precipitation.

- Nonionic Detergents

Nonionic detergents have uncharged hydrophilic head groups. As a result, they are less likely to disrupt protein-protein interactions and are particularly useful for isolating functional protein complexes. Nonionic detergents are far less denaturing than ionic detergents; however, protein aggregation may occur in the presence of these detergents.

1. Triton X-100 (Polyoxyethylene [9-10] *p-t*-octyl phenol)

Many proteins retain their activity in 1 - 3% Triton X-100. A tenfold or greater excess of Triton X-100 to membrane lipid (w/w) may be required to solubilize membrane proteins. Triton X-100 has a strong absorbance at 280 nm.

2. Triton X-114 (Polyoxyethylene [7-8] *p-t*-octyl phenol)

Triton X-114 (2%) added to a protein solution has the property of causing a separation between the detergent and the aqueous phases at temperatures above 20°C, its cloud point. Hydrophilic proteins remain in the aqueous phase while integral membrane proteins may be recovered in the detergent phase (Bordier, 1981).

3. Octyl glucoside (1-O-n-octyl-β-D-glucopyranoside)

Octyl glucoside (OG) is better defined chemically than Triton and therefore results may be more reproducible with this detergent. 20 - 45 mM OG is often sufficient to solubilize membrane proteins. OG has a high CMC (see Table 1.2) and is more easily removed from solution than is Triton.

4. Tween 20 (PEG [20] sorbitan monolaurate)

Tween 20 is commonly used to block nonspecific protein interactions in solid phase immunochemistry (ELISA, RIA, immunoblotting). Tween 20 has a very low CMC.

- Zwitterionic Detergents

Zwitterionic detergents contain head groups with both positive and negative charges. This class of detergents is more efficient than nonionic detergents at overcoming protein-protein interactions while causing less protein denaturation than ionic detergents.

1. **CHAPS** (3-[(Cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate)

   CHAPS does not interfere with ion exchange chromatography or isoelectric focusing. Proteins in solutions containing CHAPS may be frozen safely.

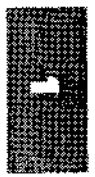
2. **Zwittergent 3-14**

Table 1.2

Characteristics of Common Detergents

Detergent	Molecular Weight	Critical Micelle Concentration	Concentration for Solubilization	Micelle Size
Sodium Dodecyl Sulfate	288.5[d]	7 - 10 mM, 0.23%[d]		18 kd
Lithium Dodecyl Sulfate	272.4	6 - 8 mM, 0.2%[a]	>10 mg/mg protein[a]	
Sodium Cholate	431[f]	3 - 10 mM, 0.2%[f]		1.8 kd
Sodium Deoxycholate	433[f]	1 - 2 mM, 0.57%[f]		4.2 kd
Triton X-100	about 628[a]	0.3 mM, 0.02%[c]	0.2 - 0.6 mg/mg protein[b]	90 kd
Triton X-114	about 543[d]	0.35 mM, 0.02%	see text (section V.B.)	
Octyl glucoside	292.4[a]	15 - 25 mM,0.5%[a]	20 - 45 mM[a]	8.0 kd
Tween 20	1230[f]	60 μM, 0.006%[f]		
Tween 80	1310[f]	10 μM, 0.0013%[f]		76 kd
Brij 35	1200[f]	90 μM, 0.01%[e]		49 kd
CHAPS	614.9[d]	4 - 8 mM, 0.5%[f]	6 - 10 mM[a]	6.0 kd
Zwittergent 3-14	364[d]	0.3 mM, 0.011%		30 kd

a - Boehringer Mannheim Catalog
b - Helenius and Simons, 1975
c - Hjelmeland and Chrambach, 1984
d - Calbiochem Catalog
e - Helenius et al., 1979
f - Harlow and Lane, 1988



C. Protocol for Membrane Protein Solubilization

- Experimental Steps (from Hjelmeland and Chrambach, 1984):

1. Prepare crude membrane fraction at a protein concentration of about 10 mg/ml in 50 mM buffer, 0.15 M KCl at 4°C.
2. Prepare detergent stock solution (10%, w/v) in the same buffer.
3. Make dilutions of the crude membrane fraction (about 5 mg/ml) in buffer containing the following amounts of detergent: 0.01%, 0.03%, 0.1%, 0.3%, 1%, 3%.
4. Stir gently for 1 hr at 4°C (avoid foaming or sonication).
5. Centrifuge at 100,000 x *g* for 1 hr at 4°C.
6. Remove supernatant and resuspend pellet in an equal volume of buffer containing the same detergent concentration.
7. Determine the protein concentration of each fraction (see Chapter 3).
8. Determine the enzyme activity in each fraction.

- General Comments

1. The detergent concentration which yields the highest soluble protein content and activity should be used for further studies. If unsatisfactory results are obtained with a variety of different detergents, mixtures of detergents may be tried. Low yields of protein activity may be improved by the addition of glycerol (25 - 50%, v/v), reducing agents (1 mM DTT or 5 mM β-mercaptoethanol), chelating agents (1 mM EDTA), or protease inhibitors (75 µg/ml PMSF, 20 µg/ml leupeptin or pepstatin; see Section VII below).

2. Sometimes, solubilization with phosphate (0.1 - 0.2 M) is successful if KCl solubilization does not work.

3. Protein solubilization very often occurs near the detergent CMC (Hjelmeland and Chrambach, 1984).

4. For separating soluble protein from integral membrane proteins, the method of Bordier (1981) using the nonionic detergent Triton X-114 may be employed. Extraction and separation in Triton X-114 may be used as a first step in the purification of a membrane protein (this step rapidly separates the membrane proteins from lysosomal proteases). Detergent exchange may be performed during ion-exchange chromatography.

5. The interactions of membrane-associated proteins with membranes may also be disrupted by exposing the membrane preparation to conditions of high salt (0.15 - 2.0 M KCl), high or low pH, high doses of chelating agents (10 mM EDTA or EGTA), or denaturing agents such as urea or guanidine HCl (6 - 10 M) (van Renswoude and Kempf, 1984).

6. The definition of a soluble protein is not always clear. The ability of a protein to remain in the supernatant after a one hour centrifugation at 105,000 x *g* is affected by both the solution density and temperature. Another test that may be applied is gel filtration chromatography (Hjelmeland and Chrambach, 1984).

VI. Protein Environment

A. Surface Effects

Dilute protein solutions often lose activity quickly, possibly via denaturation on surfaces such as glassware, but this effect can be prevented by inclusion of high levels of another protein, commonly bovine serum albumin (BSA). Ideally, to avoid introducing a "contaminating" protein, dilute protein solutions should be rapidly concentrated. However, since enzyme reactions are sometimes assayed with protein concentrations as low as 1 μg/ml which may lead to rapid inactivation, addition of BSA may be necessary. In addition, loss of the purified protein due to nonspecific adhesion onto glass surfaces (1 μg of protein is absorbed on 5 cm^2 of a glass surface) is significantly diminished when the solution is supplemented with BSA. At least 0.1 mg/ml BSA should be used in assay mixtures, while stored protein may contain as much as 10 mg/ml BSA (Scopes, 1982).

B. Temperature

An enzyme's reaction velocity roughly doubles with a temperature increase of 10°C (for example between 18°C and 28°C) although some "cold-labile" proteins are effectively inactivated at low temperature (i.e., mitochondrial ATPase). Above 30 - 40°C, proteins vary widely in their stability, most becoming inactivated, but some remaining stable even upon boiling (i.e., bacterial alkaline phosphatase).

C. Storage

As a rule, a protein's half-life is extended by storage at low temperatures. Whether the best storage conditions are at 4°C, -20°C, -80°C, or in liquid nitrogen (-200°C) depends on the protein and its intended use.

For short-term storage (one day to one week), the protein can be stored at 4°C in solution if 1) functional activity (enzyme assay, ligand binding, etc.) does not decrease during this period, 2) no degradation is visible by 2-D electrophoresis (Chapter 7), and 3) no significant amount of pelleted material is obtained by centrifuging 10 min at 20,000 x *g* (which would indicate denaturation).

For long-term storage (more than one week), several options are available depending on the stability of the individual protein. It may be advisable to test several of these methods before committing a large amount of your protein to any one of them:

1. One of the simplest methods is to store the protein at 4°C as a suspended precipitate in ammonium sulfate (Chapter 4, Section II.A.).

2. Alternatively, ammonium sulfate precipitated protein can be pelleted by centrifuging 20 min at 20,000 x *g* and then frozen in liquid nitrogen and stored at a temperature of -80°C or less.

3. If you want to freeze your protein, it is important to do it in such a way as to minimize possible phase separations which can precipitate or locally concentrate buffers and allow pH drastic pH fluctuations (+/-3 pH units). High protein concentrations (> 2mg/ml) provide some auto-buffering capacity, and freezing in liquid nitrogen reduces the time needed for freezing (Scopes, 1982). The proper method for freezing a protein in liquid nitrogen is to drop it in 50 µl droplets into liquid nitrogen from a Pasteur pipette or an automatic pipettor. Do this slowly, so individual droplets do not fuse. For large volumes, use a peristaltic

pump to automate dropping. When finished, scoop pellets out of the liquid nitrogen and quickly pour them into a storage tube so that the droplets do not thaw. Store at -80°C or in liquid nitrogen. Storing as droplets allows you to "pour" out as much protein as you need for an experiment without subjecting it to multiple freeze-thaw cycles which can be damaging.

4. A last option is to store the protein as a lyophilized powder (see Chapter 4). For many proteins, lyophilization is the safest and most convenient method of long term storage, but for a significant minority it can cause serious problems of inactivation and inability to resolubilize. Again, it is advisable to test this method on a small amount of protein before committing your entire stock.

As a general note, glycerol is often useful in stabilizing proteins. 50% glycerol is often recommended for storage, while working buffers may contain 20 - 30% glycerol without creating problems for handling (Scopes, 1982). Storage in glycerol allows for maintenance of the protein solution at very low temperatures without freezing.

VII. Protease Inhibitors

Disruption of cells for isolation of proteins may also result in the release of proteases from subcellular compartments. These proteases often need to be removed rapidly to ensure that the protein of interest remains intact. Until the protein of interest is purified, protease inhibitors should be used to retard proteolysis. Five commonly used protease inhibitors are listed below, along with indications for their use. Since protease sensitivity varies widely among different proteins, the indicated working concentration may need to be modified. Special care must be taken when diluting the protease inhibitor into the experiment buffer; since many protease inhibitors are poorly soluble in aqueous solution, they must be mixed in thoroughly and rapidly to minimize precipitation. A more complete list of proteases and protease inhibitors can be found in the Boehringer Mannheim catalog or Harris (1989).

A. Common Inhibitors

- PMSF (phenylmethylsulfonyl fluoride):
  1. inhibits serine proteases (e.g., chymotrypsin, trypsin, thrombin) and thiol proteases (e.g., papain);
  2. soluble in isopropanol to 10 mg/ml;
  3. stock solution stable over a year at room temperature;
  4. working concentration: 17 - 174 μg/ml (0.1 - 1.0 mM);
  5. unstable in aqueous solution, add fresh PMSF at every isolation or purification step.

- EDTA (ethylenediamine tetraacetic acid):
  1. inhibits metalloproteases;
  2. soluble in water to 0.5 M at pH 8 - 9;
  3. stock solution stable for over 6 months at 4°C;
  4. working concentration: 0.5 - 1.5 mM (0.2 - 0.5 mg/ml);
  5. add NaOH to adjust pH of stock solution, otherwise EDTA remains insoluble.

- Pepstatin A:
  1. inhibits acid proteases such as pepsin, renin, cathepsin D, and chymosin;
  2. soluble in methanol to 1 mg/ml;
  3. stock solution stable for 1 week at 4°C or 6 months at -20°C;
  4. working concentration: 0.7 µg/ml (1 µM);
  5. insoluble in water.

- Leupeptin:
  1. inhibits serine and thiol proteases such as papain, plasmin, and cathepsin B;
  2. soluble in water to 10 mg/ml;
  3. stock solution stable for 1 week at 4°C or 6 months at -20°C;
  4. working concentration: 0.5 µg/ml (1 µM).

- Aprotinin:
  1. inhibits serine proteases such as plasmin, kallikrein, trypsin, and chymotrypsin;
  2. soluble in water to 10 mg/ml, adjust to pH 7 - 8;
  3. stock solution stable for 1 week at 4°C or 6 months at -20°C;
  4. working concentration: 0.06 - 2.0 µg/ml (0.01-0.3 µM);
  5. avoid repeated freeze-thawing;
  6. inactive at pH >12.8.

B. A Sample Broad Range Protease Inhibitor Cocktail

35 µg/ml PMSF	- serine proteases
0.3 mg/ml EDTA	- metalloproteases
0.7 µg/ml Pepstatin A	- acid proteases
0.5 µg/ml Leupeptin	- broad spectrum

(from Boehringer Mannheim publications)

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