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PROTEIN

Second Edition

PURIFICATION

Principles,
High Resolution
Methods, and
Applications

Edited by

JAN-CHRISTER JANSON

LARS RYDÉN

Praise for the *First Edition* of *Protein Purification*:

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- Electrophoresis in gels
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- Protein mapping by two-dimensional polyacrylamide gel electrophoresis
- Protein elution and blotting methods
- Large-scale electrophoresis processes

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PREFACE

Since 1989, when the first edition of this book was launched, the development of biosciences has meant a revival of protein chemistry in the wake of the molecular biology revolution and the HUGO project. The total genome of baker's yeast is now sequenced, that of *E. coli* is not far behind, and within a not too distant future the feat of the total mapping of the human genome, which at the beginning seemed fictitious, is now within reach. This means that the attention of the world's bioscientific community will again, as in the 1960s and most of the 1970s, focus on the structure and function of the proteins. The PROTEOME era has thus begun, and with it follows the need of more efficient and more selective tools for the separation, isolation, and purification of the gene products, the proteins.

The development of new chromatographic separation media since 1989 has mainly been focused toward improvements demanded primarily by process development engineers in the biopharmaceutical industry. This has resulted in media with higher efficiencies, leading to shorter cycle times, primarily based on suspension polymerized styrene-divinylbenzene polymers with optimized internal pore size distributions, some allowing partial convective flow through the particles. This trend has received its ultimate solution in totally perfusive systems based on stacked membranes, or continuous "monolithic" columns made of cross-linked polymers, derivatized with various kinds of protein adsorptive groups. New composite media have been introduced primarily to increase the industrial applicability of size exclusion chromatography of proteins but also to increase binding capacity in, for example, ion exchange chromatography. The concept of "solid diffusion" in highly ionic group substituted composite media is still awaiting its physicochemical explanation.

The demand for systems allowing direct capture of target proteins directly from whole cultures or cell homogenates, resulting in fewer process steps and concomitantly higher yields, has led to a revival of the fluidized bed concept. However, now optimized with regard to the design of both media and columns by the introduction of the more efficient one cycle technique called expanded bed adsorption.

As long as scientists have been engaged in the isolation and purification of proteins from crude extracts, there has been a demand for media with higher adsorptive selectivities. The extremely high variability in protein surface structure as well as their wide range of functional stabilities, makes it necessary for every protein chemist to have a stock of several different separation media,

ion exchangers, hydrophobic interaction media, and a variety of general affinity media. Literature survey data presented in some of the chapters of this book reveal that on average somewhere between three and four steps are required to purify a protein to homogeneity. The hope for one-step purifications raised by the introduction of immobilized monoclonal antibodies has not yet been fulfilled. However, there is a renewed opportunity at hand to increase the selectivity of immobilized ligands in affinity chromatography and thus decrease the number of steps in the purification process. This opportunity has been raised by the recent rapid development in the design of a large variety of chemical and biological combinatorial libraries and high-speed screening technologies. It is easy to predict that over the next few years there will be an unprecedented number of new highly selective ligands, monospecific as well as group specific, introduced for the synthesis of new protein separation media.

Compared to the first edition of this book, there exists one additional chapter (Chapter 18) on large-scale electrophoretic processes. Three chapters (Chapters 15, 16, and 17) have been totally rewritten, Chapters 15 and 16 by new authors. Most other chapters have been thoroughly revised, and all have been updated regarding recent applications.

It is our hope that this new edition will receive the same overwhelmingly positive response as the first edition, and we would like to express our appreciation to Dr. Edmund H. Immergut and the staff of VCH Publishers, now John Wiley & Sons, Inc., for their patience and never-failing support of this project.

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PROTEIN PURIFICATION

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 three decades. Improvements in materials and utilization of microprocessor-based instruments have made protein separations more predictable and controllable, although to many they are still 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. Also, new electrophoretic techniques and systems for fast analysis of protein composition and purity have contributed to increased efficiency in the evaluation phase of the purification process.

In the area of chromatography, the development of new porous resin supports, new cross-linked beaded agaroses, and new bonded porous silicas has enabled a rapid growth of high-resolution techniques [high-performance liquid

chromatography (HPLC) and fast protein liquid chromatography (FPLC)] on an analytical and laboratory preparative scale as well as of industrial chromatography in columns with bed volumes of several hundred liters. The introduction of expanded bed adsorption has made it possible to rapidly isolate target proteins 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 only. Data obtained are efficiently exploited by recombinant deoxyribonucleic acid (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 based on low pressure media (90- to 100- μm beads), medium pressure media (30–50 μm), and high pressure media (5–10 μm) to satisfy 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 are discussed in this introductory chapter. There is also an overview of different protein separation techniques and their principles of operation. In the subsequent chapters, each individual technique is discussed in more detail. Finally, some basic equipment necessary for efficient protein purification work is 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.^{1–4} In *Methods in Enzymology*, especially Volumes 22, 34, 104, and 182,^{5–8} a number of useful reviews and detailed application reports can be found. Also the booklets available from manufacturers of separation equipment and media can 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 an enzyme, and the origin of this activity is often of little importance. Great care should then be taken in the selection of a suitable source for the enzyme. 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 ac-

tivities and proteins, and difficulties in handling a particular raw material. 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. One of the authors of this chapter had difficulty in obtaining guinea pig brains for the preparation of histamine *N*-methyltransferase. It turned out to be quite easy to prepare the same enzyme from pig kidney as an inexpensive and easily available source. In an attempt to study the subunit structure of human ceruloplasmin, a major difficulty was its sensitivity to proteolytic degradation. Here it was demonstrated that porcine ceruloplasmin is quite similar, is present in higher concentrations, and is not nearly as sensitive to proteases.

The traditional animal or microbial sources may today be replaced by genetically engineered microorganisms or cultured eukaryotic cells. Protein products of eukaryotic origin, cloned and expressed in bacteria such as *Escherichia coli*, may either be located in the cytoplasm or be 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 resuspended cell paste. Already at this stage, a considerable degree of purification is thus achieved by choosing a secreting strain as illustrated in Figure 1-1.

1.2.2 Extraction Methods

Some biological materials constitute themselves a clear or nearly clear protein solution suitable for direct application to chromatography columns after centrifugation or filtration. Some examples are blood serum, urine, milk, snake

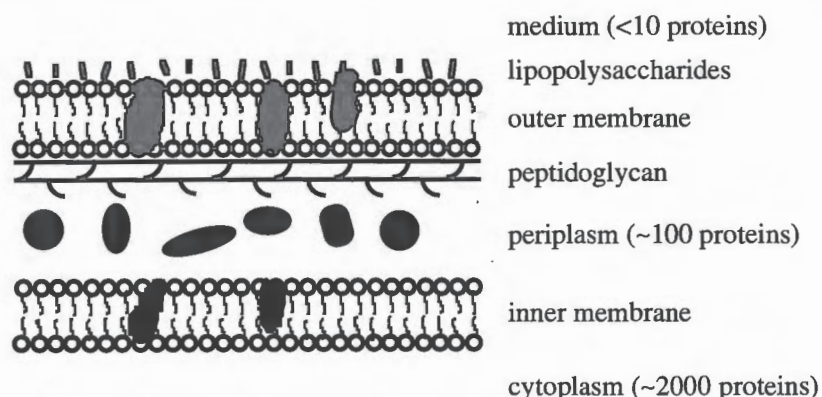


FIGURE 1-1. Location and approximate numbers of the proteins in *Escherichia coli*. (Courtesy of S. Ståhl, Royal Institute of Technology, Stockholm, Sweden.)

venoms, and—perhaps, most important—the extracellular medium after cultivation of microorganisms and mammalian cells as mentioned previously. It is normally an advantage to choose such a starting material because of the limited number of components and since extracellular proteins are comparatively stable. Some samples (e.g., urine or cell culture supernatants) are normally concentrated before purification begins.

In most cases, however, one has to extract the desired activity from a tissue or a cell paste. This means that a considerable number of contaminating molecular species are set free and that 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 article by Kula and Schutte.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. Because the number of parameters can be very large, this part of the work has to be kept within limits by proper judgment. It is, however, not recommended to accept a single successful experiment. Further investigations of, in particular, the extraction time required often pay in the long run.

Major problems in preparing a protein are in general denaturation, proteolysis, and contamination with pyrogens, nucleic acids, bacteria and viruses. These can be limited by the proper choice of extraction medium as this chapter shows. However, many of the previous problems can be reduced by short preparation times and low temperature. It is thus 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.

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

A common phenomenon when working with intracellularly expressed recombinant DNA proteins is their tendency to accumulate as insoluble aggregates (i.e., inclusion bodies, refractile 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 providing low-speed fractional centrifugation and washing of resuspended sediment. The critical step is solubilization and refolding, often combined with chromatographic purification under denaturing conditions in the presence of high concentrations of urea or guanidine hydrochloride. This area has been reviewed by Marston.¹²

1.2.3 Extraction Medium

To arrive at a suitable composition for the extraction medium, the conditions in which the protein of interest is stable must first be studied. Second, the conditions at which the protein is most efficiently released from the cells or tissue needs to be considered. The final choice is usually a compromise between maximum recovery and maximum purity. The following factors have to be taken into consideration:

1. *pH*. Normally the pH value chosen is that of maximum activity of the protein. However, it should be noted that this is not always the pH that gives the most efficient extraction or is it necessarily the pH of maximum stability. For example, trypsin has an activity optimum at pH 8 to 9 but is much more stable at pH 3, where autolysis is avoided. The use of extreme pH values, (e.g., for the extraction of yeast enzymes in 0.5 M ammonia) is sometimes very efficient and is acceptable for some proteins without causing excessive denaturation.

2. *Buffer salts*. Most proteins are maximally soluble at moderate ionic strengths, 0.05 to 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

TABLE 1-1. Buffer Salts Used in Protein Work

Buffer	pK_a -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-chloride	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 .

as such also act as buffers, and the pH should be checked after the 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.

3. *Detergents, 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, the hydrophobic interactions should be reduced 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 at all times. One is then actually purifying a protein-detergent complex. More information about detergents, including their chemical structures, can be found in Ref. 13.

Detergents are amphipatic molecules. When their concentration increases, they will eventually form micelles at the so-called critical micelle concentration (CMC). Since micelles often complicate purification procedures, particularly column chromatography, concentrations below the CMC should be used.

Instead of detergents for dissolving aggregates, chaotropic agents such as urea or guanidine hydrochloride, or moderately hydrophobic organic compounds, such as ethylene glycol, could be used. Urea and guanidine hydrochloride have proven particularly useful for the extraction and solubilization of inclusion bodies.¹²

4. *Reducing agents.* The redox potential of the cytosol is lower than that of the surrounding medium where atmospheric oxygen is present. Intracellular

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		
Lubrol PX	Nonionic		0.006
Octyl glucoside	Nonionic		0.73
Tween 80	Nonionic		0.002
Sodium deoxycholate	Anionic	Strongly denaturing	0.21
Sodium dodecyl sulfate	Anionic		0.23
3-[(3-Cholamidopropyl) dimethyl amino]	Zwitterionic		1.4
propanesulfonic acid			

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), 1,4-dithiothreitol (DTT), or mercaptoethanol (see Table 1-3). Normally 10 to 25 mM concentrations are sufficient to protect thiols without reducing internal disulfides. In other cases, a higher concentration might be needed.¹⁴

5. *Chelators or metal ions.* The presence of heavy metal ions can be detrimental to a biologically active protein mainly for two reasons. They can enhance the oxidation of thiols by molecular oxygen and they 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 to 25 mM. An alternative is ethyleneglycol-O,O'-bis(2-aminoethyl)-N,N,N',N' tetraacetic acid (EGTA), which is more specific for calcium. It should be noted that EDTA is a buffer. It is therefore best to add the disodium salt of 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. The divalent ions calcium and magnesium are trapped by EDTA and cannot be used in combination with this chelator.

6. *Proteolytic inhibitors.* The most serious threats to protein stability are the omnipresent proteases. The simplest safeguard against proteolytic degradation is normally to work fast in the cold. An alternative or added precaution is to add protease inhibitors (Table 1-4), especially in connection with the extraction step. Often there is a need for a combination of inhibitors (e.g., for both serine proteases and metalloproteases). In general, protein inhibitors are expensive chemicals that 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 system¹⁵ or by adsorption of the proteases to hydrophobic interaction adsorbents.¹⁶ Sometimes it is sufficient to adjust

TABLE 1-3. Reducing Agents

Agent	Structure
Mercaptoethanol	HS—CH ₂ —CH ₂ —OH
1,4-Dithioerythritol	$ \begin{array}{c} \text{CH}_2\text{SH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{CH}_2\text{SH} \end{array} $
1,4-Dithiotreitol	$ \begin{array}{c} \text{CH}_2\text{SH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{CH}_2\text{SH} \end{array} $

TABLE 1-4. Proteolytic Inhibitors

Inhibitor	Enzymes	Working Concentration
Diisopropyl fluorophosphate (DFP)	Serine proteases	Avoid DFP
Phenylmethylsulfonyl fluoride	Serine proteases	0.5–1 mM
EDTA	Metal-activated proteases	Around 5 mM
Cysteine reagents	Cysteine-dependent proteases	
Pepstatin A	Acid proteases	1 μ M
Leupeptin	Serine proteases	1 μ M

the pH to a value at which the proteases are inactive, but where the stability of the protein to be purified is maintained. The classic example is the purification of insulin from pancreas.

7. *Bacteriostatics.* It is wise to take precautions to avoid bacterial growth in the protein solutions. As mentioned in the previous paragraph on proteolytic inhibitors, the simplest remedy here is to use sterile-filtered buffer solutions as a routine in the laboratory. This will also reduce the risk of bacterial growth in columns. A common practice to avoid 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, (e.g., phosphate, acetate, and carbonate buffers at neutral pH values). Buffers at pH 3 and below or 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 azide at 0.001 M or merthiolate at 0.005% and alcohols such as *n*-butanol at 1%. 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 OVERVIEW OF FRACTIONATION TECHNIQUES

In early work, complex protein mixtures were fractionated mainly by extraction and precipitation methods. These methods are still used as preliminary steps for crude fractionation or to obtain more clear or more concentrated sample solutions. Preparative electrophoretic and chromatographic techniques developed during the 1950s and 1960s, made rational purification protocols possible and laid the foundation for the current situation. This section gives a short overview of the various techniques normally used in preparative biochemical work. Chapter 2 contains an introduction to chromatography, and

Chapter 12 gives an introduction to electrophoresis. Each individual chromatographic and electrophoretic separation technique is then treated in detail in subsequent chapters.

1.3.1 Precipitation, Extraction, and Centrifugation

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 property of a particular salt as a precipitation agent is described by 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 $\text{C}(\text{NH}_2)_3^+$

The *antichaotropic* salts on the left-hand side are the most efficient salting out agents. They increase the hydrophobic effect in the solution and promote protein aggregation by association of hydrophobic surfaces. The chaotropic salts on the right-hand side in the series decrease the hydrophobic effect, thus helping maintain the proteins in solution.

Organic solvents 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, proteins are chromatographed in solutions that contain up to about 50% organic solvent, often with retention of 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 6,000 or 20,000. The main advantage of PEG over

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	Polymer	Uncharged, unflammable

organic solvents is that it is more easily handled. It is inflammable, it is not poisonous, it is uncharged, and it is inexpensive. Rather low concentrations are required (often less than 25%) to precipitate most proteins. One disadvantage is that concentrated solutions of PEG are highly viscous. Polyethylene glycol can also be difficult to remove from protein solutions. However, after dilution with buffer the viscosity decreases, and since the substance is uncharged, the solution may be applied directly to an ion-exchange column to further separate the proteins and to remove the polymer.

pH adjustment has been used as a simple and inexpensive 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.

In addition to pH, another parameter that influences precipitation of proteins in salt solutions is temperature (see as follows). 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

$$\log S = B - K \cdot c$$

where S = the solubility of the protein in g/L of solution

B = intercept constant

K = salting out constant

c = 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 the 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, as a major part of the protein simply remains in solution. Reproducible results can only be achieved if all parameters mentioned previously, 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 mostly 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 to 2% agarose separates proteins mainly according to their net electric charge. Electrophore-

sis in gels such as polyacrylamide separates mainly according to the molecular size of the proteins.

Analytical gel electrophoresis requiring microgram amounts of proteins is an important tool in bioscience and biotechnology (see Chapter 12). Convenient methods for the extraction of proteins after electrophoresis have been developed, especially protein blotting (see Chapter 16), 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, for example, 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 gram 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 the 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, to allow sufficient cooling, should not exceed approximately 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, in addition to gel filtration, the mildest separation technique available for proteins. It can be recommended for special situations, but practical aspects and the excessive time required preclude routine use. Methods for large- and medium-scale preparative electrophoresis have been developed (e.g., the flowing curtain electrophoresis of Hannig¹⁹ and, more recently, the "Biostream" apparatus of Thomson²⁰). For details, see Chapter 18.

Isoelectric focusing, the other main electrophoretic technique, separates proteins according to their isoelectric points. 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 focusing²¹ 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; however, 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. However, recent developments have led to new preparative electrophoretic techniques and new types of instrumentation, as discussed in Chapter 18.

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, whereas 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 Sober²³ 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 are listed below, together with the chromatographic method where they dominate the separation:

1. Size and shape—gel filtration
2. Net charge and distribution of charged groups—ion-exchange chromatography
3. Isoelectric point—chromatofocusing
4. Hydrophobicity—hydrophobic interaction chromatography and reversed-phase chromatography
5. Metal binding—immobilized metal ion affinity chromatography
6. Content of exposed thiol groups—covalent chromatography
7. Biospecific affinities for ligands, inhibitors, receptors, antibodies, etc.—affinity chromatography

The methods often have very different requirements with regard to chromatographic conditions. This applies to ionic strength, pH, and various additives such as detergents, reducing agents, and metals. By proper 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 ion-exchange chromatography, the charge interaction is the dominant parameter, but molecular weight and hydrophobic effects can also contribute to some degree, depending on the experimental conditions.

Highly specific methods, such as those based on bioaffinity, (e.g., antibody-antigen interaction), do in some cases give a highly pure protein in a single step. Normally, however, one has to combine several chromatographic methods to

achieve complete purification of a protein from a crude biological extract. With the wide variety of chromatographic media available today, this can normally be done in a short period of time.

All of the chromatographic methods mentioned previously are treated in Part II, 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 adsorption²⁶ in which a specially designed agarose/crystalline quartz composite adsorbent, and ditto column sample distributor plate, stabilizes the fluidized bed to the extent that a plug flow situation prevails during the sample application cycle. This enables a high binding efficiency in a single sample application cycle. Stabilization is achieved by the formation of an adsorbent particle size and density gradient in the column (Fig. 1-2). The pressure drop required to keep a steady liquid flow through the expanded bed is accomplished by the design of the sample distributor plate at the column bottom inlet.

In a typical expanded bed adsorption experiment the adsorbent particles are poured into the column to a sedimented bed height of 15 cm. The particles had previously been equilibrated to the desired pH and salt concentration. The equilibration buffer is then pumped continuously upward through the bed at a flow rate of 300 cm/h, resulting in a bed expansion two- to threefold. After approximately 30 min the stabilization of the bed is complete, (i.e., the size and density gradient have been established). The sample is now applied at the same flow rate and, because of the higher viscosity, the bed expansion will increase a little. The upper adaptor of the column is kept a few centimeters above the bed level. After all the sample is applied, the column is washed with the equilibration buffer until all cells, cell debris, and nonadsorbed protein are removed. The upper adaptor is hydraulically forced downward in the column, concomitantly packing the bed. Elution of the adsorbed proteins takes place as in normal chromatography either stepwise or by applying a salt or pH gradient followed by regeneration and reequilibration. The adaptor is then hydraulically returned to its original upper position, and the bed is expanded before the next sample application cycle. The procedure is illustrated in Figure 1-3 and a photo of an experimental setup is shown in Figure 1-4.

Soon after its commercial introduction in 1993, expanded bed adsorption on ion-exchange media such as STREAMLINE® DEAE²⁷ became widely

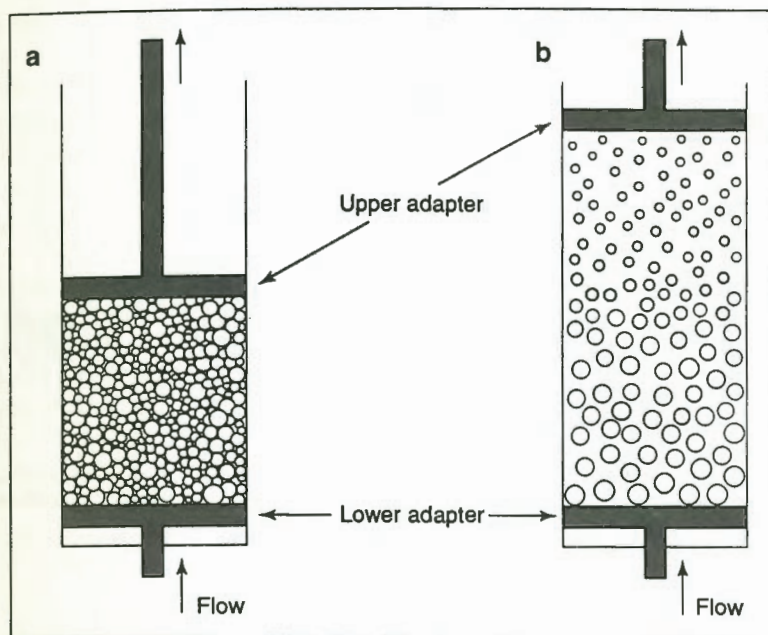


FIGURE 1-2. The difference between a packed bed (a) and an expanded bed (b) for protein adsorption. In the expanded bed, a homogeneous steady flow of liquid from the lower adaptor forces the adsorbent particles to rise to a position where their sedimentation rate is counterbalanced by the upward flow. Each size and density class of particles will find a stable equilibrium point at which they will only oscillate, neither rise nor sediment. A solid-phase gradient is thus formed, with the largest and most dense particles accumulating at the bottom and the smallest and less dense particles at the top of the expanded bed. The functional consequence of this is that a sample of whole-cell culture or crude cell homogenate will move upward by plug flow without distortion of the expanded bed, enabling efficient binding of target molecules to the gel particles in one cycle. (Reproduced by permission of author and publisher.²⁶)

accepted primarily by bioprocess engineers in the biopharmaceutical industry who saw the possibility of reducing the number of steps in the purification process, saving capital investment, and increasing product yield. Thus recombinant human serum albumin produced in the yeast *Pichia pastoris* was successfully isolated directly from a heat-treated, whole-cell culture on a 1-m-diameter STREAMLINE SP column.²⁸ Other published applications refer to the capturing of modified recombinant *Pseudomonas aeruginosa* expressed in the periplasm of *E. coli*, using STREAMLINE DEAE,²⁹ and to the purification of recombinant human placental annexin V expressed intracellularly in *E. coli* with the same adsorbent.³⁰ In 1996 more than 100 pilot scale columns for expanded bed adsorption were in operation.

Another important application area for expanded bed adsorption is the capturing of monoclonal antibodies directly from hybridoma cell cultures. To

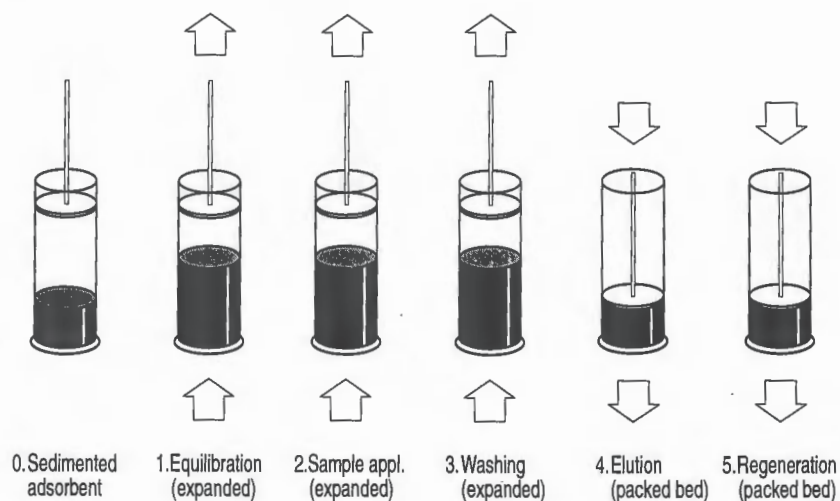


FIGURE 1-3. Schematic illustration of an expanded bed adsorption cycle.



FIGURE 1-4. Setup of an expanded bed adsorption experiment using a 200-mm-diameter STREAMLINE column. The column is supplied with STREAMLINE rProtein A for the capturing of monoclonal antibodies directly from a whole hybridoma cell culture. (Courtesy of Pharmacia Biotech AB, Uppsala, Sweden.)

this end, a specific adsorbent has been developed based on an agarose/metal alloy composite gel to which a new type of recombinant protein A has been attached through an *in vivo*-fused N-terminal cysteine. This new adsorbent, STREAMLINE rProtein A, was used for the capturing of a humanized IgG₄ directly from a 100-L fermenter.³¹

1.3.5 Membrane Chromatography

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 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.³²

1.4 FRACTIONATION STRATEGIES

1.4.1 Introductory Comments

Before attempting to design a protein purification protocol, one should collect as much information as possible about the characteristics of the protein and preferably also about the properties of the most important impurities. Useful data involve approximate molecular weight and pI, degree of hydrophobicity, presence of carbohydrate (glycoprotein) or free sulfhydryl groups, and so on. 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.

One should also establish criteria with regard to the stability of the protein to be purified. 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.

According to a study of 100 published successful protein purification procedures,³³ 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 filtrations.

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 solution suitable for chromatography 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 a solution suitable 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 be different depending on the material at hand and the purpose. If the starting material is very precious, one should favor high yield over speed and convenience. If one wants to extract several different proteins from a starting material, that will of course also influence the planning of the work. Finally, the final step is designed such that the product will be suitable for its purpose, which can vary. These aspects are 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 celite, either batchwise or on a short column.

Similarly, *lipid* material can be removed either by centrifugation, where 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 classic 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.³⁴ Another way to solve the problem is to add nucleases, which cut the nucleic acids into smaller pieces, thereby reducing the viscosity.

1.4.2.1 Clarification by Centrifugation and Microfiltration The clarification of any cell homogenate is usually no problem on a laboratory scale, where refrigerated high-speed centrifuges covering operating speeds from 20,000 to 75,000 rpm generating from about $40,000 \times g$ to about $500,000 \times g$ can

be used. A useful review of centrifugation and centrifuges in preparative biochemistry is found in Ref.³⁵ As a complement to centrifugation, tangential or cross-flow microfiltration has received increased attention, especially for large-scale applications. For a review of the advantages of cross-flow microfiltration, see Ref.³⁶

1.4.2.2 Ultrafiltration Ultrafiltration has become a widely used technique in preparative biochemistry. Ultrafiltration membranes are available with different cutoff limits for separation of molecules from 1,000 Da up to 300,000 Da. The method is excellent for the separation of salts and other small molecules from a protein fraction with a higher molecular weight and at the same time can effect a concentration of the proteins. The process is gentle, fast, and comparatively inexpensive. For a review, see Ref.³⁷

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, at the same time, 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 classic means is to make a fractional precipitation. First, bulk proteins in the solution are 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 (e.g., hydrophobic interaction adsorption of proteins in ammonium sulfate solutions and ion-exchange chromatography 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 1-5.

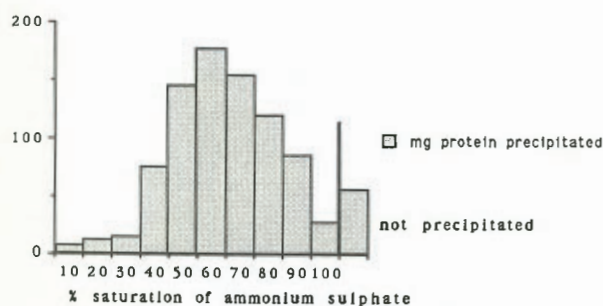


FIGURE 1-5. Example of a precipitation curve. Amount of protein precipitated by a stepwise increase of the ammonium sulfate concentration.

Of the various methods available for protein precipitation, ammonium sulfate has some disadvantages. The resulting protein solution often needs to be dialyzed to obtain an ionic strength that allows ion-exchange chromatography. This problem is avoided when using PEG. Organic solvents, particularly ethanol and acetone, often produce extremely fine, powder-like precipitates that are difficult to centrifuge and handle. They have also 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.³⁸ 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 become 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. By covalent attachment of affinity ligands to PEG molecules these can be used for affinity partitioning. This technique is dealt with in detail in Chapter 11.

1.4.3 Chromatographic Steps

1.4.3.1 Choice of Adsorbent The first information of the chromatographic behavior of a protein is often obtained most simply by preliminary analytical scale experiments (e.g., by gel filtration and by ion-exchange chromatography using salt and pH gradients). In these runs, 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.

The parallel column approach has been developed by Scopes³⁹ 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 (280-nm absorption) and the activity in the effluent were measured. Then a predetermined terminating buffer was applied to each column and again the protein and enzyme activity in the effluent were determined. A column where the bulk of the proteins, but not the activity, was adsorbed was chosen as a “minus column” while 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 a similar approach, a panel of parallel

columns was used earlier by, for example, Shaltiel⁴⁰ for the evaluation of hydrophobic adsorbents. The technique can, however, be used for any setup 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 setup 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 immobilized metal ion (IMAC) adsorbents⁴¹. 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.

1.4.3.2 Order of 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, 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 affinity chromatography 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

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(Chapter 10). This is why they should be saved for 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 techniques³³ for the early steps and others for the final steps of a protein purification process. The choice is primarily governed by the following parameters:

1. The sample volume
2. The protein concentration and viscosity of the sample
3. The degree of purity of the protein product
4. The presence of nucleic acids, pyrogens, and proteolytic enzymes in the sample
5. 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 earlier, the logical sequence of chromatographic steps would be to start with more “robust” techniques that combine a concentration effect with high chemical and physical resistance and low material cost. The obvious candidates are ion-exchange chromatography and, to some extent, hydrophobic interaction chromatography. 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 ion-exchange chromatography, 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 (Figure 1-6).

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. Different chromatographies have, in practice, widely different capacities, even though it is possible to adapt several of the 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 that have the highest capacities. To this category belong, for example, ion exchange chromatography and hydrophobic interaction, but any adsorption chromatographic method can be used to concentrate larger volumes, especially in batchwise operations.

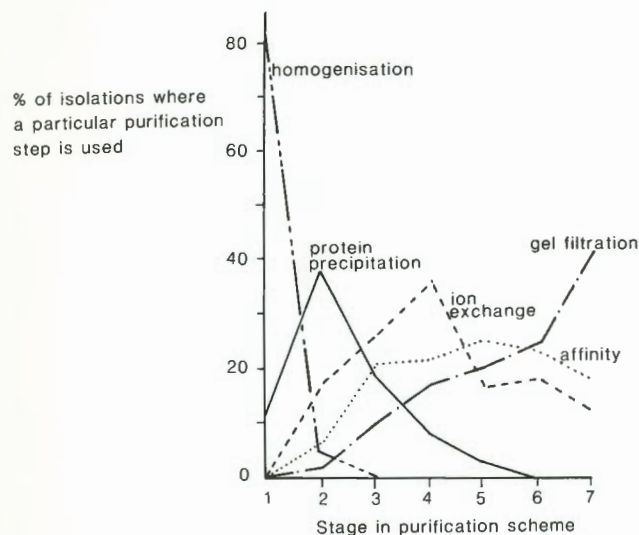


FIGURE 1-6. Analysis of the methods of purification used at successive steps in purification schemes. The results are expressed as a percentage of the total number of steps at each stage. (Adapted from Ref. 33 by permission of the authors and publisher.)

1.4.4 The Final Step

The purpose of the final step is to remove possible aggregates or target protein molecules that have been posttranslationally modified 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 low molecular weight degradation products are preferably removed by gel filtration and, if the protein is to be lyophilized, this step is also suitable for transferring the protein to a volatile buffer (see Table 1-1). This can sometimes be done by ion-exchange chromatography, but more seldom by 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 chromatographies might be adapted 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 often followed by a concentration step.

If the protein is to be used for physicochemical 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—most earlier enzyme batches apparently contained molecules in which the thiol necessary for activity was oxidized (see Chapter 9).

Proteins which after purification and formulation are intended for parenteral use in human beings should not contain endotoxins (lipopolysaccharides) or nucleic acids. The purification protocols must be designed such that these compounds are efficiently removed, and validation studies should be performed to prove this. To prepare sterile protein solutions, sterile filtration is used. For difficult to remove modifications of the target protein, more highly resolving techniques, such as reversed phase high performance liquid chromatography, are often applied (see Chapter 6).

1.5 MONITORING 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, one needs to determine the protein content 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 special literature dealing with the activity in question—hormone, enzyme, receptor, and so on.

Each preparation should be continually recorded in a purification table (Table 1-6). In combination with results from, for example, gel electrophoresis, this will serve as a guide for judging the reproducibility and the outcome of each preparation. In addition, each chromatography experiment should be accompanied by a suitable protocol such as the one exemplified in Figure 1-7. 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 Measurements of Activity

In general, biochemical activities depend on the interaction between molecules. This can be measured in different ways. The classic method of enzyme

TABLE 1-6. Examples of a Purification Table

Material	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activ. (U/ml)	Total Activ. (U)	Spec. Activ. (U/mg)	Yield (%)	Purific. (X)
Extraction	500	14	7,000	7	3,500	0.5	100	1
First purif. step	50	10	500	60	3,000	6	85	12

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

Chromatography Data Sheet

Date.....		Chrom. No.....	
Column			
Length.....	cm	V_T	ml
Diameter.....	cm	V_0	ml
C.s. area.....	cm ²	V_{salt}	ml
Packing etc.....		Column filling:	
.....		Adsorbent.....	
.....		Gel.....	
.....		Lot No.....	
.....		Preparation.....	
.....			
Equilibration of column			
Buffer system or solvent.....		Conc.....mol/l	
pH.....	Conductivity.....ohm ⁻¹ cm ⁻¹	Temp.....°C	
Detergent/Denaturant.....		Conc.....	
Bacteriostat.....		Conc.....	
Comments.....			
Sample			
Description.....		Designation.....	
Amount.....	mg	Vol.....	ml
Conc.....		A ₂₈₀ A.....	
Buffer or solvent.....			
Specific activity etc.....			
Pretreatment.....			
Elution			
Only buffer ☐		Gradient system.....	
.....			
Flowrate.....ml/h			
Prefraction.....		ml	
Fraction size		ml.....min	
Comments.....			
Analyses			
280 nm ☐		nm ☐	
pH ☐		Conductivity ☐	
Volume ☐		Other reaction.....	
Aliquote volume.....			
Procedure.....			
Comments.....			
Fate of fractions			
Pools.....			
.....			
Comments.....			

FIGURE 1-7. Example of a chromatography protocol.

catalysis is only one of these. In addition, the monitoring of components can be done in several ways, such as spectrophotometry, measurements of radioactivity, and immunological methods. Some of them are:

1. Enzyme activity by direct spectroscopy
2. Enzyme activity by secondary measurements on aliquots
3. Binding of ligand
4. Binding of antibody

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 (see, e.g., Chapter 14).

1.5.2 Determination of Protein Content

In general, a measure of protein content is obtained upon monitoring the effluent in chromatography by ultraviolet (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, as 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 protein analyses. An overestimation might result, especially if measurements of absorption at 280 nm are used, as 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.

Three main procedures for protein determination are used routinely:

1. Spectrophotometry at 280 nm
2. Colorimetry by Lowry–Folin–Ciocalteu reagent
3. 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. In the low end there is, for example, serum albumin with an 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, for example, lysozyme with an OD of 2.7 for a 1-mg/ml solution. The values are 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 are 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 has 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 they also require that buffers that are transparent in 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 methods⁴² 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 where direct UV might be impossible due to turbid solutions. The same applies to the use of Coomassie brilliant blue. This method is 5 to 10 times more sensitive than the colorimetric one but is more cumbersome to use.^{43,44}

Both of the latter two methods are destructive, whereas the UV method allows the sample to be recovered.

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, (e.g., 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 13). If an antiserum directed toward the complete mixture is available, it is also possible to 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 12.

1.6 THE FINAL PRODUCT

1.6.1 Buffer Change

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 classic way of changing the buffer composition of a protein solution is dialysis. The protein solution is included in a dialysis bag consisting of cellophane or a similar semipermeable material. Salts and low molecular weight substances ($<10,000$) 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 ($>6,000$) eluate at the void volume, whereas substances with lower molecular weights are retarded and are separated from the proteins. The method is fast, depending on the equipment and volume; the cycle time is often less than 1 h. 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. Smaller volumes of protein solutions can alternatively be concentrated by inclusion in a dialysis bag that 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. Often used polymers for this purpose are polyethylene glycol and Ficoll.⁴⁵ All chromatographic techniques which adsorb protein can also be used for the concentration of protein solutions. Especially suitable is ion exchange chromatography due to its high capacity and easy handling of the ion exchange medium. Other concentration methods that have proved useful also in large scale applications are freeze concentration⁴⁶ and concentration using dry Sephadex.⁴⁷

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 denatur-

ation, 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 $<-40^{\circ}\text{C}$. After the vacuum is applied the protein sample is gently heated such 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 is not discussed. Basic equipment includes two or three balances, one spectrophotometer, one centrifuge, and one pH meter plus stirrers and micropipettes.

A good combination of balance equipment is two preparative balances: one double-range digital balance 0 to 1200 g or 0 to 3000 g with the possibility to weigh with 10-mg resolution and the other range 0 to 120 g or 0 to 300 g with 1-mg resolution; and one analytical balance for a 0- to 150-mg interval with 0.1-mg resolution. For measuring larger amounts of material, a simpler, and therefore inexpensive, balance suffices (e.g., a balance for use in the food industry). It is important that the balances are calibrated as well as that they are serviced regularly.

Spectrophotometers for protein work should cover the 190- to 800-nm wavelength interval. Absorption around 280 nm is used routinely for estimating protein concentration, whereas light in the visible region is often needed for measuring of different enzymatic activities. A double-beam UV-VIS spectrophotometer with 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 13,000 and $50,000 \times g$ 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 $40,000 \times g$). Standard centrifuges normally need little or no service except changing 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 just mentioned are angle rotors and the flasks are normally filled completely. The special, completely tight lids, which are available for most centrifuge flasks, are strongly recommended. For small, easily centrifuged samples, a simple desk-top centrifuge without cooling often is a good complement to the larger high-speed refrigerated centrifuge.

The maximum speed of the refrigerated floor centrifuge is normally 20,000 to 21,000 rpm. A new type of centrifuge allowing speeds up to 28,000 rpm has been introduced. At speeds over 20,000 rpm, the rotor compartment can be maintained under partial vacuum. Small samples can thus be centrifuged at $100,000 \times g$.

pH determinations are critical in the protein laboratory. Buffers should be checked and titrated routinely. 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 speeds, at least one of which is equipped with a thermostat-controlled heating plate, are recommended. A variety of PTFE- and glass-coated magnets are required. For extractions, propeller stirrers with interchangeable propellers and with variable speed regulators are convenient. These should preferably be of the electronic type that compensate for variations 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 volumes 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 that is 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; this procedure should be done in the hood.

1.7.2 Equipment for Homogenization

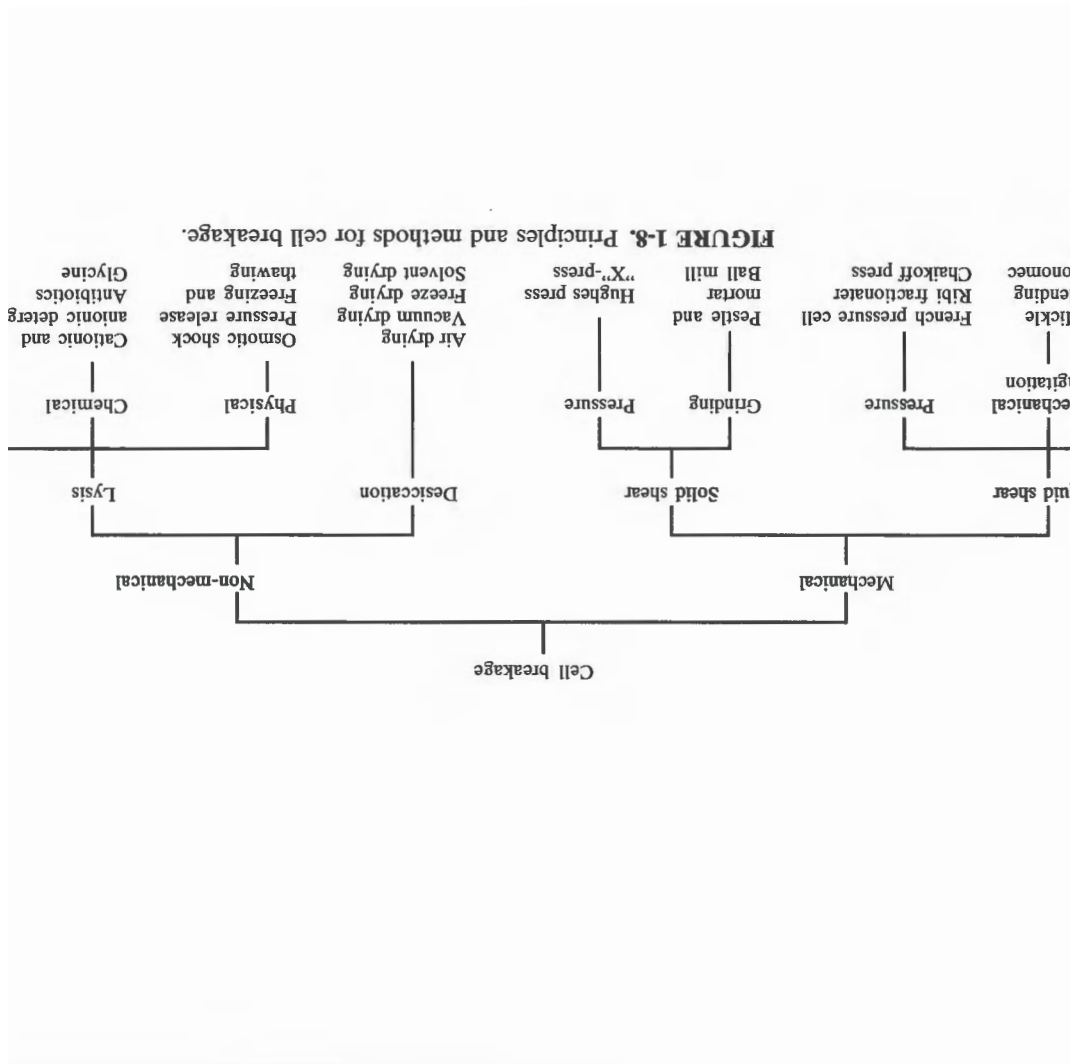
Starting materials for protein preparation can be obtained from plants, animals, and microorganisms. Plant and animal materials first have to be homogenized to individual cells but then the preparation procedures are in principle similar for the three different kinds of starting material.

Waring blenders or similar types of equipment and a "Turrax"-type apparatus are frequently used for the homogenization of plant and animal tissues. 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 such as 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 about 10% 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 eventually in combination with a Waring blender. Further homogenization is provided by Elvehjem pestle-type homogenizers. There are different types (glass, Teflon®), sizes, and also motor-driven varieties of this homogenizer.

Microorganisms are much more difficult to disintegrate. The polysaccharide cell wall withstands most homogenization procedures used for plant and animal cells. The well-cited overview by Wimpenny⁴⁸ for breakage of microorganisms is still valid for small-scale preparations (Figure 1-8). For large-scale homogenization of microorganisms, which means kilogram quantities of cell paste, the most frequently used equipment is the Dyno Mill⁴⁹ and the Manton-Gaulin⁵⁰ 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 To use packed chromatographic gel particles optimally, the column should be designed such 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 sintered metal, glass, or plastic, pore diameters, and surface structures that 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 as much as 32%. 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 (>40 cm diameter) 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 has increased to four to six. The linear flow rate of the eluent in the column feed pipe is very high in large-diameter columns and it is recommended that this jet should not directly hit 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 disk covering the support net under each pipe inlet.

The materials used in the parts of the chromatographic system that come in 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 CIP concept (cleaning in place) 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. Many HPLC columns are 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, polyethylene, 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, 1-mm-inner-diameter fluorocarbon tubing 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 for 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 connected to a programmable controller.

For HPLC, most pumps are of the piston type and are 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 and 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 normally are 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 classic way of monitoring 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. The most commonly used parameter in protein work is the absorption at 280 nm and many detectors are available for this purpose. In new diode array monitors, even a spectrum can be obtained directly on the eluate. In ion-exchange chromatography 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, chromatofocusing by 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 The introduction of the FPLC system⁵¹ 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 straightforward system approach was launched where the parts and components matched 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- μ m-diameter MonoBead ion exchangers, that 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 followers to the FPLC system have been introduced by several manufacturers. One of the latest is ÄKTAexplorer⁵¹ (Figure 1-9), 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 ion-exchange chromatography, hydrophobic interaction chromatography, reversed-phase chromatography, affinity chromatography, and gel filtration chromatography,

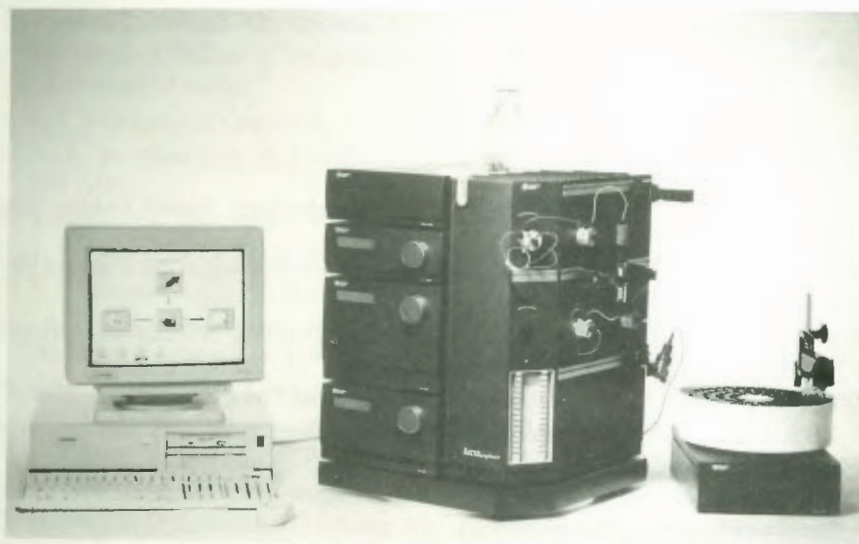


FIGURE 1-9. The ÄKTAexplorer chromatography system. (Courtesy of Pharmacia Biotech AB, Uppsala, Sweden.)

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 manual buffer preparation and titration needed for every pH change, particularly in ion exchange chromatography. The ÄKTAexplorer can also manage system control and data access via a PC network that 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 to 100 ml/min in isocratic mode and 0.5 to 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.

1.7.4 Equipment for Chromatographic and Electrophoretic Analyses

For the 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 micro- to milligram scale, and sometimes dedicated micropreparative chromatographic equipment is preferred⁵². Even more important are analyses by electrophoretic separation techniques. Equipment for the different electrophoretic techniques is described in connection with the techniques themselves in Chapters 12–18.

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PART II

Chromatography

5 Chromatofocusing

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- 5.9 References

5.1 INTRODUCTION AND BACKGROUND

Whether the goal is high-resolution separations at the analytical scale or the preparative scale isolation of biologically active macromolecules, often the most definitive separation techniques include exploitation of molecular charge properties. Thus, ion-exchange chromatography, because of its accommodating versatility and simplicity, is a frequently used separation technique in biochemistry. Similarly, electrophoretic focusing procedures, although generally more labor-intensive and cumbersome with larger sample volumes, allow macromolecular separations based on differences in net surface charge. Indeed, isoelectric focusing, as it is often referred to, is perhaps the most discriminating, high-resolution protein separation method available. During the period from 1977 to 1981, Sluyterman and colleagues offered a theoretical basis and practical means of realizing the most favorable attributes of both ion-exchange chromatography and isoelectric focusing in a single chromatographic focusing procedure.¹⁻⁵

During chromatofocusing, proteins are separated as a result of the isocratic formation of "internal" pH gradients on ion-exchange columns (Figure 5-1). Because proteins of similar net surface charge may still vary in surface charge distribution, it is even possible to resolve proteins during chromatofocusing that are not well separated by isoelectric focusing.

One of the great benefits of chromatofocusing is the ease of operation. No gradient-forming devices or mixers are required. The pH gradient is formed with a single eluent, isocratically. In the case of descending pH gradients formed using an anion-exchange stationary phase, the upper limit or starting pH (e.g., pH 8) is determined by the stationary-phase equilibration buffer. The lower or limit pH is determined by titration of the mobile phase or chromatofocusing buffer to the desired end point (e.g., pH 4). The pH gradient slope is a function of the column bed volume, buffer capacity of the matrix, and chromatofocusing buffer concentration as well as the initial and final adjusted pH of the stationary and mobile phases, respectively. The protein-focusing zone moves more slowly down the length of the column than does the focusing buffer or eluent (Figure 5-2). Repetitive protein-binding events driven by buffer flow against the resistance of the stationary phase to change pH causes the focusing phenomena to occur.

As a powerful analytical and preparative separation technique, chromatofocusing is in its infancy. The purpose of this presentation is to provide enough

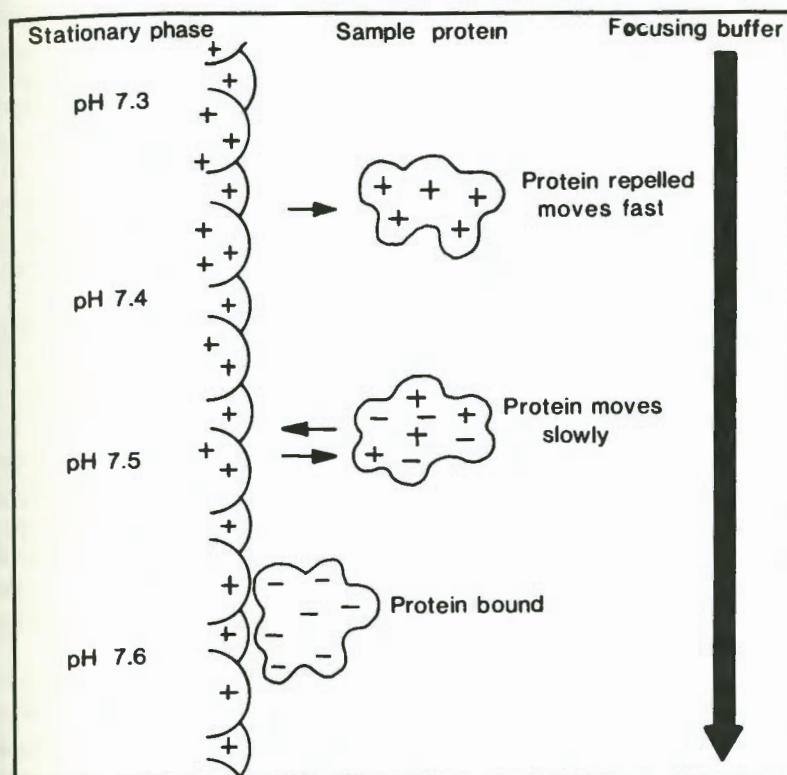


FIGURE 5-1. Illustration of the chromatofocusing process showing isocratic pH gradient formation, protein adsorption behavior, and migration of the focusing zone.

theoretical and practical guidelines to enable the successful use and further development of chromatofocusing as a high-resolution protein separation technique.

5.2 CHEMISTRY OF PROTEIN SURFACE CHARGE

The three-dimensional structure and overall surface molecular architecture of each different protein is unique. Most charged groups on proteins reside at the surface/aqueous interface. Locally, the immediate environment or milieu surrounding individual groups on the protein surface (e.g., His) may specifically affect the reactivity (e.g., pK_a) or chemical potential of that group. These surface reactive properties are influenced by both the mobile phase and the stationary phase during chromatofocusing.

The isoelectric point of a protein is defined as that pH in which the net charge is zero. Even though the majority of proteins (ca. 70%) examined to

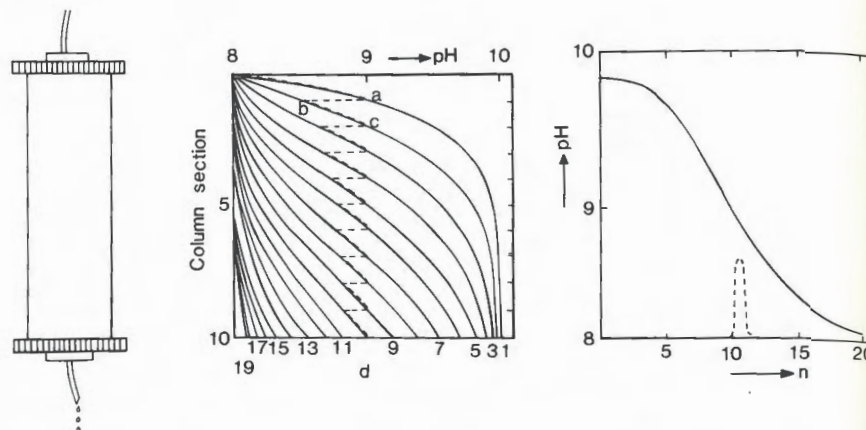


FIGURE 5-2. Formation of pH gradient in the chromatofocusing column. Each curve in the left diagram shows the gradient in the column (initial pH 10.0) after addition of a given number of unit volumes of focusing buffer (pH 8.0). The pH at the column outlet is given at the bottom of the left diagram and is used to construct the final chromatofocusing profile to the right. A sample protein with a pI of 9 in the column and the effluent is indicated by hatched lines. (Adapted from an illustration by Sluyterman and Wijdenes.²)

date have isoelectric points in the range pH 5 to 8, considerable heterogeneity exists within this region. Several well-organized and useful compilations of protein molecular weights and subunit compositions⁶ as well as apparent isoelectric points^{7,8} have been published. It is important to emphasize that even though a protein with a net zero charge will not necessarily migrate in an electric field, it nevertheless possesses a given charge distribution that may be operative during chromatofocusing as the pH of the mobile phase alters the pH of the protein and the stationary phase.

5.2.1 Factors Influencing Protein Surface Charge

Proteins and peptides are amino acid polymers (often containing carbohydrate oligomers) with titratable functional groups varying in pK from approximately 3 to 13 (Table 4-2). It is the collective contribution of different buffering groups on the protein that allow it to be workably defined as relatively basic ($pI > 7$) or acidic ($pI < 7$). In buffers with pH values above its isoelectric point, the protein will have a net negative charge and interact with a positively charged matrix (e.g., anion-exchange resin) (cf. Figure 5-1). Below its isoelectric point an overall or net positive charge will result and the protein will of course not interact with positively charged structures. Most of the individual, titratable groups are located at the protein's surface, and a gradual change in pH will result in a continuously changing surface charge distribution. These

changes can occur rapidly, in either direction, and are reversible. At pH values near the protein's isoelectric point, local charge distribution effects are important.

Several factors, both chemical and physical, can influence the protein surface charge. Intentional perturbations of this kind may include temperature, type, and concentration of ions (counterions, coions, and zwitterions) present, water structure (activity)-modifying reagents such as urea or ethylene glycol, and bound metal ions or other specific ligands, as well as detergents. In fact, the accurate determination of pH (see Section 5.6.2) is significantly influenced by solvent composition and temperature. Gelsema^{9,10} and Ui¹¹ provide a more detailed consideration of these factors relative to the measurement of pH and pI. Unintentional influences on protein charge as a function of pH may include changes in protein quaternary organization (e.g., subunit dissociation or aggregation) and protein interaction with the stationary phase or polymeric ampholytes in the chromatofocusing buffer¹² or other macromolecules present. At a given pH, the mobile-phase ionic strength can influence protein surface charge and thereby its behavior, including interaction with other proteins and the stationary phase. (see Section 4.3).

5.2.2 Ionic Strength Effects and Protein Solubility

The solubility of a protein is partially a function of surface charge and thus can vary significantly with pH. Most proteins are least soluble at their isoelectric pH values due to minimized electrostatic repulsion. Thus, low ionic strengths also influence solubility at this pH (pI). The ionic strength of chromatofocusing buffers is best kept low to favor stationary phase-solute (i.e., protein) interactions. However, relative to isoelectric focusing in an electric field, chromatofocusing is, to a certain extent, independent of small changes in ionic strength.

Protein precipitation or aggregation at the isoelectric point is for many proteins a practical problem in chromatofocusing and has hampered the wide use of the technique. It is, however, not at all a compulsory reason to avoid the technique. Isoelectric precipitation is counteracted by the fact that proteins do not elute exactly at their pI. They need to have a slight net charge to adsorb to the ion exchanger. Increased ionic strength thus counteracts precipitation both because of the increased net charge needed for adsorption and because of the salt itself. A drawback is, however, that salt in the mobile phase adversely affects the pH gradient. This effect is considerably influenced by the choice and design of the stationary phase. Decreased protein load also diminishes the risk of precipitation. In fact, it should be noted that many proteins of considerably physiological interest occur in very low amounts and the concentrations needed for precipitation are never reached during chromatofocusing. Finally, if precipitation occurs the protein might still be recovered. For example, β -lactoglobulin is only retarded on the column. The protein precipitates but is dissolved continuously as the pH decreases below its isoelectric point.

5.3 STATIONARY-PHASE DESIGN

The choice of suitable stationary phases for chromatofocusing has increased with the availability of high-capacity, weak anion-exchange materials that are compatible with large biopolymers such as proteins. Those commercially available products listed in Table 5-1 are examples of both conventional and high-performance stationary phases that have been successfully employed for chromatofocusing.

The appropriate stationary phase for chromatofocusing depends very much on the intended application. In general, for linear pH gradient formation, it is a necessary and probably sufficient requirement for the stationary phase to have a significant and even buffering capacity over the pH separation range of interest. Depending on sample loading volumes and column capacity requirements, a percentage of permanent or fixed (nontitratable) charges can aid in separations over pH ranges outside the area in which the weaker groups titrate. Thus anion exchangers contain an amount of quaternary amines and cation exchangers sulfonates. A fundamental property of the stationary phase should be the lack of nonspecific attractions for the proteins or other solutes of interest. Such interactions vary among the several types of columns currently utilized for chromatofocusing due to basic differences in their chemical and physical nature. High porosity and rigidity are also important and have been achieved with several matrices (see Table 5-1).

The choice of polymer- or silica-based stationary phases for chromatofocusing depends on, among other things, the pH range of interest. The use of silica-based stationary phases is usually discouraged for applications requiring pH values above pH 8. However, there are exceptions depending on bonded phase chemistry. Other deciding factors include available equipment, cost, useful lifetime of the material, and preference.

5.3.1 Charge, Charge Density, and Capacity

pH gradients can be formed on ion exchangers over the range in which they titrate. To achieve a chromatofocusing effect, a descending pH gradient is formed internally on anion-exchange (cationic) stationary phases and, conversely, an ascending pH gradient is formed internally using suitable cation-exchange (anionic) matrices. Most practical experience, and this chapter, involves the use of weak anion-exchange resins (primarily mixtures of secondary and tertiary amines) for the development of descending pH gradients. The charge density (meq/m^2) of various anion-exchange matrices most often reflects the chemistry and capacity of the matrix to be derivatized.

The overall charge capacity (meq/mL gel) of a given stationary phase per unit bed volume is a property of particle size and porosity (i.e., surface area). Typical figures are 15 to 30 meq/mL , half of which are strong nontitratable groups. Not all of the stated charge (binding) capacity is necessarily available for protein due to geometrical constraints. In practical terms, the determina-

1. Anion Exchange Stationary Phases Used for Chromatofocusing						
Immoblized Ligand or Stationary-Phase Composition	Particle Size (μm)	Average Pore Diameter	Reported Charge or Loading Capacity	Bonded Phase	Stationary-Phase Composition	Particle Size (μm)
WP-PEI	5, 15, and 40	250-300 Å	150-200 mg protein/g silica (40 μm)	PEI	Silica	5, 15, and 40
lker, Inc.)						
ike						
om, Inc.)						
PEI	6.5 and 7	300 Å	96 mg protein/g silica	PEI	Silica	6.5 and 7
PEI	7	500 Å		PEI	Silica	7
PEI	10	1000 Å		PEI	Silica	10
Exchanger	40-120	Porous	2-4 meq/100 ml gel	3° and 4° amines	Polymer	40-120
macia)					Sephacrose 6B	
Exchanger	40-120	Porous	0.3-5 meq/100 ml gel	3° and 4° amines	Polymer	40-120
macia)					Sephacrose 6B	
macia)					Cross-linked	
macia)					hydrophilic	
	10	Porous	15-21 meq/100 ml gel	3° and 4° amines		10

tion of the stationary-phase working capacity is influenced by the choice of protein and interaction conditions (i.e., pH, ionic strength, temperature) as well as by nonspecific adsorption phenomena. Thus on Mono Q and Mono S (Pharmacia Biotech AB), 80 mg/mL bed volume of human serum albumin and 65 mg/mL of α -lactalbumin is adsorbed. For the much larger thyroglobulin the figure decreases to 25 to 30 mg/mL.

Finally, in addition to those stationary-phase charge groups with buffering capacity that participate in the generation of the internal pH gradient, additional permanent or fixed charges (e.g., quaternary amines) have been found useful in maintaining an even protein-binding capacity over a broad pH range.

5.3.2 Ionic Adsorption Properties

The ability of weak anion-exchange matrices to participate in electrostatic interactions with biopolymers is affected by pH as well as the type (i.e., pK_a), charge, and concentration of competing counterions. Thus, the operable pH range is best kept as narrow as is consistent with good separation. Generally, the initial adjusted pH of the anion-exchange column must be above (e.g., 0.5 to 1 pH unit) the pI of proteins to be adsorbed, so the buffering capacity of the stationary-phase ligand must be adequate at this pH. Similarly, the ionic strength of the chromatofocusing buffer must be less than that required to prevent the interaction of the designated protein(s) at the loading pH. These effects are variable and depend on the actual stationary-phase/mobile-phase system utilized. Table 5-2 gives recommended column equilibration buffers and elution buffers for developing pH gradients over several different ranges for use with Pharmacia Polybuffer Exchanger (PBE) and Mono P gels. In practice, both anionic and cationic as well as zwitterionic buffer constituents have been used successfully. Sometimes one good experimental effort provides acceptable results independent of accepted theory.

5.3.3 Buffering Capacity of the Stationary Phase

Anion-exchange columns and packing materials with suitable and even buffering capacity over a given pH range may be used effectively for chromatofocusing in that same pH range. The column or material in question can be titrated with acid (e.g., 0.5 to 2 *N* HCl) or base (e.g., 0.5 to 2 *N* NaOH) in the presence of elevated ionic strengths to calculate the buffering capacity (β) as originally outlined by Van Slyke.¹⁴ Stationary phases with high and even buffering capacities over a fairly wide pH range have been developed specifically for chromatofocusing and are available commercially (see Table 5-1). Also, other cationic stationary phases developed for high-performance ion-exchange chromatography have been found to have excellent buffering capacities and pH gradient-generating qualities (Figure 5-3).

Certain columns have been carefully developed for a wide range of chromatofocusing applications, that is, pH ranges from pH 8 or 9 to pH 4. Yet, it is

Buffer Substances for Focusing in Different pH Ranges			
Starting Buffer	Eluent	Dilution Factor	Approximate (in Column) Pregradient
0.25 M triethylamine pH 11, HCl	Pharmalyte 8-10.5 pH 7.0 HCl	1:45	2.0
0.25 M ethanolamine pH 9.4, HCl	Polybuffer 96 pH 6.0, CH ₃ COOH	1:10	1.5
0.25 M Tris pH 8.3, CH ₃ COOH	30% polybuffer 96 70% polybuffer 74 pH 5.0, CH ₃ COOH	1:10	2.0
0.25 M imidazole pH 7.4, HCl	Polybuffer 74 pH 4.0, HCl	1:8	2.5
0.25 M piperazine pH 5.5, HCl	Polybuffer 74 pH 4.0, HCl	1:10	3.0

and on instructions given in Pharmacia booklet (Ref. 13). Polybuffers are oligomeric ampholyte buffers titrating between pH 9

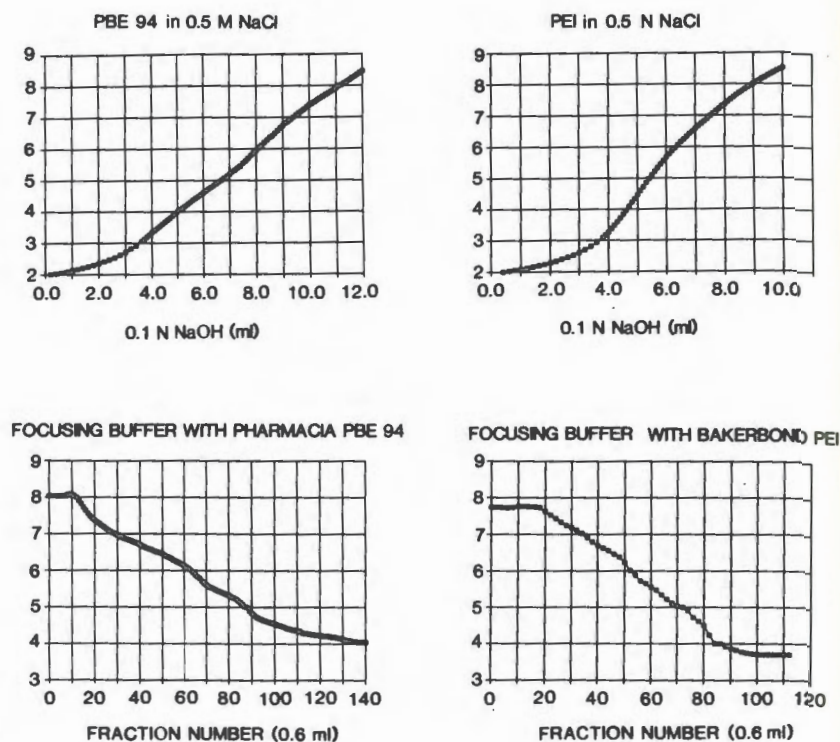


FIGURE 5-3. Titration curves of (1) a stationary phase specifically designed for chromatofocusing (Pharmacia Polybuffer Exchanger PBE 94) and (2) a silica-based high-performance anion-exchange stationary phase (J. T. Baker, Inc., Bakerbond WP-PEI). The characteristics of the pH gradients formed internally with a chemically defined focusing buffer are also illustrated for the two stationary phases.

difficult to maintain wide-range pH gradients that are linear over more than about 3 pH units. Narrow pH gradients (less than 2 to 3 pH units) may also be formed on columns with a buffering capacity limited to that same pH region. Thus, several other column types, as yet unexploited for chromatofocusing, may be found suitable for certain applications. It also needs to be emphasized that the stationary-phase buffering capacity and the pH gradient formed isocratically during separation need not necessarily be linear as long as a satisfactory or optimal separation of the proteins is obtained without it.

5.3.4 Column Volume and Geometry

The smallest possible chromatofocusing bed volume consistent with sample load requirements and resolution should be employed to minimize focusing buffer volumes and the time required for analysis. In general terms, the amount of buffering groups on the stationary phase and column volume defines the

buffering capacity of the column. This determines the concentration and/or volume of focusing buffer required to generate a pH gradient of defined slope. Excessive column bed volumes also prolong column regeneration times. Good-quality analytical separations have been achieved using Pasteur pipettes or other narrow (e.g., 0.4 to 0.5 cm) diameter open columns packed with 2 to 4 ml bed volumes of Pharmacia Polybuffer Exchanger 94. An average chromatofocusing column is, however, 15 to 30 cm long. The pH gradient becomes less steep as it travels through the column, but after a certain distance further lengthening of the column does not change the result.

5.4 MOBILE-PHASE DESIGN

The single column eluent or mobile phase necessary to develop a particular pH gradient during chromatofocusing, like the mobile phase operative during isoelectric focusing, is actually a carefully designed set of buffering constituents. Collectively, these constituents make up one of the more complex buffer systems used in chromatography today. Relatively simple in concept, it is instructive to understand the mobile phase as an important variable contributing greatly to the efficiency and outcome of the desired separation.

5.4.1 Buffering Capacity of the Mobile Phase

It was Van Slyke¹⁴ who first described the concept of buffering capacity (β) and showed it to be an additive property of weak acids and bases in solution:

$$\beta = \frac{dB}{dpH}$$

where dB is the increment of base (B) added to the buffer solution and dpH is the incremental change in pH which results.

In the context of protein-focusing techniques, Svensson^{15,16} first provided the theoretical calculations for using the buffering capacity of "carrier" ampholytes to create natural pH gradients in an electric field. The lack of simple ampholytes suitable for this purpose limited the use of isoelectric fractionation until Vesterberg¹⁷ reported the synthesis of polyampholytes with the necessary isoelectric properties. The similarities of commercially available polyampholytes used for isoelectric focusing and chromatofocusing are not coincidental and the reader is urged to consult one of the many reviews on the subject (e.g., Ref. 18) for a more thorough appreciation of the synthesis and properties of polyampholytes.

Polyampholytes are designed to provide an even buffering capacity (β) over the pH range in which separation is to take place. Figure 5-4 illustrates the titration curve of Pharmacia Polybuffers 96 and 74. This curve represents

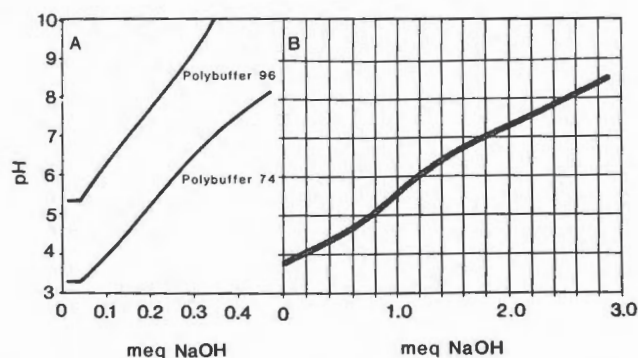


FIGURE 5-4. Titration curve of polymeric (polyampholyte) buffers (left) as well as of a simple nonpolymeric chromatofocusing buffer (right). Polybuffer 96 titrates between pH 9 and 6, whereas Polybuffer 74 titrates between pH 7 and 4.

the buffering action of titratable constituents with numerous successive pK_a values.

A generic alternative to oligomeric ampholyte focusing buffers is the use of chemically defined, nonpolymeric (so-called "simple") focusing buffers. Such universal or wide range buffer systems have existed for many years.¹⁹⁻²¹ Several attempts have been made to extend this philosophy and formulate useful, "simple," focusing buffer systems for chromatofocusing.²²⁻²⁶ Figure 5-4 also shows the titration curve of one such focusing buffer. Some examples of the utility of these focusing buffer systems are provided in Section 5.7.1 (also see Figure 5-5). Although earlier attempts to prepare simple, chemically defined, focusing buffers for electrophoretic focusing procedures²⁷⁻³⁰ have been met with only partial acceptance, perhaps due to perceptions of electrofocusing theory (e.g., Ref. 18), the development of simple chromatofocusing buffer systems is expected to continue. There are presently no theoretical or practical limitations to the use and further improvement of nonpolymeric (i.e., very low molecular mass), chemically defined chromatofocusing buffer systems, especially for large-scale applications. For buffer use in general, Perrin and Dempsey³¹ have published a useful book that provides a simplified overview of key considerations in buffer preparation and use.

In practice, pH gradient engineering for chromatofocusing is complicated by the contribution of stationary-phase effects.

5.4.2 Physical Properties of Chromatofocusing Buffer Constituents

A desirable chromatofocusing buffer will have sufficient and even buffering capacity over the pH range of interest. The ionic strengths have to be very low in order to give a separation according to isoelectric point, and thus to favor protein or solute interaction with the charged stationary phase during focusing. The light (UV)-absorbing properties should be minimal to permit

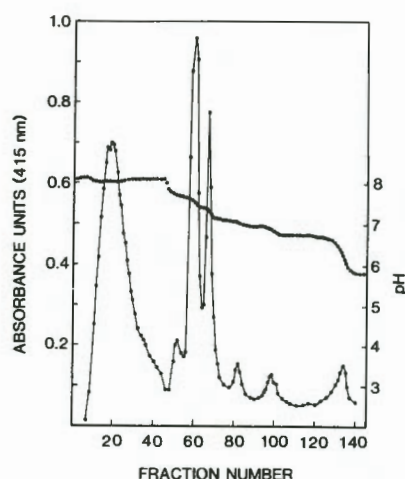


FIGURE 5-5. Fractionation of a commercial preparation of hemoglobin (15 mg) using the nonpolymeric focusing buffer described in Refs. 23 and 24 with Pharmacia Polybuffer Exchanger 94 as the stationary phase (1.0×5 cm). Two-minute fractions were collected at a flow rate of 0.5 to 0.6 mL/min. The average pH gradient slope ($\Delta\text{pH}/\text{mL}$) from pH 7.75 to 6.75 was 0.01 to 0.02.

an adequate signal-to-noise ratio while monitoring the protein or solute elution (e.g., at 280 nm). The most successful chromatofocusing buffer should be composed of constituents that (1) are easily separated from most proteins and (2) do not interact with high affinity or irreversibly with the protein(s) being separated. In particular, the structure and/or function of the protein should not be practically or irreversibly compromised. The separation of proteins from polyampholyte chromatofocusing buffers is treated in more detail in Section 5.6.6.

The precise physical and chemical properties of oligoampholyte (oligomeric) focusing buffers are variable and poorly defined. The degree to which they interact with proteins remains the subject of considerable debate.^{12,18} Even chemically defined, low molecular weight buffer constituents may interact with proteins.^{28,29} Presumably, however, with defined chromatofocusing buffers these constituents can be identified and deleted or replaced if necessary. The degree to which these considerations pose a practical barrier must be determined on an individual base. There are strong arguments for the use and continued development of small, "noninteractive" focusing buffer systems.²²⁻³⁰

5.5 MECHANISM(S) OF SEPARATION

This section briefly evaluates existing theories of internal pH gradient formation on stationary phases, as well as the postulated protein separation mecha-

nisms. The aim is to understand better the utility of given experiment variables for the solution of a given separation or analytical problem.

5.5.1 Theory of pH Gradient Formation

The initial assumptions (all-or-none model) and first approximation equations as originally outlined by Sluyterman and colleagues²⁻⁵ provide a logical approach to the underlying principles generally utilized to explain the generation of internally developed pH gradients during chromatofocusing. They derived the following equation for final eluted pH (pH_f) when the two phases are mixed. The buffering capacity per defined unit (e.g., column length) of stationary phase is β_s and mobile phase is β_m

$$\text{pH}_f = \frac{(\beta_s)\text{pH}_s + (\beta_m)\text{pH}_m}{(\beta_s) + (\beta_m)} \quad (5-1)$$

In the simple case when β_s and β_m are assumed to be equal, Eq. (5-1) becomes

$$\text{pH}_f = \frac{\text{pH}_s + \text{pH}_m}{2} \quad (\beta_s = \beta_m) \quad (5-2)$$

That is, if the buffering capacities of the mobile phase and stationary phase are equal and additive, upon mixing, the resulting pH will reflect the simple average. To best illustrate the consequences of this assumption, Figure 5-6 shows the theoretical pH profiles generated using this equation. To begin, the pH of the stationary phase (pH_s) was adjusted to pH 8. The column (e.g., 100 individual units or sections) pH is indicated during the sequential addition of 100 aliquots of focusing buffer at pH 4, where each aliquot of focusing buffer is equal in buffering capacity to one unit or section of the column. The internal pH values of column sections 1, 10, 25, 50, and 75 are shown as a function of the number of focusing buffer aliquots added. Except for the extremes at either end, a series of linear pH gradients are developed.

These data reveal an important, experimentally verified, point. The slope of the pH gradient depends on the number of column sections for a given amount of focusing buffer. Also, if the buffering capacity of the mobile phase is increased relative to that of the stationary phase, the pH gradient will be greater in slope. Another important assumption for the model predicted by Eq. (5-2), aside from equal and additive buffering capacities for the stationary and mobile phases, is that of even (linear) and parallel buffering capacities over the pH ranges of interest. However, upon physical (electrostatic or hydrophobic) interactions between mobile-phase buffer constituents (e.g., polyampholytes) and the stationary phase, the functional buffering capacities (pK_a values) may be altered. Indeed, an unequal distribution of buffer constituents between mobile and stationary phases can result in uneven variations in the

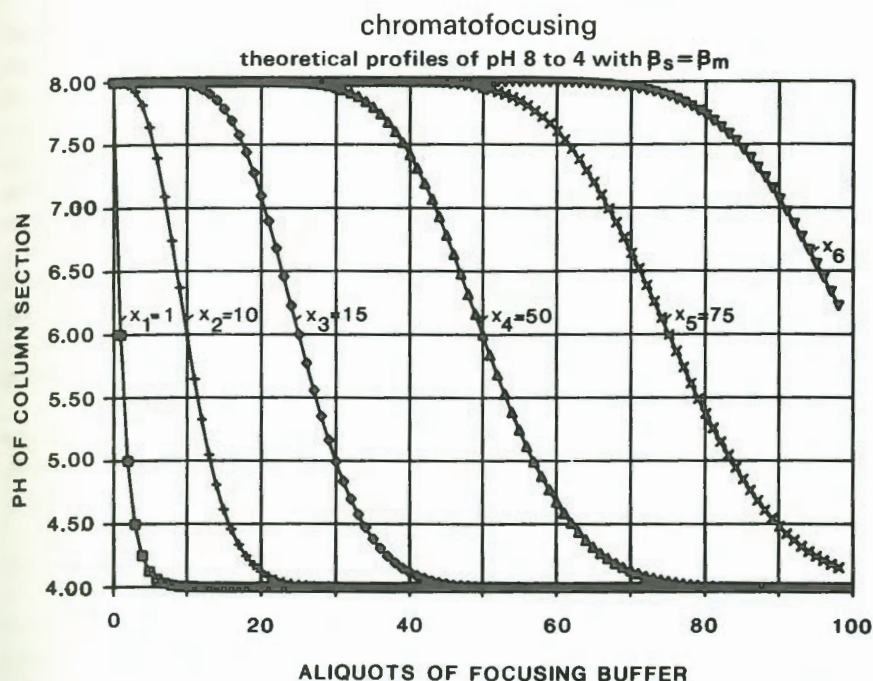


FIGURE 5-6. Theoretical pH profiles generated from pH 8 to pH 4 as a function of focusing buffer (volume) for each of 100 aliquots as described in the text. The pH gradient of the final column eluate is partially shown (far right). An equal buffering capacity of focusing buffer and stationary phase is assumed per unit volume. x_n is the number of column sections alternatively, column length.

column buffering capacity as a function of pH. So, while the buffering capacities of soluble buffering agents are additive, the buffering capacities of interacting mobile and stationary phase components may not always be additive. Nevertheless, it has been shown that the contribution of stationary-phase and mobile-phase buffering capacities alone can indeed be additive and account for the pH gradient formation predicted by Eq. (5-1). This was demonstrated using chemically identical (or similar), nonamphoteric buffering groups in both mobile and stationary phases.²⁵

Despite the theoretical uncertainties involved, it has been shown that chemically defined mixtures of simple (nonpolymeric) buffer reagents,^{22,23} nonamphoteric buffers,²⁵ and heterogeneous, polymeric ampholytes (e.g., Polybuffers 96 and 74) can be used to internally generate similar pH gradients.

5.5.2 Control of Separation Variables and Resolution

In practical terms, the most efficient means of controlling solute or protein separation efficiency (i.e., resolution) during chromatofocusing is by altering

the shape and/or slope of the pH gradient generated. This may be accomplished for a given pH range by altering the stationary-phase bed volume (e.g., column length) or changing the relative concentration of focusing buffer. Thus an increase in column length will only initially make the gradient more shallow, as explained earlier. The possibility of increasing the concentration of focusing buffer is also limited. A too high buffer strength that does not match the stationary phase will not give an even gradient. Its steepness will increase toward the end of the run.

Alternatively, the initial column equilibration pH (starting pH for gradient) and/or the final adjusted pH of the focusing buffer (limiting pH for gradient) may be adjusted to provide the desired pH gradient slope ($\Delta\text{pH}/\text{mL}$). Protein resolution can be excellent with chromatofocusing. Protein peaks separated by 0.01 pH units have been reported.²⁶

The specific elution pH of a given protein can also be affected by the inclusion of mobile-phase modifiers such as urea, metals, and detergents. These influence the way proteins in the sample behave in the separation.

5.5.3 Ampholyte Displacement Effects

Before the development of chromatofocusing, polyampholytes had been used to displace proteins from conventional anion exchangers. Ampholyte displacement chromatography, apparently first reported by Leaback and Robinson,³² can be distinguished from chromatofocusing under specifically defined conditions. In particular, the formation of a pH gradient does not always appear to be necessary for the displacement of proteins when high concentrations of competing polyampholytes are used. Discussions have been presented to distinguish, theoretically and practically, chromatofocusing from ampholyte displacement chromatography.³³⁻³⁸

However, uncertainties remain as to the precise separation mechanism(s) operable in either case. Under the conditions thus far used for chromatofocusing, the distinction appears to be more apparent than real as these two protein separation processes do not necessarily have to be mutually exclusive. For example, as the pH approaches that point at which the protein interaction with the stationary phase becomes weaker, competitive binding of polyampholytes will act more effectively to displace the protein. Stated another way, as pH effects begin to decrease the affinity of protein for the stationary phase, competitive binding of ampholytes or other displacers may become a significant factor. The most basic polyampholytes (high pI values) elute first and others break through in order of their relative basicity. In practice, the separation mechanism may be irrelevant if purification is the desired end point. If the investigation is analytical or comparative in nature, unexpected elution behavior must be reconciled with the dominant elution mechanism.

5.5.4 Comparison with Isoelectric Focusing

It is important to emphasize that proteins may elute during chromatofocusing at pH values close to or well away from (e.g., 1 to 2 pH units) their isoelectric

points. The several possible explanations for this phenomena are based on one of the major mechanistic differences between chromatographic focusing and electrophoretic focusing techniques, namely, the stationary phase.

Individual proteins may vary in their distribution and density of surface charge even if they are similar in their isoelectric points. Thus, differential interaction with the charged stationary phase is still possible. Because the slope of the titration curve for individual proteins (Δ surface charge/ Δ pH) can vary significantly for a given difference between pI and pH, proteins may have markedly different affinities for the stationary phase. The interaction of a protein with a charged column matrix may itself alter the conformational response of that protein to changes in environmental pH. These processes must occur repeatedly during chromatofocusing because the focusing effect is the result of proteins repeatedly adsorbing to and desorbing from the charged matrix. It is during these interactions (at or near the pH of adsorption/desorption) that both concentration and composition of polyampholytes or salts may affect the elution of proteins differentially.

It has been postulated^{2,3} that a Donnan potential between the interior and the exterior of the stationary-phase particles might decrease the elution pH of the proteins from chromatofocusing columns. During chromatofocusing on highly charged (positive), porous matrices, due to electrostatic repulsion, positively charged species in solution (including hydrogen ions, basic ampholytes, etc.) may be excluded and increase the pH of the buffer immediately surrounding the surface of the porous matrix.

To summarize, in contrast to electrophoretic focusing techniques, chromatofocusing relies on interfacial (solid/solution phase) molecular interactions that are influenced, in a collective manner, by such factors as pH, ionic strength, charge attraction and repulsion, and competitive binding reagents (displacers), as well as any "nonspecific," adsorption properties the stationary phase may have. It is therefore inappropriate to refer to elution pH as pI. However, it is not trivial to measure the pH on an isoelectric focusing gel, especially in the presence of various additives. Thus, it may also be questionable to equal the measured pH value and the actual pI.

5.6 ANALYTICAL AND PREPARATIVE CHROMATOFOCUSING TECHNIQUES

Chromatofocusing is a separation procedure useful for the analysis of protein surface charge heterogeneity as well as alterations in the surface charge properties of individual proteins at different stages of purification and in unfractionated biological fluids and extracts. However, in addition to the small-scale analysis and separation of proteins or protein isoforms, chromatofocusing can be useful on a larger scale for preparative protein purifications. Chromatofocusing, as a sample concentrating procedure, is particularly useful at early or intermediate stages in the protein isolation scheme. Since proteins are eluted from anion-exchange columns during the formation of descending internal

pH gradients, the protein is not subjected to pH levels below that just required for elution.

It is also possible to collect fractions or individual protein peaks into pH neutralizing buffer solutions to preserve bioactivity (see Section 5.7.1).

The pH gradient formed during chromatofocusing can be monitored continuously along with sample elution during the separation experiment. Thus, gradient slope and shape may be modified as needed to alter separation efficiency (see Section 5.7.1). It is not always desirable to use linear pH gradients. In fact, nonlinear gradients can be predesigned to maximize the separation of given components.

5.6.1 Analytical to Preparative Scale Applications

Substantial increases in sample volume, sample protein concentrations and/or column bed volume can often bring changes (e.g., pH gradient slope) not easily anticipated from analytical scale operations. Depending on sample volumes or quantities of protein needed, utilizing available high-performance stationary phases with rapid analysis times to make replicate "analytical" scale separations under familiar conditions may be more advantageous than altering column dimensions to achieve the desired end product. There are, however, no theoretical or practical reasons why chromatofocusing cannot be used on a large scale to address difficult separation problems.

Since chromatofocusing is indeed a focusing procedure, unlike zonal elution chromatography, a large part of the bed volume can be utilized during sample loading. Isocratic pH gradient formation greatly simplifies equipment requirements (i.e., controllers and mixers) for large volume applications. The continued development and commercial availability of suitable, inexpensive focusing buffers may further reduce the cost of large-scale chromatofocusing separations.

Large sample volumes can bring unexpected changes in protein elution behavior if sample contaminants or buffer constituents, even if present at the same concentrations as for analytical scale operations, build up in the sample loading zone. EDTA is an example of one common buffer constituent that causes no problems when present in minor quantities (small sample volumes) but can create serious problems with larger sample volumes. EDTA binds to most anion-exchange stationary phases used for chromatofocusing and elutes in a pH-dependent manner with absorbance at 280 nm,

5.6.2 pH Measurements

Particularly for analytical or comparative experiments by chromatofocusing, accurate and reproducible pH measurements are essential. Aside from pH electrode type and stability, the most immediate concerns are the temperature of separation and pH measurement (they should be the same), dissolved CO₂,

and mobile-phase additives such as urea, glycerol, or anything else that affects the H^+ activity of aqueous eluents (see Refs. 9–11).

Flowthrough pH electrodes are convenient but may not be as accurate or reproducible as needed for sensitive analytical measurements. Of course, if the primary concern is resolution and separation, pH measurements are of less concern.

5.6.3 Sample Preparation and Column Loading

Proper sample preparation and column loading procedures will help increase resolution, sample recovery, and useful column lifetime. It is necessary to remove all sample components that are insoluble (e.g., precipitates) or immiscible (e.g., lipid layers or micelles) in the chosen sample and column equilibration buffer. Brief centrifugation at high speeds (10,000 to 15,000 g; 10 to 15 min) or filtration through inert, porous (0.22 or 0.45 μm) membranes (e.g., Gelman or Millipore) will normally suffice to ensure the application of fully soluble sample components if these procedures are performed at the temperature of chromatography. After equilibration of the sample into the column equilibration buffer, difficulties with protein solubility, when encountered, may be dealt with in several different ways. It is also possible to load the sample in focusing eluent buffer after that the gradient has developed on the column. Very diluted samples can of course also be used.

5.6.4 Solubility of Proteins

Sample protein solubility during chromatofocusing, depending on the protein, may become a necessary consideration. Protein solubility during chromatofocusing is most probably influenced by pH as well as by the concentration and type of salts (i.e., buffer and counterions) present (see Section 5.2.2). Several mobile-phase (focusing buffer) modifiers are helpful in minimizing solubility problems, and also increase resolution during protein separation. The low ionic strength column and sample equilibration buffers utilized during chromatofocusing sometimes induce protein aggregation even at the start pH, which may be well above (e.g., 0.5 to 2 or more pH units) the isoelectric point of the protein of interest. In this case, the addition of urea or nonionic detergent to the sample can sometimes prevent aggregation until protein adsorption to the stationary phase and/or gradient development begins and aggregation may no longer be a problem.

In fact, adding concentrations of urea from 1 to 6 *M* in the sample preparation buffer and both the column equilibration and focusing buffer is an effective, probably underutilized, means of optimizing sample solubility and resolution during chromatofocusing. Many proteins retain their tertiary and/or quaternary structure as well as their activity in relatively high concentrations of urea, especially at 4°C. Low concentrations of detergents may also be utilized to maintain protein solubility without compromising biological activ-

ity. Triton X-100, Nonidet P-40, and Tween 80 are all examples of nonionic detergents (available from Sigma) that have been used effectively during chromatofocusing. CHAPS (Cal Biochem) is a zwitterionic detergent prepared from cholic acid that has also been found useful in preventing aggregation and maintaining high recoveries during chromatofocusing. Low concentrations (4 to 10%, w/v) of zwitterions (e.g., betaine or taurine) and glycerol have been found to be effective solubilizing/stabilizing agents for some proteins during chromatofocusing.

5.6.5 Inclusion of Mobile-Phase Modifying Reagents

The mobile phase includes the chromatofocusing column equilibration buffer and the focusing buffer used to generate the internal pH gradient. Since the protein migration zone or focusing zone proceeds slowly down the length of the chromatofocusing column relative to the flow of buffer, the sample protein may be exposed to 10 to 20 column volumes of buffer before finally eluting. Thus, specific buffer constituents required to maintain protein structure (or solubility) and/or function must be included in the focusing buffer. Fortunately, most of them can be included. In fact, in contrast to focusing in an electric field where current (conductivity) is important, even relatively high concentrations of charged components can be included and maintained during chromatofocusing. A good example is the inclusion of 10 mM sodium molybdate in steroid receptor chromatofocusing buffers. The effect of molybdate on the stability of receptor structure and function is easily reversible but could be maintained during chromatofocusing. Other salts can be included (up to 20–30 mM) to influence protein solubility or prevent aggregation.

Urea, detergents, and glycerol have been especially useful in chromatofocusing buffers with “sticky” proteins.

There is a problem with mobile-phase modifiers when used to analyze surface charge heterogeneity or when comparisons of surface charge are being made. These additives can cause significant error in pH measurements. They can also cause major shifts in protein elution pH, which vary in a protein-dependent manner. Again, if purification is the only objective, elution pH may not be a major concern.

5.6.6 Sample Recovery

Some proteins can be encountered that are surprisingly resistant to pH-dependent elution during chromatofocusing. This depends very much on the properties of the individual protein and, just as much, on the properties of the stationary phase chosen for chromatofocusing. The number of different types of protein present (e.g., crude mixtures vs purified samples) often influences recovery simply due to the mass action of adsorption. Highly purified proteins can often be further purified or analyzed in the presence of carrier proteins to saturate so-called nonspecific adsorption sites. Alternatively, mo-

bile phase additives (e.g., detergents) have also been effectively utilized to prevent adsorption.

If the protein being analyzed or purified is a receptor or other ligand-binding protein whose activity is determined by the association with ligand (e.g., steroid-receptor complex or metal-protein complex), it is important that the stationary phase have little or no affinity for the ligand in question. For example, [³H]estradiol was found to bind to SynChropak AX-300, AX-500, and AX-1000 silica-based anion-exchanged columns and elute in a pH-dependent manner.³⁹⁻⁴² However, no steroid interaction was evident using Bakerbond PEI (J. T. Baker) as the stationary phase.

The separation of proteins from focusing buffer constituents (most commonly polyampholyte buffers such as Pharmacia Polybuffers 74 and 96) can normally be achieved by size exclusion chromatography (e.g., Sephadex G-50 or G-75 Fine), precipitation with ammonium sulfate, hydrophobic interaction chromatography, or other types of affinity chromatography. However, certain polyampholyte properties^{12,18} and reported difficulties in protein-polyampholyte separations^{12,23} indicate that some caution should be used in defining adequate removal of focusing buffer constituents from the specific protein of interest.

5.6.7 Column Regeneration

In general, sample recovery and column maintenance require the routine use of high salt concentrations after completion of the pH gradient to help ensure quantitative elution of adsorbed material. A gradual rise in back pressure is an alert to a growing column contamination problem. Postanalysis column washing and/or column regeneration procedures are best performed with reversed flow, if possible. Typically, 1 to 2 *M* solutions of neutral salt (e.g., NaCl) should be adequate, although more chaotropic salts can be used, especially if hydrophobic adsorption is suspected. A useful practice is to utilize 1 to 2 *M* concentrations of equilibrating buffer adjusted to the upper or starting pH to facilitate column regeneration. The removal of more difficult proteins can often be accomplished with 8 *M* urea or 6 *M* guanidine-HCl. Lipoproteins and lipids can be removed with one or more of several different organic solvents. Ethylene glycol (e.g., 50%), diethyl ether, dimethylformamide, or dimethyl sulfoxide (e.g., 10 to 50%), acetonitrile (up to 100 ± 0.1% trifluoroacetate), ethanol, methanol, and other solvents can be used regularly. Care should be taken to ensure that all salts are washed away (Milli Q water) and that as many proteins as possible are removed before using organic solvents to avoid precipitation. Multiple injections of 0.1 *M* HCl and/or 0.1 *M* NaOH are also an effective cleaning procedure. Columns may be stored in 20% methanol as a preservative. Sodium azide should be avoided. The use of clean buffers (especially containers) will reduce the risk of bacterial contaminations of "clean" columns.

5.7 APPLICATIONS

5.7.1 Analysis of Surface Charge Heterogeneity and Purification of a Labile DNA Regulatory Protein: The Estrogen Receptor

The utilities of chromatofocusing are revealed by the examples provided below illustrating the characterization and purification of various steroid receptor protein isoforms. Because chromatofocusing is preparative even at the analytical scale and because the procedure is amenable to the inclusion of various structure-modifying reagents, it has been extremely useful in efforts to better define receptor, structures and their functional relationships.

The intracellular receptor protein(s) for the female sex hormone estradiol has been evaluated for surface charge heterogeneity and partially purified by chromatofocusing using several different types of stationary phases, including conventional open columns of polymeric anion-exchange gels^{39,40} and high-performance polymeric⁴¹ and silica-based^{23-26,39,42-44} anion-exchange matrices. During these investigations, both polyampholytes and nonpolymeric, chemically defined focusing buffers have been utilized to generate similar pH gradients and receptor elution profiles.^{23,24,26} One goal of the original investigations⁴² was to develop a rapid, high-resolution protein separation technique to better evaluate and purify the different forms of steroid receptor proteins known to exist in the presence and absence of (1) natural steroid ligand,⁴³ (2) receptor structure-stabilizing metal ions,^{42,43} and (3) receptor structure modifying reagents known to induce exposure of the receptor's DNA-binding site.⁴⁴

The steroid receptor proteins are identified by their ability to bind specific steroid ligands with high affinity. To extract and radiolabel the estrogen receptor proteins, endocrine target tissues (e.g., uterus or mammary glands) are homogenized in 2 to 4 volumes of buffer and centrifuged (>100,000 g, 60 min) to obtain particulate-free cytosol. The cytosol is then labeled with [³H]estradiol-17 β or [¹²⁵I]iodoestradiol-17 β in the presence and absence of a radioinert, estrogen receptorspecific competitor (diethylstilbestrol) to identify receptor from nonspecific estrogen-binding proteins.⁴² Cytosol preparations (10 to 20 mg protein/mL) containing what are considered to be biologically relevant concentrations of receptor protein (typically from as low as 5 to 10 fmol up to several hundred femtomole receptors per milligram cytosol protein) are equilibrated into chromatofocusing column equilibration buffer using minicolumns of Sephadex G-25. The chromatofocusing column equilibration buffer usually consists of 25 mM Tris-HCl, pH 8.0 (4°C), containing dithiothreitol (1 mM) and glycerol (10 to 20%, v/v). Where noted, 10 mM sodium molybdate, 6 M urea, detergent (CHAPS), and other receptor structure modifying reagents are included in the sample buffer, stationary-phase equilibration buffer, and focusing buffers. All procedures, including equilibration buffer and focusing pH determinations, are carried out at 0 to 4°C. Buffers and samples are filtered to remove particulate contaminants using Millipore

0.45- μm HAWP filters. Buffers are degassed before final pH adjustments and use. Sample aliquots of 0.5 to 2.0 mL (5 to 20 mg total protein) are usually loaded onto the column prior to initiation of the pH gradient. The formation of the internal pH gradient is initiated using either polyampholytes (Pharmacia Polybuffers 96 and 74) or other nonpolymeric, chemically defined focusing buffers²²⁻²⁴ at flow rates of 0.5 to 1.0 mL/min. The pH of collected fractions (1.0 min) is determined immediately upon elution using a Corning Model 125 pH meter equipped with a microcombination calomel electrode. Flowthrough pH electrodes (Phoenix Electrodes, Houston, TX) have also been used. The protein elution profile is monitored at 254 or 280 nm using a Beckman Model 153 or Model 166 analytical flowthrough UV detector (8- μL flow cell) or other suitable spectrophotometer. The elution profile of labeled estrogen receptor proteins was monitored by liquid scintillation counting (30 to 38% efficiency) or gamma counting (60 to 80% efficiency).

The estrogen receptor elution profile shown in Figure 5-7 is typical of that obtained for the human uterine estrogen receptor chromatofocused in the

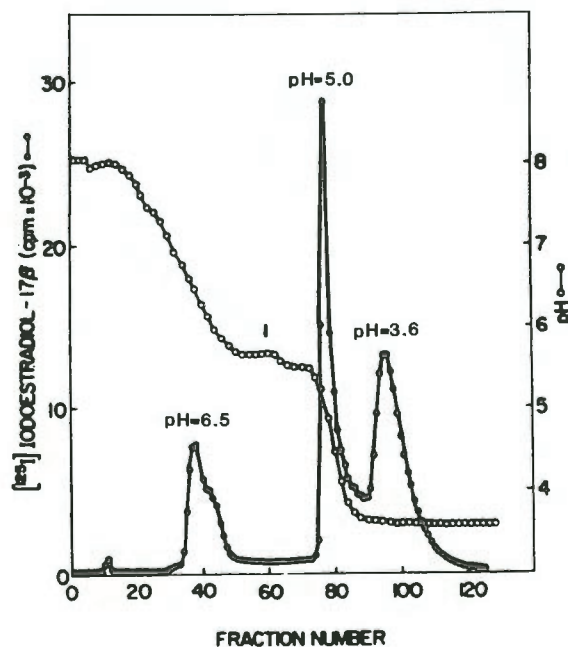


FIGURE 5-7. Open column chromatofocusing of human uterine estrogen-binding proteins on PBE 94. Cytosol was prepared from a fresh specimen of postmenopausal human uterus and labeled with 10 nM [^3H]estradiol-17 β . Termination of the labeling reaction and details of chromatofocusing on PBE 94 are described elsewhere.³⁹ Arrows mark initiation of the primary pH gradient [Polybuffers 96 and 74 (30:70) diluted 1:15, pH 4 at 0°C] and secondary eluent (Polybuffer 74 diluted 1:15, pH 3 at 0°C), respectively.

absence of structure-stabilizing or structure-destabilizing mobile-phase reagents. Open columns (0.7 cm i.d. $\times$ 9 cm) of PBE 94 were used with Polybuffers 96 and 74. Relatively small bed volumes of chromatofocusing gel were utilized to minimize separation times. The rather labile steroid receptor proteins are stabilized (reversibly) by the presence of relatively high concentrations of molybdate. Figure 5-8 illustrates an important property of chromatofocusing discussed earlier, namely, the ability to maintain continuous concentrations of mobile-phase modifiers or stabilizers in both column equilibration and focusing buffers. It shows the elution behavior of human uterine estrogen receptor proteins chromatofocused using SynChropak AX-500 high-performance anion-exchange columns in the absence and presence of 10 mM sodium molybdate. The practical use of simple, chemically defined focusing buffers is shown in Figure 5-9 for the separation of calf uterine estrogen receptor forms showing surface charge heterogeneity. Polyethyleneimine-derivatized high-performance silica-based columns (Bakerbond WP-PEI) from J. T. Baker Inc. were used for the experiments

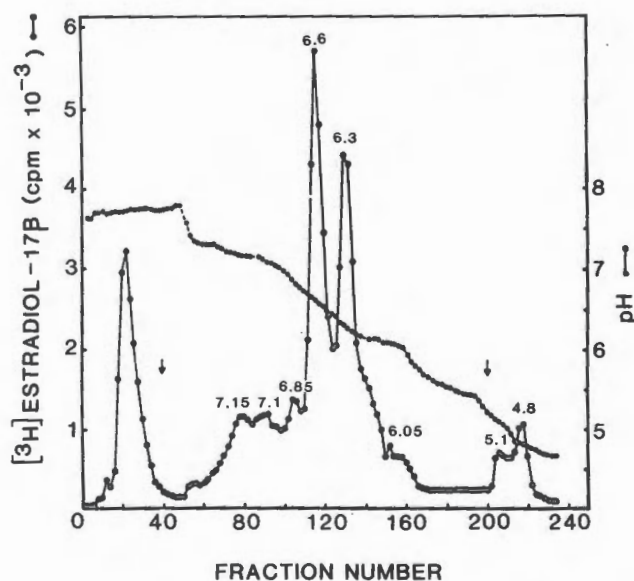
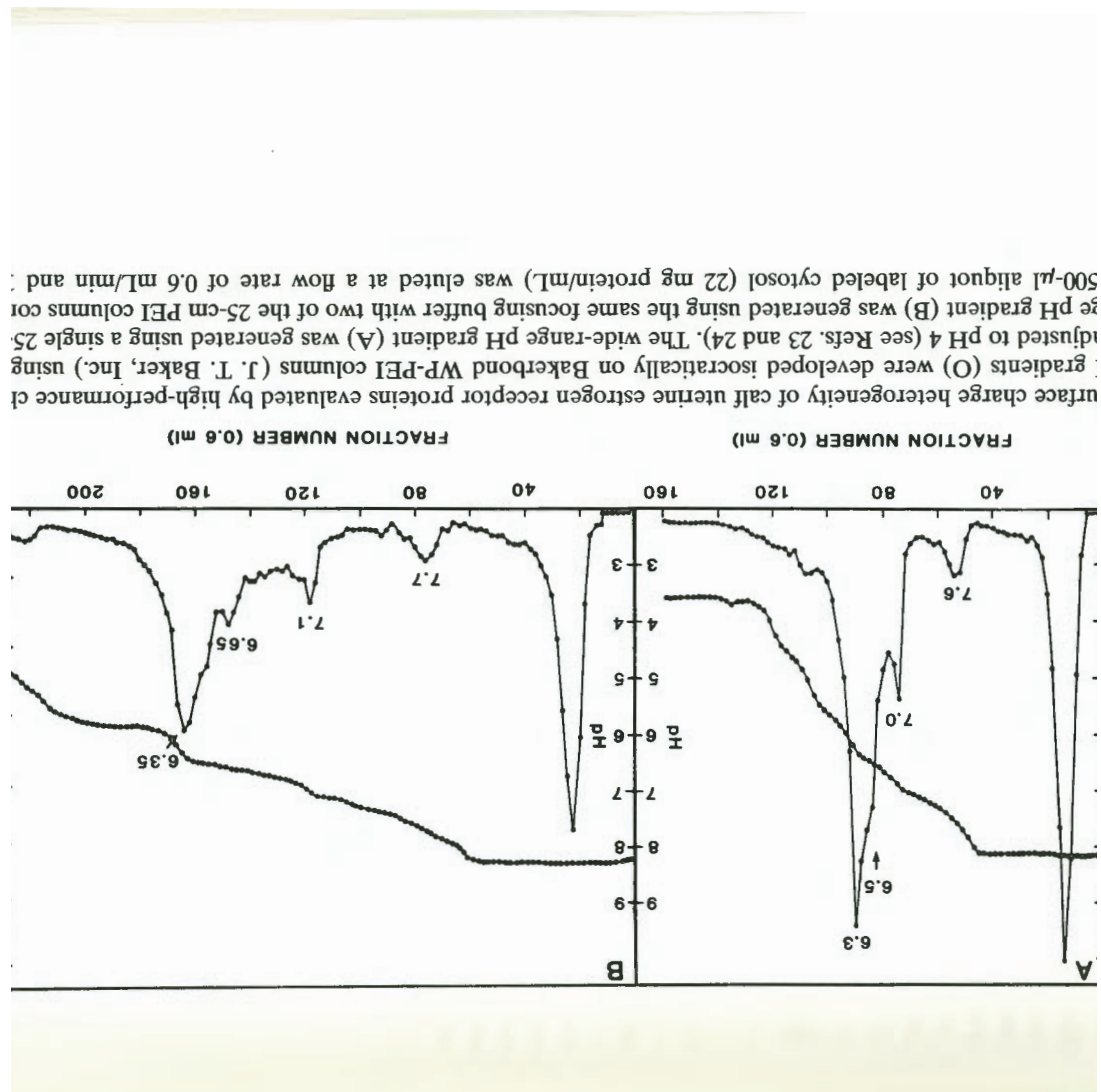


FIGURE 5-8. Identification of molybdate-stabilized receptor species by HPLC chromatofocusing on AX-500 in the continued presence of molybdate. Human uterine cytosol was prepared and labeled with $[^{125}\text{I}]\text{iodoestradiol-17}\beta$ in the presence of 10 mM sodium molybdate. Receptor preparations (2 to 4 mg) were eluted from the AX-500 column with a biphasic pH gradient. The primary eluent was a 30:70 mixture of Polybuffers 96 and 74, diluted 1:10 with 20% glycerol containing 10 mM sodium molybdate and adjusted to pH 5.0. The secondary eluent (initiated at arrow) was Polybuffer 74, diluted 1:10 with 20% glycerol (no molybdate) and adjusted to pH 3.5. The recovery of activity in this representative experiment was 91%.



surface charge heterogeneity of calf uterine estrogen receptor proteins evaluated by high-performance cationic isoelectric focusing on Bakerbond WP-PEI columns (J. T. Baker, Inc.) using gradients (O) were developed isocratically on Bakerbond WP-PEI columns (J. T. Baker, Inc.) using adjusted to pH 4 (see Refs. 23 and 24). The wide-range pH gradient (A) was generated using a single 25-cm PEI column (J. T. Baker, Inc.) and the narrow-range pH gradient (B) was generated using the same focusing buffer with two of the 25-cm PEI columns connected in series. A 500- μ l aliquot of labeled cytosol (22 mg protein/mL) was eluted at a flow rate of 0.6 mL/min and

shown here with the defined focusing buffer. Since the estrogen receptor proteins have high-affinity binding sites from polyanions and are also relatively hydrophobic, one purpose of these experiments was to determine if polymeric ampholytes in the polybuffers previously utilized to evaluate receptor surface charge were themselves influencing receptor heterogeneity. These profiles also illustrate nicely how increased column buffering capacity can be used with a single focusing buffer to decrease the pH gradient slope and increase resolution. The average gradient slope ($\Delta\text{pH}/\Delta\text{mL}$) was decreased from approximately 0.1 to 0.03 over the separation pH range by doubling the column length. The use of this same nonpolymeric focusing buffer can be used to generate a narrow pH gradient on a polymer-based chromatofocusing column (Pharmacia PBE 94) to fractionate a commercial source of hemoglobin (Figure 5-5).

The unliganded form of any steroid receptor protein is even more labile than the steroid-bound form. The separation and recovery of unliganded estrogen receptor (aporeceptor) forms with apparently unaltered biological activity (i.e., steroid binding) were possible by collecting receptor fractions, directly into pH-neutralizing buffer solutions containing radiolabeled steroid.

Figure 5-10 shows how the inclusion of 6 *M* urea in both column equilibration buffers and focusing buffers was utilized to differentially affect the chromatofocusing elution properties of unfractionated cytosolic versus DNA affinity-purified calf uterine estrogen receptor forms. In this manner, by differential chromatofocusing, two previously uncharacterized receptor forms were identified, separated, and partially purified for further analysis.

pH gradient engineering can also be effectively utilized to maximally separate and purify protein isoforms. Affinity-purified estrogen receptors, indistinguishable by sedimentation or size exclusion analyses, were resolved into two major subfractions using a biphasic descending pH gradient.

5.7.2 Analysis and Preparative Scale Isolation of Heterogeneous Forms of Cyanobacterial Ferredoxin-NADP⁺ Oxidoreductase

Serrano⁴⁵ achieved nice results using chromatofocusing as both an analytical and a preparative tool to evaluate and separate molecular heterogeneous forms of ferredoxin-NADP oxidoreductase (FNR) (EC 1.18.1.2) from the nitrogen-fixing cyanobacterium *Anabaena* sp. strain 7119. The cells were grown in culture, harvested, washed, and stored at -20°C before use. The cells were then thawed and disrupted by exposure to buffered solutions containing a 2% solution of the nonionic detergent Triton X-100. Duplicate batches of cells were processed in the presence and absence of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). All of the FNR enzyme activity was recovered in the supernatant liquid after centrifugation at 40,000 *g* for 2 min. The FNR (M_r 33,000 to 38,000) was purified by chromatography on DEAE-cellulose (Whatman DE-52) and 2',5' ADP-Sepharose 4B (Pharmacia).

Chromatofocusing was utilized to analyze molecular heterogeneity of the FNR purified in the presence and absence of PMSF. A column (1 $\times$ 25 cm)

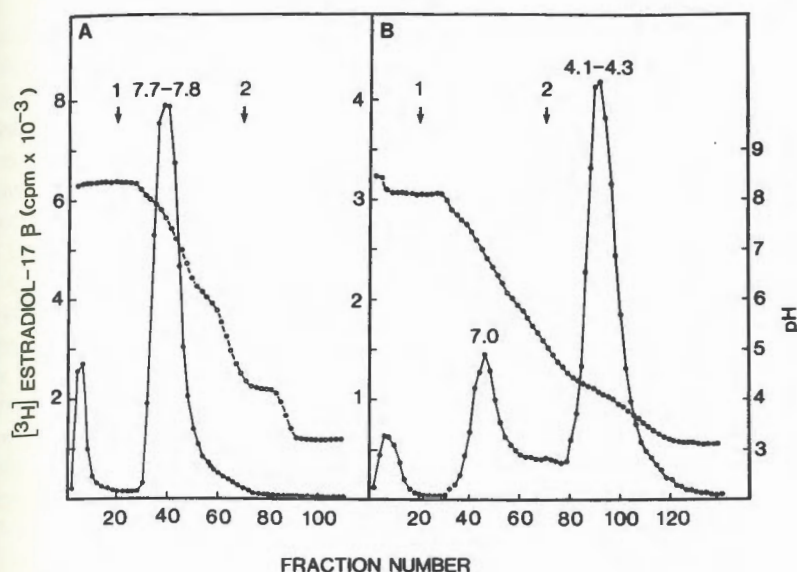


FIGURE 5-10. Chromatofocusing to evaluate urea effects on the surface charge properties of estrogen-receptor complexes purified by DNA affinity chromatography. Purified estrogen-receptor complexes were analyzed by chromatofocusing on PBE 94 in both the absence (A) and the presence (B) of 6 M urea. Nonspecific estrogen-binding components were absent after receptor purification by DNA affinity chromatography in the presence of 6 M urea. The specific elution pH of estrogen-receptor forms is indicated.

of Pharmacia PBE 94 was equilibrated with 25 mM piperazine-HCl buffer, pH 5.3. Samples of the purified FNR (3 to 13 mg in 5 mL) were dialyzed against the PBE column equilibration buffer before application. The focusing pH gradient was initiated by elution (12 mL/h) with 10-fold diluted Polybuffer 74 adjusted to pH 4 with HCl. As shown in Figure 5-11, a narrow (shallow slope) pH gradient was quite effective in the separation of (nicked) FNR enzyme generated by purification in the absence of the protease inhibitor PMSF. The one minor and three major peaks of FNR resolved (elution pH 4.3 to 4.6) were recovered in milligram quantities for subsequent characterization by SDS-PAGE, isoelectric focusing, and amino acid analysis. The high-resolution and preparative nature of chromatofocusing was clearly used in this example.

5.7.3 Isolation of Human Very-Low-Density Lipoproteins

In this example, Weisweiler and co-workers⁴⁶ utilized highly porous (>500 Å), monodisperse polymer particles (10 μm) developed by Ugelstad⁴⁷ and derivatized by Pharmacia (Mono P) for chromatofocusing to improve their separations of human very-low-density lipoproteins (VLDL). Serum was pre-

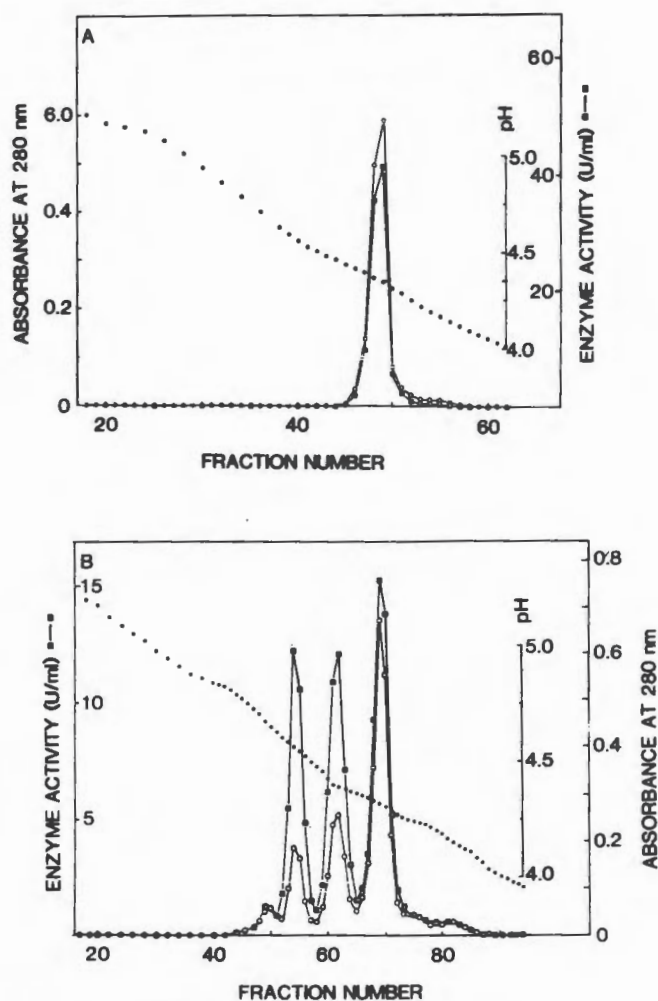


FIGURE 5-11. (A) Column chromatofocusing on PBE 94 of *Anabaena* sp. strain 7119 FNR purified in the presence of the protease inhibitor PMSF. A sample containing 3.5 mg of protein was applied to a PBE 94 column (1 × 25 cm), and the enzyme was eluted by using a pH gradient (●) generated with Polybuffer 74-HCl, pH 4.0 (7.5 μ mol/pH unit/mL). Fractions of 2 ml were collected. Absorbance at 280 nm (○) and enzyme activity (■) were measured for each fraction. (B) Column chromatofocusing on PBE 94 of *Anabaena* sp. strain 7119 FNR purified in the absence of the protease inhibitor PMSF. A sample containing 13.5 mg of protein, purified from the same batch of cells used in A, was chromatographed under the conditions described for A.

pared from hypertriglyceridemic patients by a combination of low-speed centrifugation and ultracentrifugation (160,000 *g* for 24 h) at 4°C. VLDL was removed from the top of the supernatant, dialyzed against 0.01 *M* ammonium bicarbonate buffer (pH 8.6), and lyophilized. Lipids were removed by extraction with diethyl ether and ethanol/diethyl ether. The VLDL apolipoproteins were solubilized in the chromatofocusing equilibration buffer, which consisted of 0.025 *M* bis-tris (pH 6.3) containing 6 *M* urea. Sample protein (up to 10 mg) was applied to a 4-mL prepacked Mono P column (5 mm i.d. × 20 cm) in column equilibration buffer, and the descending pH gradient was developed with 30 mL of (10-fold diluted) Polybuffer 74 (pH 4.0) at a flow rate of 1 mL/min. Figure 5-12 shows the chromatofocusing elution profile of the VLDL apolipoproteins. Seven peaks were resolved and collected. Polybuffers and urea were removed by dialysis and gel filtration (Sephadex G-50 fine). Overall recovery was reported to be 78%. Individual VLDL protein fractions were identified by double immunodiffusion in 2% agar using monoclonal antibodies before further analysis by isoelectric focusing and SDS-PAGE. The speed of VLDL apolipoprotein separation by chromatofocusing (30 min) on MonoBeads (Mono P) was considered a significant improvement over previously utilized, more time-consuming procedures.

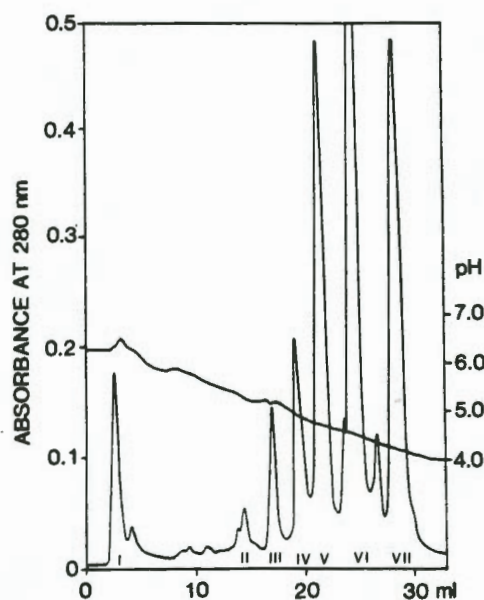


FIGURE 5-12. Fractionation of soluble VLDL apolipoproteins by chromatofocusing on 10- μ m monodisperse beads. Apolipoproteins (approximately 8 mg protein) were applied to a prepacked column (pH 6.3) in 6 *M* urea (Mono P). The pH gradient was developed with 30 mL of 1/10 diluted polybuffer 74 (pH 4.0) containing 6 *M* urea. The absorbance at 280 nm and the pH of each fraction are as shown. Seven fractions were collected as indicated.

5.8 SELECTED OTHER APPLICATIONS

Steroid sulfotransferase from human fetal liver was purified approximately 1,800-fold by a combination of ion-exchange chromatography, chromatofocusing, and affinity chromatography.⁴⁸ A 90-kDa glucoamylase from *Aspergillus niger*, a glycoprotein, was purified by a combination of ion-exchange chromatography, chromatofocusing, and Con A affinity chromatography.⁴⁹ The major lignin peroxidase isoenzymes from the basidiomycete *Phanerochaete chrysosporium* were purified to high homogeneity by a one-step separation by chromatofocusing with a recovery of over 80%.⁵⁰ Chromatofocusing was used for the large-scale purification of thymosin β -4 and thymosin β -9 from bovine lung tissue (2 kg). The method developed is rapid and convenient and can be used for the isolation of thymosin β -4 and homologous peptides from various tissues.⁵¹ A 32-kDa juvenile hormone from the hemolymph of the silkworm, *Bombyx mori* (pI 4.9), was purified by a combination of gel filtration, ion-exchange chromatography, chromatofocusing, and hydroxyapatite chromatography.⁵² The β -hemolysin from *Staphylococcus aureus* was purified 38,000-fold to homogeneity in just two steps using glass bead chromatography and chromatofocusing.⁵³ Two novel chitinases from *Streptomyces griseus* were purified by a procedure based on ammonium sulfate precipitation, hydrophobic interaction chromatography, and chromatofocusing. Both enzymes had a molecular mass of approximately 27 kDa, but had different pI values (7.7 and 7.3).⁵⁴ Six glycoforms of plasminogen 2 were purified by a combination of lectin affinity chromatography and chromatofocusing on the basis of their sialic acid content.⁵⁵ Finally, an extracellular dextranase from *Penicillium notatum* was purified from a crude enzyme powder extract by sequential anion- and cation-exchange chromatography and chromatofocusing. Two highly purified fractions were obtained with molecular masses of 55.8 and 50.1 kDa and isoelectric points at pH 4.9 and pH 4.75, respectively.⁵⁶

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