PART I

INTRODUCTION

1

INTRODUCTION TO PROTEIN PURIFICATION

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1.1 INTRODUCTION

The development of techniques and methods for the separation and purification of biological macromolecules such as proteins has been an important prerequisite for many of the advancements made in bioscience and biotechnology over the past five decades. Improvements in materials, utilization of computerized instruments, and an increased use of *in vivo* tagging have made protein separations more predictable and controllable, although many still consider purification of non-tagged proteins more an art than a science. However, gone are the days when an investigator had to spend months in search of an efficient route to purify an enzyme or hormone from a cell extract. This is a consequence of the development of new generations of chromatographic media with increased efficiency and selectivity as well as of new automated chromatographic systems supplied with sophisticated interactive software packages and data bases. New electrophoresis techniques and systems for fast analysis of protein composition and purity have also contributed to increasing the efficiency of the evaluation phase of the purification process.

In the field of chromatography, the development of new porous resin supports, new crosslinked beaded agaroses, and new bonded porous silicas has enabled rapid growth in high resolution techniques (high performance liquid chromatography, HPLC; fast protein liquid chromatography, FPLC), both on an analytical and laboratory preparative scale as well as for industrial chromatography in columns with bed volumes of several hundred liters. Expanded bed adsorption enables rapid isolation of target proteins, directly from whole cell cultures or cell homogenates. Another field of increasing importance is micropreparative chromatography, a consequence of modern methods for amino acid and sequence analysis requiring submicrogram samples. The data obtained are efficiently exploited by recombinant DNA technology, and biological activities previously not amenable to proper biochemical study can now be ascribed to identifiable proteins and peptides.

A wide variety of chromatographic column packing materials such as gel-filtration media, ion exchangers, reversed phase packings, hydrophobic interaction adsorbents, and affinity chromatography adsorbents are today commercially available. These are identified as large diameter media (90–100 μm), medium diameter media (30–50 μm) and small diameter media (5–10 μm) in order to satisfy the different requirements of efficiency, capacity, and cost.

However, not all problems in protein purification are solved by the acquisition of sophisticated laboratory equipment and column packings that give high selectivity and efficiency. Difficulties still remain in finding optimum conditions for protein extraction and sample pretreatment, as well as in choosing suitable methods for monitoring protein concentration and biological activity. These problems will be discussed in this introductory chapter. There will also be an overview of different protein separation techniques and their principles of operation. In subsequent chapters, each individual technique will be discussed in more detail. Finally, some basic equipment necessary for efficient protein purification work will be described in this chapter.

Several useful books covering protein separation and purification from different points of view are available on the market or in libraries (1–3). In "Methods of Enzymology," for example, in older volumes 22, 34, 104, and 182 (4–7), but particularly in the most recent volume, 463 (8), a number of very useful reviews and detailed application reports will be found. The booklets available from manufacturers regarding their separation equipment and media can also be helpful by providing detailed information regarding their products.

1.2 THE PROTEIN EXTRACT

1.2.1 Choice of Raw Material

In most cases, interest is focused on one particular biological activity, such as that of an enzyme, and the origin of this activity is often of little importance. Great care should therefore be taken in the selection of a suitable source. Among different sources there might be considerable variation with respect to the concentration of the enzyme, the availability and cost of the raw material, the stability of the enzyme, the presence of interfering activities and proteins, and difficulties in handling a particular raw material. Very often it is compelling to choose a particular source because it has been described previously in the literature. However, sometimes it is advantageous to consider an alternative choice.

Traditional animal or microbial sources have today, to a large degree, been replaced by genetically engineered microorganisms or cultured eukaryotic cells. Protein products of eukaryotic orgin, cloned and expressed in bacteria such as *Escherichia coli*, may either be located in the cytoplasm or secreted through the cell membrane. In the latter case they are either collected inside the periplasmic space or they are truly extracellular, secreted to the culture medium. Proteins that accumulate inside the periplasmic space may be selectively released either into the growth medium by changing the growth conditions (9), or following cell harvesting and washing of the resuspended cell paste. At this stage, a considerable degree of purification has already been achieved by choosing a secreting strain as illustrated in Figure 1.1. In connection with the cloning, the recombinant protein may be equipped with an "affinity handle" such as a His-tag or a fusion protein such as Protein A, glutathione-S-transferase, or maltose binding protein in order to facilitate purification. The handle is often designed such that it can be cleaved off using highly specific proteolytic enzymes. Proteins of eukaryotic origin, and some virus surface proteins are often

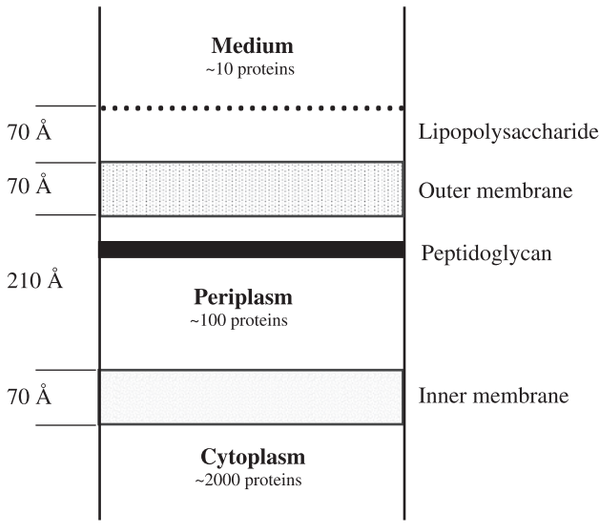


Figure 1.1 Location and approximate numbers of proteins in *E. coli*.

glycosylated why eukaryotic host cells have to be chosen for their production.

1.2.2 Extraction Methods

Some biological materials themselves constitute a clear or nearly clear protein solution suitable for direct application to chromatography columns after centrifugation or filtration. Examples include blood serum, urine, milk, snake venoms, and—perhaps most importantly—the extracellular medium after cultivation of microorganisms and mammalian cells, as mentioned above. It is normally an advantage to choose such a starting material because of the limited number of components and also because extracellular proteins are comparatively stable. Some samples, such as urine or cell culture supernatants, are normally concentrated before purification begins.

In most cases, however, it is necessary to extract the activity from a tissue or a cell paste. This means that a considerable number of contaminating molecular species are set free, and proteolytic activity will make the preparation work more difficult. The extraction of a particular protein from a solid source often involves a compromise between recovery and purity. Optimization of extraction conditions should favor the release of the desired protein and leave difficult-to-remove contaminants behind. Of particular concern is to find conditions under which the already extracted protein is not degraded or denatured while more is being released.

Various methods are available for the homogenization of cells or tissues. For further details and discussions the reader is referred to the paper by Kula and Schütte (10). The extraction conditions are optimized by systematic variation of parameters such as the composition of the extraction medium (see below), time, temperature, and type of equipment used.

The proper design of an extraction method thus requires preliminary experiments in which aliquots are taken at various time intervals and analyzed for activity and protein content. The number of parameters can be very large, so this part of the work has to be kept within limits by applying proper judgment. However, it is not recommended to accept a single successful experiment. Further investigations of the required extraction time, in particular, often pays in the long run. The number of optimization experiments can be reduced considerably by using chemometrics (multivariate analysis), for which there are computer programs available (www.chemometrics.com/software).

The major problems confronted when preparing a protein are in general denaturation, proteolysis, and contamination with pyrogens, nucleic acids, bacteria, and viruses. These can be limited by appropriate choice of the extraction medium, as we shall show. However, we can already state that many of the above problems can be reduced by short preparation times and low temperatures. It is therefore good biochemical practice to carry out the first preparation steps as fast as possible and at the lowest possible temperature. However, low temperatures are not always necessary and are sometimes inconvenient. The working temperature is therefore one of the parameters that should be optimized carefully, especially if a preparation is to be done routinely in the laboratory or if it is going to be scaled up to pilot or production scale.

The extract must be clarified by centrifugation and/or by filtration before submission to column chromatography. A preparative laboratory centrifuge is normally sufficient for this step.

A common phenomenon when working with intracellularly expressed recombinant proteins is their tendency to accumulate as insoluble aggregates known as inclusion bodies, which have to be solubilized and refolded to recover their native state. At first glance, the formation of insoluble aggregates in the cytoplasm might be considered a major problem. However, as the inclusion bodies seem to be fairly well defined with regard to both particle size and density (11), they should provide a unique means for rapid and efficient enrichment of the desired protein simply by low speed fractional centrifugation and washing of the resuspended sediment. The critical step is solubilization and refolding, often combined with chromatographic purification under denaturating conditions in the presence of high concentrations of urea or guanidine hydrochloride. This area is treated in more detail in Chapter 13 and has recently been reviewed by Burgess (12).

1.2.3 Extraction Medium

To arrive at a suitable composition for the extraction medium it is necessary initially to study the conditions at which the

protein of interest is stable and secondly, where it is most efficiently released from the cells or tissue. The final choice is usually a compromise between maximum recovery and maximum purity. The following factors have to be taken into consideration: pH, buffer salts, detergents/chaotropic agents, reducing agents, chelators or metal ions, proteolytic inhibitors, and bacteriostatics.

1.2.3.1 pH Normally, the pH value is chosen such that the activity of the protein is at a maximum. However, it should be noted that this is not always the pH that gives the most efficient extraction, nor is it necessarily the pH of maximum stability. For example, trypsin has an activity optimum at pH 8–9, but is much more stable at pH 3, where autolysis is avoided. The use of extreme pH values, for example, for the extraction of yeast enzymes in 0.5 M ammonia, is sometimes very efficient and is acceptable for some proteins without causing too much denaturation.

1.2.3.2 Buffer Salts Most proteins are maximally soluble at moderate ionic strengths, 0.05–0.1, and these values are chosen if the buffer capacity is sufficient. Suitable buffer salts are given in Table 1.1.

An acceptable buffer capacity is obtained within one pH unit from the pK_a values given. The proteins as such also act as buffers, and the pH should be checked after addition of large amounts of proteins to a weakly buffered solution. Some extractions do not give rise to acids and bases and thus do not need a high buffer capacity. In other cases this might be necessary, and occasional control of the pH value of an extract is recommended.

1.2.3.3 Detergents and Chaotropic Agents In many extractions the desired protein is bound to membranes or particles, or is aggregated due to its hydrophobic character. In these cases one should reduce the hydrophobic interactions by using either detergents or chaotropic agents (not both!). Some of the commonly used detergents are listed in Table 1.2. Several of them do not denature globular proteins or interfere with their biological activity. Others, such as sodium dodecyl sulfate (SDS), will do that. Quite often it is not necessary to continue using a detergent in the buffer after the first step(s) in the purification, so its use is restricted to the extraction medium. In other cases it might be necessary to use a detergent throughout the whole preparation process, leading to the final purification of a protein–detergent complex. More information about detergents, including their chemical structures, can be found in Reference 13.

TABLE 1.1 Buffer Salts Used in Protein Work

Buffer	pK values	Properties
Sodium acetate	4.75	
Sodium bicarbonate	6.50, 10.25	
Sodium citrate	3.09, 4.75, 5.41	Binds Ca^{2+}
Ammonium acetate	4.75, 9.25	Volatile
Ammonium bicarbonate	6.50, 9.25, 10.25	Volatile
Tris-hydrochloride	8.21	
Sodium phosphate	1.5, 7.5, 12.0	
Tris-phosphate	7.5, 8.21	

Buffer concentration refers to total concentration of buffering species. Buffer pH should be as close as possible to the pK_a value, and not more than one pH unit from the pK_a.

TABLE 1.2 Detergents Used for Solubilization of Proteins

Detergent	Ionic Character	Effect on Protein	Critical Micelle Concentration, % w/v
Triton X-100	Nonionic	Mild, nondenaturing	0.02
Nonidet P 40	Nonionic	Mild, nondenaturing	0.012
Lubrol PX	Nonionic	Mild, nondenaturing	0.006
Octyl glucoside	Nonionic	Mild, nondenaturing	0.73
Tween 80	Nonionic	Mild, nondenaturing	0.002
Sodium deoxycholate	Anionic	Mild, denaturing	0.21
Sodium dodecyl sulfate, SDS	Anionic	Strongly denaturing	0.23
CHAPS	Zwitter-ionic	Mild, denaturing	1.4

Detergents are amphipathic molecules. When their concentration increases they will eventually aggregate; that is, they will form micelles at the so-called critical micelle concentration (CMC). Because micelles often complicate purification procedures, in particular column chromatography, detergent concentrations below the CMC should be used.

Instead of using detergents to dissolve aggregates, chaotropic agents such as urea or guanidine hydrochloride, or moderately hydrophobic organic compounds such as ethylene glycol, can be tried. Urea and guanidine hydrochloride have proven particularly useful for the extraction and solubilization of inclusion bodies (12).

1.2.3.4 Reducing Agents The redox potential of the cytosol is lower than that of the surrounding medium where atmospheric oxygen is present. Intracellular proteins often have exposed thiol groups and these might become oxidized in the purification process. Thiol groups can be protected by reducing agents such as 1,4-dithioerythritol (DTE), dithiothreitol (DTT) or mercaptoethanol (Table 1.3). Normally, 10–25 mM concentrations are sufficient to protect thiols without reducing internal disulfides. In other cases a higher concentration might be needed (14). Ascorbic acid is sometimes added to polyphenol containing crude plant extracts in order to avoid oxidation and miscoloration.

1.2.3.5 Chelators or Metal Ions The presence of heavy metal ions can be detrimental to a biologically active protein,

TABLE 1.3 Reducing Agents

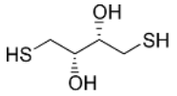
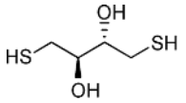
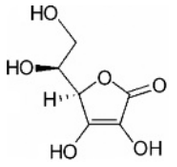
Agent	Structure
Mercaptoethanol	HS—CH_2—CH_2—OH
1,4-Dithioerythritol (DTE)	
1,4-Dithiothreitol (DTT)	
Ascorbic acid	

TABLE 1.4 Proteolytic Inhibitors

Inhibitor	Enzymes Inhibited	Working Concentration
Diisopropyl fluorophosphate (DFP)	Serine proteases	(avoid DFP)
Phenylmethylsulfonyl fluoride (PMSF)	Serine proteases	0.5–1 mM
Ethylenediamine tetraacetate (EDTA)	Metal-activated proteases	~5 mM
Cysteine reagents	Cysteine-dependent proteases	(varying)
Pepstatin A	Acid proteases	1 μM
Leupeptin	Serin proteases	1 μM

for two main reasons. They can enhance the oxidation of thiols by molecular oxygen and can form complexes with specific groups, which may cause problems. Heavy metals can be trapped by chelating agents. The most commonly used is ethylenediamine tetraacetic acid (EDTA) in the concentration range 10–25 mM. An alternative is ethylene glycol tetraacetic acid (EGTA), which is more specific for calcium. It should be noted that EDTA is a buffer. It is therefore best to add EDTA before final pH adjustment. The chelating capacity of EDTA increases with increasing pH.

In other cases, stabilizing metal ions are needed. Many proteins are stabilized by calcium ions. However, the divalent ions calcium and magnesium are trapped by EDTA and cannot be used in combination with this chelator.

1.2.3.6 Proteolytic Inhibitors The most serious threats to protein stability are the omnipresent proteases. The simplest safeguard against proteolytic degradation is normally to work quickly at low temperatures. An alternative, or added, precaution is to make use of protease inhibitors (Table 1.4), especially in connection with the extraction step. Often there is a need for a combination of inhibitors, for example, for both serine proteases and metalloproteases. In general, protein inhibitors are expensive, which may limit their use in large-scale applications. Proteolysis can also be reduced by rapid extraction of the fresh homogenate in an aqueous polymer two-phase system (15) or by adsorption of the proteases to hydrophobic interaction adsorbents (16). Sometimes it is sufficient to adjust the pH to a value at which the proteases are inactive, but where the stability of the protein to be purified is maintained. A classical example is the purification of insulin from the pancreas.

1.2.3.7 Bacteriostatics It is wise to take precautions to avoid bacterial growth in protein solutions. The simplest remedy here is to use sterile filtered buffer solutions as routine in the laboratory. This will also reduce the risk of bacterial growth in columns. A common practice for avoiding bacterial growth in chromatographic columns is to allow the column to flow at a reduced rate, even when it is not in operation. Some buffers are more likely than others to support bacterial growth, such as phosphate, acetate, and carbonate buffers at neutral pH values. Buffers at pH 3 and below or at pH 9 and above usually prevent bacterial growth, but may occasionally allow growth of molds.

Whenever possible it is recommended to add an antimicrobial agent to the buffer solutions. Often used are sodium azide at 0.001 M or merthiolate at 0.005%, or alcohols such as *n*-butanol at 1%. Sodium azide has the drawback that it is a nucleophilic substance and binds metals. In cases where these substances may interfere with activity measurements or the chromatography itself, it is always possible to add the substances to solutions of the protein to be stored.

1.3 AN OVERVIEW OF FRACTIONATION TECHNIQUES

In early work, complex protein mixtures were fractionated mainly by adsorption and precipitation methods. These methods are still used today as preliminary steps for initial fractionation or for concentration of sample solutions. Preparative electrophoretic and chromatographic techniques developed during the l950s and 1960s made possible rational purification protocols and laid the foundation for the situation we have today. The following sections give a short overview of the various techniques normally used in preparative biochemical work. Chapter 2 contains an introduction to chromatography, including a historical review, and Chapter 15 gives an introduction to electrophoresis. Each individual chromatographic and electrophoretic separation technique is then treated in detail in subsequent chapters.

1.3.1 Precipitation

Precipitation of a protein in an extract may be achieved by adding salts, organic solvents, or organic polymers, or by varying the pH or temperature of the solution. The most commonly used precipitation agents are listed in Table 1.5. The strength of a particular ion as a precipitation agent is shown by its position in the so-called Hofmeister series:

Anions: PO_4^{3-}, SO_4^{2-}, CH_3COO^-, Cl^-, Br^-, NO_3^-, ClO_3^-, I^-, SCN^-

Cations: NH_4^+, K^+, Na^+, guanidine $C(NH_2)_3^+$

The so-called *antichaotropic* ions to the left are the most efficient salting out agents. They are efficient water molecule binders, thus increasing the hydrophobic effect in the solution and promoting protein aggregation by facilitating the association of hydrophobic surfaces. The chaotropic salts on the right-hand side in the series decrease the hydrophobic effect, and thus help maintain the proteins in solution.

Polar organic solvents such as ethanol promote the precipitation of proteins due to the decrease in water activity in the solution as the water is replaced by organic solvent. They have been widely used as precipitation agents, especially in the fractionation of serum proteins. The following five variables are usually kept under control: concentration of organic solvent, protein concentration, pH, ionic strength, and temperature (17). Low temperature during the precipitation operations is often necessary to avoid protein denaturation; the addition of an organic solvent decreases the freezing point of the solution and temperatures below 0°C can be used. In reversed phase chromatography, some proteins can be chromatographed in solutions that contain up to ~50% organic solvent, with retention of their biological activity.

Organic polymers function in a way similar to that of organic solvents. The most widely used polymer is polyethylene glycol (PEG), with an average molecular weight of either 6000 or 20,000. The main advantage of PEG over organic solvents is that it is more easily handled. It is unflammable, not poisonous, uncharged, and relatively unexpensive. Rather low concentrations are required (often less than 25%) to precipitate most proteins. One disadvantage is that high concentration solutions of PEG are viscous. PEG can also be difficult to remove from protein solutions. However, after dilution with buffer the viscosity decreases, and because the substance is uncharged, the solution may be applied directly to an ion exchange column to further separate the proteins, simultaneously removing the polymer.

TABLE 1.5 Precipitation Agents

Agent	Type	Properties
Ammonium sulfate	Salt	Easily soluble, stabilizing
Sodium sulfate	Salt	
Ethanol	Solvent	Flammable, risk of denaturation
Acetone	Solvent	Flammable, risk of denaturation
Polyethylene glycol (PEG)	Polymer	Uncharged, unflammable

pH adjustment has been used as a simple and cheap way to precipitate proteins. Proteins have their lowest solubility at their isoelectric point. This is sometimes used in serum fractionation and also in the purification of insulin.

Besides pH, another parameter that influences precipitation of proteins in salt solutions is temperature (see below). Keeping the salt concentration constant and varying the temperature is another way of fractionating a protein solution.

The salting out of a protein can be described by the equation

$$\log S = B - Kc$$

where S is the solubility of the protein in g/L of solution, B is an intercept constant, K is the salting out constant, and c is salt concentration in mol/L.

The value of B depends on the salt used, the pH, the temperature, and the protein itself; K depends on the salt used and the protein. It should be stressed that addition of a salt or another precipitating agent to a protein solution only decreases the solubility of the proteins. This is why a very dilute protein solution for precipitation may lead to low recovery, because a major part of the protein simply remains in solution. Reproducible results can only be achieved if all the parameters mentioned above, including the protein concentration, are kept constant.

Centrifugation is used routinely in the protein purification laboratory to recover precipitates. It can also be used to separate two immiscible liquid phases. Another application is density gradient centrifugation. Today this is used predominantly for the fractionation of subcellular particles and nucleic acids. An alternative is the use of liquid–liquid phase extraction, which seems to offer several advantages over the more classical methods.

1.3.2 Electrophoresis

Electrophoresis in free solution or in macroporous gels such as 1–2% agarose separates proteins mainly according to their net electric charge. Electrophoresis in gels such as polyacrylamide separates mainly according to the molecular size of the proteins.

Today, analytical gel electrophoresis requiring microgram amounts of proteins is an important tool in bioscience and biotechnology (see Chapter 15). Convenient methods for the extraction of proteins after electrophoresis have been developed, in particular protein blotting (see Chapter 18), making the technique micropreparative. There are also many instances where a very small amount of protein is

sufficient for the analysis of size and composition as well as the primary structure. Finally, there are cases where the starting material is extremely limited, such as protein extracts from small amounts of tissue (biopsies, etc.). In these cases, the protein "extract" might be just large enough for gel electrophoretic analysis.

Larger scale (milligrams to grams of protein) electrophoresis was an important method for the fractionation of protein extracts during the 1950s and early 1960s. It was carried out using columns packed with, for example, cellulose powder as a convection depressor, as in the "Porath column" (18). An innate limitation of preparative column electrophoresis is the joule heat developed during the course of the experiment. This means that the column diameter, if it is to allow sufficient cooling, should not exceed ~3 cm. Several hundred milligrams of protein can, however, be separated on such columns. Column zone electrophoresis has the advantage of allowing a precise description of the separation parameters involved and is, besides gel filtration, the mildest separation technique available for proteins. It can be recommended for special situations, but practical aspects and the excessive time required precludes its routine use. Methods for large and medium scale preparative electrophoresis have been developed, such as the flowing curtain electrophoresis of Hannig (19) and, more recently, the "Biostream" apparatus of Thomson (20).

Isoelectric focusing, the other main electrophoretic technique, separates proteins according to their isoelectric points (see Chapter 16). This technique gives very high resolution, but presents major difficulties as a preparative large or medium scale technique. Special equipment is required to allow cooling during the focusing. Proteins often precipitate at their isoelectric point, and this precipitate can contaminate the other bands when a vertical Sephadex™ bed or column with sucrose gradient is used as an anticonvection medium in the focusing experiment. Modern equipment for preparative isoelectric focusing (21) avoids these problems by dividing the separation chamber into smaller compartments. Another solution is to carry out the isoelectric focusing in a horizontal trough of sedimented gel particles such as Sephadex (22). Here, precipitation in one zone will not disturb the other bands. On the other hand, the recovery of proteins is more tedious.

For routine preparative protein fractionation the electrophoretic techniques have become less important than chromatography. Ion exchange chromatography depends on parameters similar to those for electrophoresis. Chromatofocusing fractionates proteins largely according to their isoelectric points and would therefore appear to be a more convenient alternative to preparative isoelectric focusing.

1.3.3 Chromatography

Separation by chromatography depends on the differential partition of proteins between a stationary phase (the chromatographic medium or the adsorbent) and a mobile phase (the buffer solution). Normally, the stationary phase is packed into a vertical column of plastic, glass, or stainless steel, and the buffer is pumped through this column. An alternative is to stir the protein solution with the adsorbent, batchwise, and then pour the slurry onto an appropriate filter and make the washings and desorptions on the filter.

Column chromatography has proved to be an extremely efficient technique for the separation of proteins in biological extracts. Since the development of the first cellulose ion exchangers by Peterson and Sober (23) and of the first practical gel filtration media by Porath and Flodin (24, 25) a wide variety of adsorbents have been introduced that exploit various properties of the protein for the fractionation. The more important of these properties, together with the chromatographic method for which they dominate the separation, are as follows:

1. Size and shape (gel filtration/size exclusion chromatography, SEC).
2. Net charge and distribution of charged groups (ion exchange chromatography, IEC).
3. Isoelectric point (chromatofocusing, CF).
4. Hydrophobicity (hydrophobic interaction chromatography, HIC; reversed phase chromatography, RPC).
5. Metal binding (immobilized metal ion affinity chromatography, IMAC).
6. Content of exposed thiol groups (covalent chromatography, CC).
7. Biospecific affinities for ligands, inhibitors, receptors, antibodies, and so on (affinity chromatography, AC).

The methods often have very different requirements with regard to chromatographic conditions., including ionic strength, pH, and various additives such as detergents, reducing agents, and metals. By appropriate adjustment of the buffer composition, the conditions for adsorption and desorption of the desired protein can be optimized. It should be stressed that the result of a particular chromatographic separation often depends on more than one parameter. In IEC, the charge interaction is the dominant parameter, but molecular weight and hydrophobic effects can also contribute to some degree, depending on the experimental conditions and type of solid phase used. In recent years the concept of multimodal, or mixed-mode adsorption chromatography, has received an increasing amount of attention, with several new products emerging on the market (see Chapter 4 for more detailed information).

Highly specific methods, such as those based on bioaffinity (e.g., antibody–antigen interaction) or those based on the use of *in vivo* fused tags such as $(His)_6$ or glutathione-S-transferase (GST), do in some cases give a highly pure

protein in a single step. Normally, however, several chromatographic methods have to be combined in order to achieve maximal purification of a protein from a crude biological extract. With the wide variety of chromatographic media available today, in combination with a modern computerized chromatography system, adequate purification can normally be achieved within a few days to a couple of weeks.

In recent years, columns containing a continuous, homogeneously porous solid phase have become available. See Chapters 2 and 9 for more information about these so-called monolithic column types. As in membrane adsorption techniques, the main advantage is the considerably reduced diffusion restriction, allowing high efficiency and also high flow rates. The main disadvantage of both these techniques is the concomitant smaller surface area per unit adsorption medium volume, which will restrict the nominal column binding capacity.

All of the chromatographic methods mentioned above are treated in Part II of this book, which begins with a general description of the concepts used in protein chromatography.

1.3.4 Expanded Bed Adsorption

The problem of removing cells and cell debris from large volumes of whole cell cultures or cell homogenates has encouraged the development of technologies for the direct adsorption of target molecules from such feed stocks. In a fluidized bed, the adsorbent particles are subject to an upward flow of liquid that keeps them separated from each other. The resulting increased voidage allows the unhindered passage of cells and cell debris. In a typical fluidized bed there is a total mixing of particles and sample in the reactor, leading to incomplete adsorption of the target molecules unless the feed stock is recycled. Expanded bed adsorption is a special case of fluidized bed adsorption (26), and is primarily applied in a pilot- or production-scale environment (26–29).

1.3.5 Membrane Adsorption

The main argument for utilizing modified membranes as media for protein adsorption is to solve the problem of mass transport restriction in standard chromatography due to the slow diffusion of proteins in the pores of the large gel particles. In membranes, most pores allow convective flow, and the mass transport resistance is therefore minimized to film diffusion at the membrane matrix surface. The result is a more efficient adsorption–desorption cycle of target solutes, allowing considerably higher flow rates and thus considerably shorter separation times. The area has been reviewed by Thömmes and Kula (30). See Chapter 12 for more data regarding membrane separation.

1.4 FRACTIONATION STRATEGIES

1.4.1 Introductory Comments

Before attempting to design a purification protocol for a particular protein, as much information as possible should be collected about the characteristics of that protein and preferably also about the properties of the most important known impurities. Useful data include approximate molecular weight and pI, degree of hydrophobicity, presence of carbohydrate (glycoprotein) or free –SH. Some of this information might be obtained already on a DNA level, if nucleotide sequence data are available, but is otherwise often collected easily by preliminary trials using crude extracts.

Criteria with regard to the stability of the protein to be purified should be established. Important parameters affecting structure are temperature, pH, organic solvents, oxygen (air), heavy metals, and mechanical shear. Special concern should be addressed to the risk of proteolytic degradation. Finally, it is the amount of protein to be purified per batch, and the required degree of purity, that to a high degree governs the techniques and methods used in the purification process.

According to a study of 100 published successful protein purification procedures (31), the average number of steps in a purification process is four. Very seldom can a protein be obtained in pure form from a single chromatographic procedure, even when this is based on a unique biospecificity. In addition to the purification steps there is often a need for concentrations and sometimes changes of buffers by dialysis or membrane ultrafiltration.

The preparation scheme can be described as consisting of three stages:

1. The preliminary or initial fractionation stage (often called the capturing stage).
2. The intermediate purification stage.
3. The final polishing stage.

The purpose of the initial stage is to obtain a stable, particle-free solution suitable for chromatography. This is achieved by clarification, coarse fractionation, and concentration of the protein extract. The purpose of the final stage is to remove aggregates and degradation products and to prepare the protein solution for the final formulation of the purified protein.

Sometimes one or two of these stages coincide. An initial ion exchange adsorption step can thus serve as a preliminary fractionation applied directly to the protein extract, or a gel filtration can give a product that is suitable as a final product, However, as the purposes of the three stages are different it is useful to discuss them separately.

The design of the preparation scheme will differ depending on the material at hand and the purpose of the purification.

If the starting material is very precious, one should favor high yield over speed and convenience. In cases where several different proteins are to be extracted from a single starting material, this of course also affects the planning of the work. Finally, the final step is designed so that the product will be suitable for its purpose, which can vary. These aspects will be discussed below.

1.4.2 Initial Fractionation

There are many methods for the clarification of protein solutions. Extracts of fungal or plant origin often contain phenolic substances or other pigments. These can be removed by adsorption to diatomite (diatomaceous earth, Celite), either batchwise or on a short column. In order to prevent oxidation and miscoloration, small amounts of ascorbic acid can be added to the crude plant extract.

Similarly, lipid material can be removed either by centrifugation, as the lipids will float, and one thus needs to extract the protein solution from below, or by a chromatographic procedure. Lipids adsorb to a number of materials. Aerosil, a fused silica, has been used for the adsorption of lipids, but agarose is sometimes a simple choice.

Contamination with nucleic acids can, in some cases, especially when preparing proteins from bacteria, constitute a problem due to their high viscosity. The classical way to solve this problem is to precipitate the nucleic acids. Streptomycin sulfate and polyethylenimine have been used as precipitants, as have protamine sulfate and manganese salts (32). Another way to solve the problem is to add nucleases, which cut the nucleic acids into smaller pieces, thereby reducing the viscosity. Another problem with nucleic acids or degradation products of nucleic acids is that, due to their low isoelectric points, they still are negatively charged at low pH. Anion exchangers strongly adsorb nucleic acids and are thus difficult to regenerate. The solution to this problem can in some cases be to perform two consecutive adsorption steps. The first is executed at a pH below the pI of the target protein, thus preventing it from binding to the ion exchanger. Often, a fairly small amount of the ion exchanger is required in this step, which is why it is economically motivated to discard the contaminated gel. In the second step using the same anion exchanger, the pH is increased to a value above the pI of the target protein, resulting in binding and subsequent elution using either a stepwise or continuous salt gradient.

1.4.2.1 Clarification by Centrifugation and/or Microfiltration The clarification of any cell homogenate is usually no problem on a laboratory scale, where refrigerated high speed centrifuges operating at speeds from 20,000 rpm to 75,000 rpm, generating from $\sim$40,000g to $\sim$500,000g can be used. A useful review of centrifugation and centrifuges in preparative biochemistry is found in Reference 33. As a complement to centrifugation, in recent years, tangential or cross-flow microfiltration has received increased attention, especially for large-scale applications. For a review of the advantages of cross-flow microfiltration we suggest Reference 34. The area is also treated in more detail in Chapter 12.

1.4.2.2 Ultrafiltration Ultrafiltration has become a widely used technique in preparative biochemistry. Ultrafiltration membranes are available with different cut-off limits for separation of molecules from 1 kDa up to 300 kDa. The method is excellent for the separation of salts and other small molecules from a protein fraction with higher molecular weight and at the same time can effect a concentration of the proteins. The process is gentle, fast, and inexpensive. Ultrafiltration is treated in more detail in Chapter 12.

1.4.2.3 Precipitation Crude extracts are seldom suitable for direct application to chromatographic columns. Preparative differential centrifugation seldom results in a sufficiently clear solution. This is one reason why it is often necessary to use other means for clarification that simultaneously concentrate the solution and remove most of the bulk proteins. Such an initial fractionation step should also result in the removal of proteases and membrane fragments that sometimes bind the protein of interest in the absence of detergents. The classical means is to make a fractional precipitation. Bulk proteins in the solution are first precipitated together with residual particulate matter, and then the protein of interest can be precipitated from the resulting supernatant solution. Sometimes the protein of interest is allowed to remain in the mother liquor solution for direct application to chromatographic columns, for example, hydrophobic interaction adsorption of proteins in ammonium sulfate solutions and IEC of proteins in PEG mother liquors. The most commonly used precipitating agents are listed in Table 1.5, together with some of their properties. A typical precipitation curve is shown in Figure 1.2.

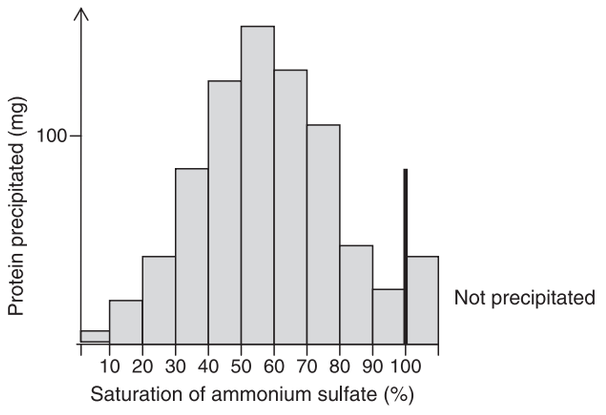


Figure 1.2 Example of a precipitation curve, showing the amount of protein precipitated with a stepwise increase in ammonium sulfate concentration.

Of the various methods available for protein precipitation, the classical ammonium sulfate has some disadvantages. The resulting protein solution often needs to be dialyzed to obtain an ionic strength that allows IEC. This problem is avoided when using PEG. Organic solvents, in particular ethanol and acetone, often produce extremely fine powder-like precipitates that are difficult to centrifuge and handle. They have also often been shown to cause partial denaturation of proteins, which can, for example, prevent subsequent crystallization. This is why organic solvents are not recommended as first-choice precipitating agents.

1.4.2.4 Liquid–Liquid Phase Extraction A radically different way of making an initial fractionation is by partitioning in an aqueous polymer liquid–liquid two-phase system (35). These systems often contain PEG as one phase constituent and another polymer, such as dextran or even salt, as the other. Under favorable conditions it is possible to obtain the protein of interest in the upper, normally the PEG phase. The contaminating bulk protein, as well as particles, will be collected in the lower phase and can be removed by centrifugation. Particles sometimes stay at the interphase and are thus also removed in the centrifugation step. Aqueous polymer two-phase systems have been shown to be effective tools for plasma membrane proteomics (36). By covalent attachment of affinity ligands to PEG molecules these can be used for affinity partitioning.

1.4.3 The Chromatographic Steps

1.4.3.1 Choice of Adsorbent Preliminary separation conditions for known proteins are easily extracted using data bases available over the Internet. For unknown (e.g., nonrecombinant) proteins, information regarding their chromatographic behavior can only be obtained by preliminary analytical-scale experiments, for example by gel filtration and by IEC using salt and pH gradients. Using these techniques, approximate values of molecular size and ionic properties such as isoelectric points are obtained, information that is fundamental to the further planning of the work. A more thorough survey of the behavior of the protein on various adsorbents can then be done using a panel of adsorbents. This can be carried out either in a panel of parallel columns or using tandem columns.

A classical parallel column approach was developed by Scopes (37) for a panel of dye adsorbents. In this case he used up to 20 small columns containing various dye adsorbents. The columns were equilibrated with a predetermined application or starting buffer. A small volume of the protein extract was applied to each column and the protein content (A_{280} absorption) and activity in the effluent measured. A predetermined terminating buffer was then applied to each column, and the protein and enzyme activity in the effluent were then determined. A column where the bulk of the proteins, but not the activity, was adsorbed was chosen as a "minus column," and an adsorbent where the reverse happened was chosen as a "plus column." These two columns in combination effected a considerable purification of the desired substance in the actual preparation. In an earlier but similar approach, a panel of parallel columns was used by Shaltiel (38) for the evaluation of hydrophobic adsorbents. The technique can, however, be used for any set-up of adsorbents such as different ion exchangers, the same ion exchanger under different conditions, thiol-gels, metal-chelating gels, and so on. The elution of the columns can also be performed with more than two elution buffers. The purpose, however, is to get a quick idea of the behavior of a previously unknown protein and thus the set-up should not be enlarged beyond what can be handled easily in the laboratory.

If the adsorbents used have well defined and continuously increasing adsorption capacities for proteins, in general the panel can also be arranged as tandem columns. This approach was used by Porath and co-workers for the immobilized metal ion (IMAC) adsorbents (39). Here, three columns (e.g., Zn, Fe, and Cu) were connected in series and a sample was pumped through all of them. After washing with starting buffer, the three columns were disconnected and eluted separately, mostly using gradients. The approach requires that the first column adsorbs few of the proteins present, whereas the last adsorbs almost all of them. This technique is not as generally applicable as the use of parallel columns.

In Chapter 20, the use of high throughput methods in the development of industrial-scale protein purification processes will be discussed.

1.4.3.2 The Order of the Chromatographic Steps A priori, one would expect that the order in which the different chromatographic steps are applied in a protein purification protocol is of minor importance. The total purification factor should be constant and the product of the factors obtained in each individual step should be independent of the other steps of the protocol. In the ideal case, where each chromatographic technique is utilized optimally with regard to the resolution and recovery, that is, within the linear regions of the adsorption isotherms (see Chapter 2), with adequate sample volume to column volume ratios, and with no adverse viscosity effects, this is probably true. However the real-life situations are always far from ideal or at least such that adaptation to ideality becomes highly impractical. For example, a fractionation gel filtration step can be optimized to give very high resolution (Chapter 3), but only at the cost of time and sample volume. To choose fractionation gel filtration as the first step, when the sample volume might be much larger than the total volume of the column, means repetitive injections and excessive and impractical total process times, which would probably also be deleterious to the proteins in the sample solution. Likewise, to choose AC on immobilized monoclonal antibodies as the first step would

probably result in an extraordinarily high purification factor. However, the high cost of such adsorbents prohibits the use of large columns, which makes repeated injections of sample in smaller columns almost mandatory. This leads to long process times and the risk of product losses and/or modifications due to proteolytic attack. Proteolytic activity can also threaten the stability and life length of the actual immunosorbent. Furthermore, protein-based adsorbents are difficult to maintain to a sufficiently high degree of hygiene. There are limitations with regard to means for regeneration (washing) and sterilization (Chapter 9). This is why they should be saved for the later steps of the purification protocol.

The consequence of these considerations is that there are a number of practical rather than theoretical reasons why one should choose certain chromatographic techniques (31) for the early steps and others for the final steps of a protein purification process. The choice is primarily governed by the following parameters:

- the sample volume
- the protein concentration and viscosity of the sample
- the degree of purity of the protein product
- the presence of nucleic acids, pyrogens, and proteolytic enzymes in the sample
- the ease with which different types of adsorbents can be washed free from adsorbed contaminants and denatured protein.

The last parameter governs the life length of the adsorbent and, together with its purchasing price, the material cost of the particular purification step.

In light of what has been said above, the logical sequence of chromatographic steps would start with more "robust" techniques that combine a concentration effect with high chemical and physical resistance and low material cost. The obvious candidates are IEC and, to some extent, HIC. As the latter often requires the addition of salt for adequate protein binding, it is preferably applied after salt precipitation or after salt displacement from IEC, thereby excluding the need for a desalting step. Thereafter, the protein fractions can preferably be applied to a more "specific" and more expensive adsorbent. The protocol is often finished with a gel filtration step (Fig. 1.3).

It is advisable to design the sequence of chromatographic steps in such a way that buffer changes and concentration steps are avoided. The peaks eluted from an ion exchanger can, regardless of the ionic strength, be applied to a gel filtration column. This step also functions as a desalting procedure, which means that the buffer used for the gel filtration should be chosen so as to allow direct application of the eluted peaks to the next chromatographic step. The different chromatographies have, in practice, widely different capacities, even though it is possible to adapt several of the

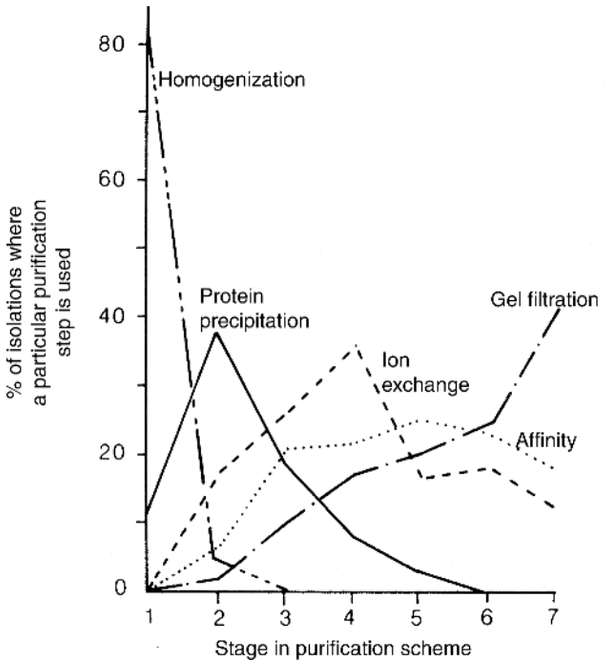


Figure 1.3 Analysis of the methods of purification used at successive steps in the purification schemes. The results are expressed as a percentage of the total number of steps at each stage. Adapted from Reference 31 by permission of the authors and publisher.

methods to a larger scale. However, in the initial stages of a purification scheme it is most convenient to start with the methods that allow the application of large volumes and which have the highest capacities. To this category belong IEC and hydrophobic interaction, but any adsorption chromatographic method can be used to concentrate larger volumes, especially in batchwise operations.

1.4.4 The Final Step

The purpose of the final step is to remove possible aggregates or degradation products and to condition the purified protein for its use or storage. The procedure will thus be different depending on the fate of the protein. Aggregates and degradation products are preferably removed by gel filtration. If the protein is to be lyophilized, this step is also suitable for transferring the protein to a volatile buffer (Table 1.1). This can sometimes be done by IEC, but more seldom by the other forms of chromatography. If the protein solution is intended to be frozen, stored as a solution, or used immediately the requirements for specific buffer salts might be less stringent.

Several of the adsorption chromatography steps might be designed in such a way that they result in peaks of reasonably high protein concentration. This is an advantage when gel filtration is chosen as a final step. Gel filtration will always result

in dilution of the sample and is therefore often followed by a concentration step.

If the protein is to be used for physical-chemical characterization, especially for molecular weight studies, gel filtration has the advantage of giving a protein solution of defined size and also in perfect equilibrium with a particular buffer. Biospecific methods, by definition, give a product that is homogeneous with respect to biological activity. This was taken advantage of for papain, where the enzyme eluted from a thiol column was twice as active as any previous preparation. Many of the early enzyme preparations apparently contained molecules in which the thiol necessary for activity was oxidized (see Chapter 8).

Proteins that, after purification and formulation, are intended for parenteral use in human beings must not contain endotoxins (lipopolysaccarides, LPS) or nucleic acids. The purification protocols must be designed so that these compounds are efficiently removed, and validation studies should be performed to prove this. To prepare sterile protein solutions, aseptic filtration is used.

1.5 MONITORING THE FRACTIONATION

Proper analysis is a prerequisite for successful protein purification. Most important is the establishment of a reliable assay of the biological activity. In addition, the protein content should be determined in order to be able to assess the efficiency of the different steps. It is beyond the scope of this chapter to go into details of the particular assay methods. This is covered by the special literature dealing with the activity in question—hormone, enzyme, receptor, and so on.

We recommend that each preparation be continually recorded in a purification table (Table 1.6). In combination with results from gel electrophoresis, for example, this will serve as a guide for judging the reproducibility and outcome of each preparation. In addition, each chromatography experiment should be accompanied by a suitable protocol such as the one exemplified in Figure 1.4. However, the need for measurements of biological activity and protein concentration—especially the latter—should not be allowed to delay the preparation, and in many cases it is sufficient to save aliquots for analysis at one's convenience.

1.5.1 Assay of Biological Activity

In general, biochemical activities depend on the interaction between molecules. This can be measured in different ways. The classical method of enzyme catalysis is only one of these. In addition, the monitoring of the components can be done in several ways, such as spectrophotometry, measurements of radioactivity, and immunological methods. Examples of these include the following:

- enzyme activity by direct spectroscopy
- enzyme activity by secondary measurements on aliquots
- binding of ligand
- binding of antibody.

The immunological methods require that the protein studied has already been purified once to allow production of an antibody or an antiserum by immunization. The detection of the antigen–antibody precipitate can be done at almost any sensitivity down to the extreme sensitivity afforded by the use of sandwich techniques and radioactively or enzymatically labeled reagents (e.g., Chapters 17 and 18).

1.5.2 Determination of Protein Content

In general, a measure of protein content is obtained upon monitoring the effluent in chromatography by UV absorption. However, it is not always easy to relate these measurements to the protein content. In fact, the only certain measure of protein content is total amino acid analysis after hydrolysis. Strictly speaking, even this latter analysis suffers from some shortcomings, because tryptophan and cysteine normally have to be analyzed separately.

Large deviations from true protein values sometimes occur in the first steps in a purification scheme. The extract itself often contains substances that interfere with the protein analyses. An overestimation might result, especially if measurements of absorption at 280 nm are used, because the solutions are often turbid and absorbing substances of nonprotein origin are present. This in turn will make the calculated values of specific activity erroneous.

TABLE 1.6 Example of a Purification Table

Material	Volume (mL)	Protein (mg/mL)	Total Protein (mg)	Activity (U)	Total Activity (U)	Spec. Activity (U/mg)	Yield (%)	Purif. Factor (fold)
Extract	500	14	7000	7	3500	0.5	100	1
First purif. step	50	10	500	60	3000	6	85	12

Activity (e.g., enzyme activity) is expressed as units, and specific activity as units per mg of protein (U/mg).

Chromatography Data Sheet

Date_____ **Chrom. No**_____

Column
Length _____ V_T _____ ml Column filling:
Diameter _____ V_0 _____ ml Adsorbent_____
C.s. area _____ V_{salt} _____ ml Gel_____
Packing etc _____ Lot No_____
_____ Preparaion_____
_____ _____

Equilibration of column
Buffer system or solvent_____ Conc_____mol/l
pH_____ Conductivity_____ Temp_____ ^oC
Detergent/Denaturant_____ Conc_____
Bacteriostat_____
Comments_____

Sample
Description_____ Designation_____
Amount_____mg Vol_____ml Conc_____ A_{280}_____A _____
Buffer or solvent_____
Specific Activity etc_____
Pretreatment_____

Elution
Only buffer ☐ Gradient system _____
_____
_____
Flow rate_____ml/h Prefraction_____ml Fraction size_____ml_____min
Comments_____

Analyses
280 nm ☐ pH ☐ Conductivity ☐ Volume ☐
Other reaction_____ Aliquote volume_____
Procedure_____
Comments_____

Fate of fractions
Pools_____
_____
Comments_____

Figure 1.4 Example of a chromatography protocol.

Three main procedures for protein determination are used routinely:

- spectrophotometry at 280 nm
- colorimetry by Lowry–Folin–Ciocalteau reagent
- dye binding with Coomassie Brilliant Blue G-250.

Each of these methods has its advantages and its disadvantages. UV-absorption measurements require knowledge of the extinction coefficient of the protein(s) to be measured. These vary widely. For example, in the low end there is serum albumin, with an optical density (OD) of 0.6 for a 1 mg/mL solution, and the extreme parvalbumin, with no

absorption at all in the 280 nm band. At the other extreme there is lysozyme, with an OD of 2.7 for a 1 mg/mL solution. These values arise due to a corresponding variation in the content of the aromatic amino acids tryptophan and to a lesser extent tyrosine. As a rule of thumb it is convenient to assume a mean extinction of 1.0 for a 1 mg/mL solution, and this is often sufficient for practical purposes. When the protein is purified, the extinction coefficient and the wavelength for maximum extinction should, however, be determined on a solution by spectral and amino acid analysis.

An alternative to measurements at 280 nm is the low wavelength measurements at 225 nm or below. This absorption is due to the peptide bond, which has a maximum at 192 nm but still a considerable absorption at 205 nm (50% of maximum or an OD of 31 for a 1 mg/mL protein solution) and at 220 nm an OD of 11. These measurements are, of course, even more sensitive to contamination and also require that buffers that are transparent in the low UV regions are used. The use of sensitive UV monitors at these wavelengths in chromatographic equipment thus allows an extreme sensitivity, but their use is not possible unless great care is taken to avoid contaminants and impurities in the buffer salts.

The Lowry methods (40) are less problematic but also less sensitive. Aliquots for analysis should have protein concentrations of 0.1 mg/mL or more. It is often a good alternative in the beginning of a purification procedure where direct UV measurement might be impossible due to turbid solutions. The same applies to the use of Coomassie Brilliant Blue. This method is 5–10 times more sensitive than the colorimetric one, but is more cumbersome to use (41, 42). The latter two methods are both destructive, whereas the UV method allows the sample to be recovered.

A complementary treatment of this topic can be found in Chapter 4, "Ion Exchange Chromatography" (Section 4.9.1).

1.5.3 Analytical Gel Electrophoresis

The gel electrophoresis techniques allow the investigator to get an idea of the complexity of the sample and, in particular, what the main contaminating species are. By using sieving electrophoresis, for example in the presence of SDS, and isoelectric focusing, a considerable amount of information about the sample can be obtained in a couple of runs. The amount of each component, its molecular weight, its isoelectric point and even its titration curve can be obtained (see Chapter 16). If an antiserum directed towards the complete mixture is available one can also see whether some of the components are immunologically related and thus also structurally related by means of crossed immunoelectrophoresis.

The gel electrophoresis techniques are introduced in Chapter 15.

1.6 THE FINAL PRODUCT

1.6.1 Buffer Exchange

The high resolution chromatographic steps for protein fractionation usually result in a product that is not directly suitable for the intended use, storage, or distribution. The salt content may be too high, the pH of the protein solution may be unsuitable for long-term storage, the concentration of the protein may be too low, or the solution may contain desorption agents from an affinity chromatography step that must be removed before the protein can be used.

The classical way of changing the buffer composition of a protein solution is dialysis. The protein solution is here included in a dialysis bag consisting of cellophane or a similar semipermeable material. Salts and low molecular weight substances can diffuse through the membrane, whereas high molecular weight material remains within the dialysis bag. The bag is placed in a larger stirred vessel containing the desired buffer, which is changed several times.

A faster way of changing the buffer composition of protein solutions is by gel filtration on, for example, Sephadex G25 or BioGel™ P-6 equilibrated in the desired buffer. Proteins and other high molecular weight substances (>6000) elute at the void volume, whereas substances with lower molecular weights are retarded and thus separated from the proteins. The method is fast and, depending on the equipment and volume, the cycle time is often less than one hour. As in every type of column chromatography, the protein solution must not contain particles or colloidal material.

Ultrafiltration (diafiltration) is a third way of changing the buffer composition of a protein solution. With this technique the protein solution is diluted with the desired buffer, concentrated to the original volume, diluted again, and so on. After a number of cycles the original buffer has in practice changed to the dilution buffer. The last concentration cycle may be driven longer so that the protein solution after the buffer change is concentrated.

1.6.2 Concentration

Concentration is another operation often required after the final step in a protein purification procedure. Ultrafiltration is the most frequently applied technique for this purpose (see Chapter 12). Smaller volumes of protein solutions can alternatively be concentrated by inclusion in a dialysis bag, which is covered with a high molecular weight substance that cannot penetrate the dialysis bag but creates an osmotic pressure that drives the liquid out through the dialysis membrane. Polymers that are often used for this purpose are PEG and Ficoll™ (43).

All chromatographic techniques that adsorb protein can also be used for the concentration of protein solutions. Especially suitable is IEC because of its high capacity and

easy handling of the ion exchange medium. Other concentration methods that have also proved useful in large-scale applications are freeze concentration (44) and concentration using dry Sephadex (45).

1.6.3 Drying

Most biological processes occur in water solution, and one way to stop these is to freeze the protein sample. For minimum risk of inactivation or denaturation a storage temperature of −70°C or below is required. If the protein under study cannot stand repeated freezing and thawing, storage in aliquots is recommended. Another way to stop biological processes is to remove the water. The method used most for biologically active proteins is freeze-drying or lyophilization. In this method the protein solution is frozen below the eutectic point of the solution to ensure that all liquid is frozen. The frozen solution is then placed in a chamber that can be set under high vacuum. In the chamber or connected to the chamber is a condensing surface, a cold trap, with a temperature of less than −40°C. After the vacuum is applied, the protein sample is gently heated so that it does not melt to speed up the sublimation of water to the condenser. Normally, all proteins maintain their biological activity and are fully recovered upon adding water. A technique often used for commercially available biochemicals is to lyophilize aliquots of protein solution in ampoules.

1.7 LABORATORY EQUIPMENT

1.7.1 General Equipment

Laboratories for the preparation and separation of proteins may look very different, from the well-equipped special laboratory serving many research groups to the small laboratory with few people and limited resources. The large laboratory, in addition, often has dedicated service groups for special analyses, and so on.

For the successful preparation and separation of proteins, certain basic equipment is needed. Standard laboratory glassware will not be discussed. In the category of basic equipment will be two or three balances, one spectrophotometer, one centrifuge, one pH meter, as well as stirrers and micropipettes.

A good combination of balance equipment comprises two preparative balances—one a double-range digital balance 0–1200 g or 0–3000 g, with the facility to weigh with a resolution of 10 mg, and the other with the range 0–120 g or 0–300 g, with a resolution of 1 mg—and one analytical balance for the interval 0–150 g with a resolution of 0.1 mg. For the measurement of larger amounts of material, a simpler, and therefore cheaper, balance will suffice, for example, a balance for use in the food industry. It is also important that the balances are calibrated and that they are serviced regularly.

Spectrophotometers for protein work should cover the wavelength interval 190–800 nm. Absorption around 280 nm is used routinely for estimating protein concentration, whereas light in the visible region is often needed for measurement of different enzymatic activities. A double-beam UV–vis spectrophotometer equipped with an adjustable slit and a thermostated cuvette holder is a good choice. It is important that the wavelength setting is correctly calibrated and that the instrument shows low drift. Regular servicing of the instrument is recommended.

A refrigerated floor centrifuge is standard in a preparative protein laboratory. The largest rotor should take six flasks of 500 mL, whereas the smallest rotor should accommodate eight tubes of 50 mL. The maximum *g*-force for those rotors are normally about 15,000*g* and 50,000*g* at the tube bottom, respectively. An additional rotor for six flasks of 250 mL will increase the flexibility of the centrifuge. Some centrifuges accommodate zonal rotors with accessories for continuous operation, allowing larger volumes containing relatively low contents of fine particles to be centrifuged at high *g*-forces (up to 40,000*g*). The standard centrifuges normally need no or little service except changing of motor brushes. If the rotors are carefully maintained, including thorough cleaning after each run when liquid is found inside the rotors, they will function for many years. The rotors mentioned above are angle rotors and the flasks are normally filled completely. The special, completely tight lids that are available for most centrifuge flasks are strongly recommended. For small, easily centrifuged samples, a simple desk-top centrifuge without cooling is often a good complement to the larger high speed refrigerated centrifuge.

pH determinations are critical in the protein laboratory. Buffers should routinely be checked and the pH adjusted if necessary (alkaline buffers may absorb carbon dioxide from air if stored over extended periods of time). For certain enzyme assays equipment for monitoring the formation or consumption of protons is needed. The pH meter can then be equipped with an automatic burette that adds acid or base to hold the pH constant, and the consumption of acid or base is recorded. Combination electrodes are generally the most convenient. The linearity of the electrode changes with time and for accurate measurements the electrode has to be calibrated on both sides of the interval within which the pH will be determined.

At least two types of stirrers are integral parts of the basic laboratory equipment. First, magnetic stirrers with variable speed, at least one of which is equipped with a thermostat-controlled heating plate, are recommended. A variety of polytetrafluoroethylene (PTFE)- and glass-coated magnets are required. For extractions, propeller stirrers with interchangeable propellers and variable speed regulators are convenient. These should preferably be of the electronic type, which compensate for variation in load. Stirrers where the speed is controlled only with a variable resistor should be avoided.

Pipetting small volumes, up to 1 mL, is a standard laboratory procedure. Micropipettes of different types, most commonly with adjustable volume and with disposable tips, are used. Two or three pipettes of different sizes are normally sufficient. Check that the length of the pipette allows samples to be collected from the bottom of the longest type of test tube used in the laboratory.

For personnel safety the laboratory must have a ventilated hood or fume cupboard where procedures involving handling or preparation of hazardous substances can be safely performed. Even such a simple and common procedure as dissolving sodium hydroxide in water creates aerosols that are very irritating, and this procedure should be done in the hood.

1.7.2 Equipment for Homogenization

Even if the majority of starting material for protein purification is today obtained from fermentation of microorganisms and animal cell lines (recombinant or nonrecombinant), plant and animal tissues are sometimes chosen. For these, "Waring Blenders" or similar types of equipment and "Turrax"-type apparatuses are frequently used for the homogenization. The material is first cut into pieces with a knife or a pair of scissors to a size that suits the type of blender used. At the same time, the tissue or organ is trimmed and unwanted material like fat, ligaments, and vessels are discarded before grinding the material in the blender. The blender can be used both dry and wet. Plant material like seeds, for example, can be ground dry if the time of grinding can be kept short. Dry ice can be added to keep the temperature low. Wet grinding prevents the formation of harmful dust particles and the heat formed in the grinding procedure is dissipated by the liquid. In most homogenization equipment only a small part of the energy input is normally used for the disintegration procedure, the rest of the energy is lost as heat. A common household meat grinder is also useful in many instances, in combination with a Waring blender. Further homogenization is provided by the Elvehjem pestle-type homogenizers. There are different types (glass, Teflon®), sizes, and also motor-driven varieties of this homogenizer.

Microorganisms such as bacteria and yeast are much more difficult to disintegrate. The polysaccaride and proteoglycan cell wall withstands most homogenization procedures used for plant and animal cells. The well-cited overview by Wimpenny (46) for breakage of micro-organisms is still valid for small-scale preparations (Fig. 1.5). For large-scale homogenization of microorganisms, which means kilogram quantities of cell paste, the most frequently used equipment

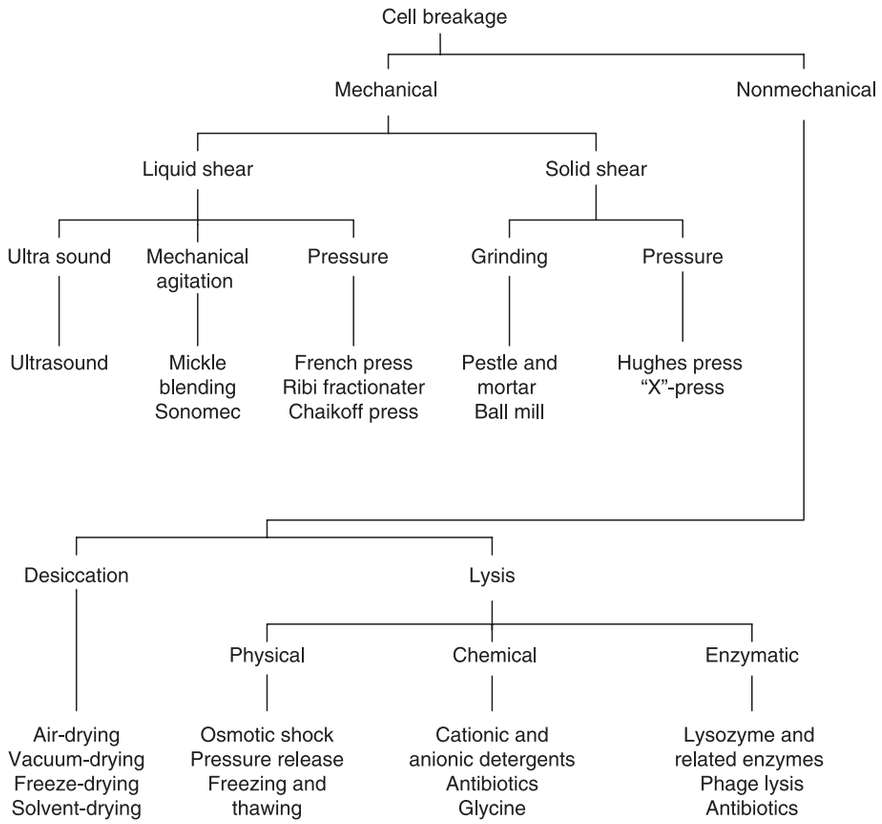


Figure 1.5 Principles and methods for cell breakage.

is the Dynomill (47) and the Manton–Gaulin (48) homogenizer. Both are designed for continuous operation and are available in various sizes for the breakage of from 100 g up to several kilograms of microbial cell paste per hour.

1.7.3 Equipment for Chromatography

1.7.3.1 Column Design In order to utilize the packed chromatographic gel particles optimally, the column should be designed so that it does not significantly contribute to band spreading or peak distortion. Most modern columns are of the closed type, with two fixed end pieces or with one fixed end piece and one adjustable adaptor. Sometimes it is convenient to use two adjustable adaptors, one at each end of the column. The end pieces or adaptors are equipped with porous frits made of, for example, sintered metal, glass, or plastic, the pore diameters and surface structures of which should be optimized to avoid clogging and abrasion of the chromatographic particles. The frits should be easily exchangeable. For larger-diameter columns the frits have to be combined with the flow distribution system. The prime requisites of such a system are that its volume should be negligible compared to the total column volume and that there should be no pressure drop in the system between the eluent inlet and the column wall. In many modern columns for low and medium pressure chromatography, the frits have been replaced by a combination of a fine mesh (10 μm) polyamide fabric and a coarse mesh (e.g. 0.93×0.61 mm) polypropylene support net. The flow distribution layer will then be only 1 mm in depth and the support net will reduce the dead volume by more than 30%. There is no indication that this uniform support net affects the sample application. This is the way many very large-diameter industrial columns are designed. For the largest columns (diameter, >400 mm) the fine mesh nylon fabric is in most cases replaced by a high quality stainless steel mesh. In most large-diameter industrial columns, the number of sample and eluent inlets and outlets have been increased to between four and six. The linear flow rate of the eluent in the column feed pipe is very high in large-diameter columns and it is recommended to prevent this jet from directly hitting the fine mesh fabric (when using a frit this problem is less pronounced), which could cause flow inhomogeneity and band distortion. The effect is reduced by the application of a small disc covering the support net under each pipe inlet.

The materials used in the parts of the chromatographic system that come into contact with the eluent should preferably be noncorrosive and compatible with all normal protein samples and eluent buffers/solvents used. However, they should also preferably be resistant to the conditions applied in the cleaning and maintenance of the packed gel particles. This includes high concentrations of sodium hydroxide and solvents such as alcohols. The cleaning-in-place (CIP) concept has become an important part of process design in biotechnology. In many instances, sterilization by autoclaving is preferred. Most laboratory column tubes are made of borosilicate glass or plastics with end pieces and adaptors made of polypropylene, nylon, or fluorocarbon. HPLC columns are normally made of stainless steel to withstand the mechanical stress.

The column is connected to the pump and to the monitors by small-bore tubing made of stainless steel or titanium in HPLC systems, or fluorocarbon, polypropylene, polyethene, or nylon in medium or low pressure systems. The inner diameters of the tubings should be optimized for each application. They should be as small as possible without too much contribution to the pressure drop of the system. To avoid zone mixing, the tube length should be reduced as much as possible. Ideally, the column should be attached directly to the monitor cell on top of the fraction collector. In normal laboratory systems fluorocarbon tubing with an inner diameter of 1 mm is recommended.

1.7.3.2 Pumps and Fraction Collectors The traditional laboratory-scale pumps for low pressure systems (0.1 MPa) are of the peristaltic type, of which several brands are on the market. Care must be taken to keep the rollers and pumping tube clean. Otherwise, the tube life length is severely decreased. Peristaltic pumps are available with flow rates of a few milliliters per hour to cubic meters per hour. The pumping tube is available in several different materials, but for the protein work silicone tubing is recommended. Pumps with three or more channels can be used for gradient formation. The normal way to form a gradient is to use two connected vessels, with stirring in the output mixing vessel, In medium and high pressure chromatography, gradients are usually formed using two pumps with individually adjusted speed connected to a programmable controller.

For HPLC, most pumps are of the piston type and made of stainless steel or titanium, as are the sample injectors. In most cases they function according to the reciprocal displacement principle and are capable of pressures up to 35 MPa or more. Among medium pressure (5 MPa) pumps is the positive displacement type, in which a fluorocarbon-sealed piston made of titanium or stainless steel is allowed to move in a thick-walled borosilicate glass tube. When the piston reaches the end of the tube, a valve switches over to a second identical piston-equipped glass tube filled with eluent, which will continue feeding the column with eluent at the same pressure while the first is filled. Unless proper damping is provided, a pressure transient will occur, giving rise to spikes in the UV-monitor recordings at each piston change.

Traditional fraction collectors consist of a large plastic or metallic ring with holes bored at the periphery, moved by an electric motor controlled by a timer. This type of fraction collector requires a relatively large bench area. Alternatives are compact designs with the holes for the test tubes arranged

in a spiral or with the test tubes placed in racks. These collectors are controlled by microprocessors, and different tube sizes and fraction collection times can be programmed in advance. In large-scale chromatography, when the results are normally predictable from pilot experiments on a small scale, only a few fractions are needed and the fractions are obtained by using magnetic valves connected to the controlling equipment.

1.7.3.3 Monitoring Equipment The classical way to monitor a chromatographic experiment is to take fractions from the outlet of the column in a fraction collector and analyze each tube manually for the different substances one wishes to determine. This is very time-consuming work, and flow monitors connected directly to the column eluate have largely replaced this practice today. The most commonly used parameter in protein work is absorption at 280 nm, and a lot of detectors are available for this purpose. In new diode array monitors, a spectrum can even be obtained directly on the eluate. In IEC, the ionic strength is of interest and conductometers with flow cells are available. The pH of the eluate can also be monitored in, for example, by chromatofocusing using continuously working monitors. Even complicated enzyme assays can be made directly on-line by the use of autoanalyzers. Here a small part of the process stream is shunted through the autoanalyzer. This part of the sample is normally destroyed during the analysis.

1.7.3.4 Chromatography Systems During the last decade the use of stand-alone (nonsystem) components has been in decline, and very few laboratories are using these "separates" nowadays. So many new scientists expect a complete out-of-box solution to perform their protein purification. Within the systems market there is a broad product offering in terms of levels of automation and sophistication.

The introduction of the FPLC System (GE Healthcare Life Sciences, Uppsala) in 1982 meant a new way of thinking in the design of dedicated chromatography equipment for protein separation and purification. For the first time, a microprocessor-controlled and straightforward system approach was launched in which the parts and components were matching the performance of a new chromatography material. Parts in contact with sample and buffers are made of glass, plastic, or titanium to minimize corrosion and nonspecific adsorption of the sample components. In combination with prepacked columns containing the 10-μm-diameter MonoBead™ ion exchangers, which give high performance separations at moderate pressure drops, the FPLC System became extremely popular and has since been installed in several thousand biochemical laboratories.

Many successors to the FPLC System have been introduced by several manufacturers. In 1998, ÄKTAexplorer™ (GE Healthcare Life Sciences, Uppsala) was launched, which is a preassembled chromatography system configured for fast and easy development and optimization of a variety of purification methods using the "Adviser" included in its UNICORN™ software. This contains method development templates for media screening and method scouting, covering IEC, HIC, RPC, AC, and gel filtration chromatography, as well as purification protocols for recombinant proteins from *E. coli*, peptides, and oligonucleotides. Included is also a general purpose method for column cleaning. When a prepacked column is selected from those listed in the data base, the system automatically sets the running parameters to those best suited to that particular column. This allows direct and optimal use of the column without any previous special knowledge or experience. An automatic buffer preparation function covering a broad pH range eliminates the manual buffer preparation and titration needed for every pH change, particularly in IEC. ÄKTAexplorer can also manage system control and data access via a PC network, which gives a complete overview of operations. Results can be automatically saved on a server, and evaluation and generation of reports can be made locally or at remote PCs. The pumps have an exceptionally wide flow rate operation range (0.01–100 mL/min in isocratic mode and 0.5–100 mL/min in gradient mode) at pressures of up to 10 MPa, previously not covered by a single chromatography system. The whole system is mounted on a swivel platform and requires minimal bench space.

Following the successful introduction of the ÄKTAexplorer system, GE Healthcare Life Sciences AB introduced ÄKTAprimeplus, ÄKTAxpress and, recently, ÄKTAavant. ÄKTAprimeplus can be considered to be an "entry-level" complete instrument for simple, push-button purification, thus ideal for new protein scientists. ÄKTAxpress meets the needs of the scientist who must obtain high purity proteins but does not have the time or

Figure 1.6 ÄKTA™ avant chromatography system. From GE Healthcare Life Sciences AB, Uppsala, Sweden. (See color insert.)

skill to construct multistep purification schemes. Thus it offers the user a very simple interface to create sophisticated two- or three-step purifications employing affinity, ion exchange, and gel filtration techniques.

ÄKTAavant (Fig. 1.6) is a new generation of ÄKTA instruments. It is designed for the process developer who realizes that speed (productivity), security of operation, and scalability are critical factors. To this end, ÄKTA avant is equipped with an integrated "Design of Experiments" tool, which assists the user in designing statistically robust experiments to screen, optimize and test their "design and operating space." Additionally, all buffers, using scalable buffer recipes, are titrated automatically and hence multiple pH environments can be automatically created for screening work.

A new software called UNICORN 6 enables the user to create chromatography methods with greater levels of complexity in quicker time than before. The temperature-controlled fraction collection is now built into the chassis of the instrument (giving greater control and minimizing the footprint), and all columns have a unique matrix code (Unitag^{TM} that is scanned into the system, providing full column tracking history to give greater operational safety. ÄKTAavant has been designed around, and optimized for, modern purification media intended to be used at a production scale. A new feature is also a flow velocity and pressure relationship function.

1.7.4 Equipment for Chromatographic and Electrophoretic Analyses

For characterization of the product at various stages of a protein preparation, in principle the same separation techniques that are used for preparation of the protein can be used. The techniques are, however, scaled down to the microgram-to-milligram scale, and sometimes a dedicated micropreparative chromatographic equipment is preferred (49). Even more important are analyses by electrophoretic separation techniques. Equipment and methods for the different electrophoretic techniques are described in connection with the techniques themselves in Chapters 15–19.

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