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Third Edition

PROTEIN PURIFICATION

Principles, High Resolution Methods, and Applications

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edited by
Jan-Christer Janson

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PROTEIN PURIFICATION

PREFACE TO THE THIRD EDITION

Most will agree that the major achievement in bioscience since 1998, when the second edition of this book was published, is sequencing of the human genome. Rather than diminishing interest in proteins, this has led to a revival in protein exploration and an intensive search for better understanding of molecular processes in health and disease. During this time, industrial exploitation of proteins in healthcare has hardly declined. The application of monoclonal antibodies targeted against rheumatoid arthritis and cancer has been booming, many second- and third-generation biopharmaceuticals have been approved, and modern technologies for vaccine production based on protein engineering and cell culture are being developed on a wide front.

There are approximately 21,000 protein-encoding genes, and the human proteome is much larger than this. Although mapping the genome revealed what was in the box, the jigsaw puzzle is far from complete. Several major research projects exemplify the revitalized interest in proteins. One is the Protein Atlas initiative (www.protematlas.org), aimed at providing a comprehensive database of high resolution microscopic images identifying proteins in normal and cancer tissues. Others involve an ever-widening range of refined tools exploiting protein profiling micro arrays, surface plasmon resonance, mass spectrometry, ELISA, quantitative 2D electrophoresis, and so on. Many technologies are aimed at parallel processing of thousands of targets, and this is profoundly changing the way structural biology projects are managed. Streamlined, miniaturized, automated high throughput (HTP) protocols are becoming the standard, but there is still a fundamental need for protein expression and purification, not least for X-ray structural studies. Many "proteomic" projects exploit high throughput purification of tagged proteins or antibodies.

On the industrial side, particular in healthcare, protein production is rapidly maturing. Platform technologies are being applied both upstream and downstream, allowing faster and leaner implementation as well as better control. Expression of monoclonal antibodies in mammalian cells is at the multigram per liter level, with cell densities of more than twenty million per milliliter, specific productivity over 20 picograms per cell per day, in bioreactors with capacities up to 20,000 liters. This several-hundred-fold increase in productivity has changed the pressures on downstream purification, resulting in the development of very high capacity chromatography media for product capture and highly selective media (frequently "multimodal") for polishing. Downstream purification of biopharmaceuticals uses platform modules for assuring virus safety and for removal of host cell proteins, aggregates and critical contaminants. Regulatory agencies are encouraging greater understanding and control of production processes, a quality by design (QbD) doctrine, and the use of modern risk management techniques and experimental design—all of which is impacting the development of purification methods.

Compared to the second edition of this book, four chapters have been deleted (Chromatofocusing, Affinity Partitioning, Immunoelectrophoresis, and Large-Scale Electrophoresis). Three chapters have been totally rewritten by new authors: Chapter 5 (High Resolution Reversed-Phase Liquid Chromatography of Proteins), Chapter 15 (Electrophoresis in Gels), Chapter 16 (Conventional Isoelectric Focusing in Gel Slabs and Capillaries and Immobilized pH Gradients). Six new chapters have been added: Chapter 10 (Affinity Ligands from Chemical Combinatorial Libraries), Chapter 11 (Affinity Ligands from Biological Combinatorial Libraries), Chapter 12 (Membrane Separations), Chapter 13 (Refolding of Inclusion Body Proteins from *E. coli*), Chapter 14 (Purification of PEGylated Proteins), and Chapter 20 (High Throughput Screening Techniques in Protein Purification). These new chapters have been written by leading experts in their respective fields. All other chapters have been thoroughly revised and updated regarding recent

applications. A new section on the history of protein chromatography has been added to Chapter 2 (Introduction to Chromatography).

It is my hope that the third edition will receive the same overwhelmingly positive response as the first and second editions, and I would like to express my appreciation to all contributing authors and to Ms Anita Lekhwani and her staff at John Wiley & Sons, Inc., Hoboken, New Jersey, for their patience and never-failing support of this project.

JAN-CHRISTER JANSON

PREFACE TO THE SECOND EDITION

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.

JAN-CHRISTER JANSON
LARS RYDÉN

PREFACE TO THE FIRST EDITION

Over the last two decades the scientific community has witnessed an unprecedented expansion within the biosciences and biotechnology. This expansion has been to a large extent driven by advances in several key areas, most notably recombinant DNA technology, hybridoma and cell culture techniques and, finally, in biochemical separation methods. This book is a description of the current status of one of these areas: modern techniques for protein purification and analysis.

The research on which the progress in separation techniques is based has been conducted both in university departments, devoted to basic research, and in industrial laboratories whose main concern is the development of new equipment and tools. In many cases the two communities have cooperated to their mutual benefit. In fact, a great number of the products now available for the separation and purification of proteins, such as chromatographic media with a wide range of selectivities and efficiencies, as well as equipment for electrophoretic separation and analysis, were originally developed in a university setting. This book is also the result of a joint effort between university researchers, in particular at Uppsala University, and the research staff of a company, Pharmacia LKB Biotechnology. Although it is thus a product of this condition of mutual benefit, the ambition has not been to give a selective description of methods or materials from a single commerical source, but rather to give an unbiased account of all key techniques in the field.

Today it is to a great extent possible to base the separation of proteins on knowledge of their molecular properties, structural as well as functional. Suggestions on how to solve a separation problem can best be made if data on protein structure and function, including particular structural details, is available. Conversely, results from the application of a particular separation method can often be interpreted in terms of molecular properties of the protein under study. Throughout the text of this book, separation results are related to protein properties, often in a detailed manner. We are the first generation to be on the verge of rational protein management.

Starting with this general concept, we have aimed at providing students, teachers and research workers in biomedicine, bioscience and biotechnology with a concise and practical treatise covering, in a single volume, all important chromatographic and electrophoretic techniques used in preparative and analytical protein chemistry. The book contains a general introductory chapter on protein preparative work, Chapter 1, where the key concepts are introduced. Similarly, a general introduction to chromatography is given in Chapter 2 and an introduction to analytical electrophoresis in Chapter 12. The major chromatographic and electrophoretic techniques are presented in individual chapters, including one chapter on affinity partitioning in aqueous polymer two-phase systems.

No single person can today be even close to acquiring the amount of experience necessary to describe with confidence the wealth of techniques and methods which makes up the arsenal for protein separations. We have thus chosen to produce a multi-author volume recruiting expertise from the entire field. All chapters have, however, been thoroughly worked through by the editors to achieve a reasonable uniformity of style and organization. Each chapter deals first with the theory and underlying principles of each separation technique, followed by a section on methodology, and ends with a number of representative application examples described in detail.

The preparation of this book has been a matter of several years. We would like to thank the authors for their cooperation, from the first planning stage to the last phase of updating and addition. We would also like to thank our editors at VCH Publishers in New York, in particular Dr. Edmund H. Immergut who took the first initiative and who followed the project up to its realization. The management and staff of Pharmacia LKB Biotechnology are thanked for their cooperation and support which allowed the selling price to be considerably reduced. Many staff members have made invaluable contributions to the final result, which are

gratefully acknowledged. We also thank Elizabeth Hill and Ursula Snow for their contributions in the early phase of the project; Inger Galvér, Gull-Maj Hedén, Inga Johansson and Madeleine de Sharengrad for secretarial work; Bengt Westerlund for handling the computer programmes for the chemical structures; Uno Skatt and Lilian Forsberg for producing a number of the illustrations; and David Eaker and John Brewer for keeping our freedom with the English language within limits. Finally, we would like to add that we are well aware that much of our own efforts, occasional achievements and sometimes hardwon experience, as well as that of several of the other authors of this book, spring from the tree planted long ago by The Svedberg and Arne Tiselius, and later kept alive by Jerker Porath and Stellan Hjertén and many of their colleagues and pupils through fifty years of separation science at Uppsala University. We offer this book as the latest fruit of this tree, hopefully to be enjoyed by many.

JAN-CHRISTER JANSON
LARS RYDÉN
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PART I

INTRODUCTION

1

INTRODUCTION TO PROTEIN PURIFICATION

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Protein Purification: Principles, High Resolution Methods, and Applications, Third Edition. Edited by Jan-Christer Janson

1.1 INTRODUCTION

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

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

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

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

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

1.2 THE PROTEIN EXTRACT

1.2.1 Choice of Raw Material

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

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