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PROTEIN PURIFICATION

Second Edition

Principles, High Resolution Methods, and Applications

Edited by

JAN-CHRISTER JANSON

LARS RYDÉN

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Principles, High-Resolution Methods, and Applications

SECOND EDITION

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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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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 orgin, 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 resuspensed 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

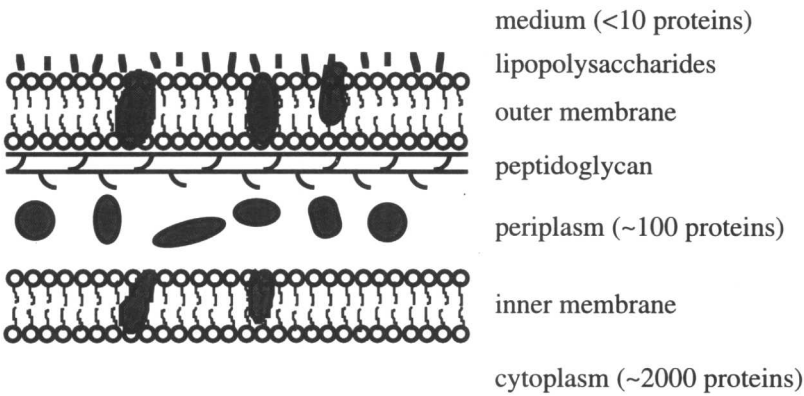

FIGURE 1-1. Location and approximate numbers of the proteins in *Escherichia coli.* (Courtesy of S. Ståhl, Royal Institute of Technology, Stockholm, Sweden.)

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 denaturated 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 denaturating 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.

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.