Protein Analysis and Purification

Second Edition

Ian M. Rosenberg

Protein Analysis and Purification

Benchtop Techniques

Second Edition

Birkhäuser
Boston • Basel • Berlin

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Library of Congress Cataloging-in-Publication Data
Rosenberg, Ian M., 1949–
Protein analysis and purification / Ian M. Rosenberg.
p. cm.
Originally published: Boston: Birkhäuser, c1996.
Includes bibliographical references and index.
ISBN 0-8176-4340-0 (alk. paper)—ISBN 0-8176-4341-9 (softcover : alk. paper)
1. Proteins—Analysis—Laboratory manuals. 2. Proteins—Purification—Laboratory manuals. I. Title.
QP551.R785 2004
572'.6—dc22 2004049926

Printed on acid-free paper.

Birkhäuser ®

ISBN 0-8176-4340-0 (hardcover) ISBN 0-8176-4341-9 (softcover)

Printed in the United States of America. (BS/HAM)

9 8 7 6 5 4 3 2 1 SPIN 10961834 (hardcover) SPIN 10988541 (softcover)

To Mireille, Ella, and Dina

Preface

How one goes about analyzing proteins is a constantly evolving field that is no longer solely the domain of the protein biochemist. Investigators from diverse disciplines find themselves with the unanticipated task of identifying and analyzing a protein and studying its physical properties and biochemical interactions. In most cases, the ultimate goal remains understanding the role(s) that the target protein is playing in cellular physiology.

It was my intention that this manual would make the initial steps in the discovery process less time consuming and less intimidating. This book is not meant to be read from cover to cover. The expanded Table of Contents and the index should help locate what you are seeking. My aim was to provide practically oriented information that will assist the experimentalist in benchtop problem solving.

The appendices are filled with diverse information gleaned from catalogs, handbooks, and manuals that are presented in a distilled fashion designed to save trips to the library and calls to technical service representatives. The user is encouraged to expand on the tables and charts to fit individual experimental situations.

This second edition pays homage to the computer explosion and the various genome projects that have revolutionized how benchtop scientific research is performed. Bioinformatics and *In silico* science are here to stay. However, the second edition still includes recipes for preparing buffers and methods for lysing cells.

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Acknowledgments

I would like to thank Paula Callaghan for giving me the opportunity to correct the first edition and bring this manual into the new millennium. Thanks also go to Barbara Chernow who has guided me through the final steps of this project, and special thanks to my accountant.

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☠ **Toxic substances are used.** Exercise caution when handling. Refer to Appendix A for further safety tips.

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An Overview of This Manual

Introduction

This book is designed to be a practical progression of experimental techniques an investigator may follow when embarking on a protein purification or protein analysis project. The protocols may be performed in the order laid out or may be used independently. The aim of the book is to assist a wide range of researchers, from the novice to the frustrated veteran, in the choice and design of experiments that are to be performed to provide answers to specific questions. The manual describes standard techniques that have been shown to work, as well as some newer ones that are beginning to prove important. By following the prominently numbered steps, one can work through any protocol, whether it's a new technique or a task that has been previously performed for which a quick review or updated methodology is needed.

Some topics are introduced with a brief description and a reference. When appropriate, the researcher is encouraged to follow this lead to learn more about a specific technique.

This manual will assist the experimentalist in designing properly controlled experiments. The basic premise remains the same: ask questions and test hypotheses. Sometimes the investigator must make a decision on what conditions to use. Since scientific experimentation is an iterative process, one might have to perform the same experiment three different ways in order to reach a satisfactory conclusion.

There will be no advice for dealing with specific pieces of equipment other than encouragement to read the manual, if you can find it. Throughout all manipulations try to be objective. Be on the lookout for unexpected findings. You will learn the most from surprising results, and they are often the beginning of the next project. It is never possible to record too much in your lab notebook. Treasure the rare moments when everything "works". Do not get discouraged. Remember, things will not always run smoothly.

During the selective survey of methodology, some specific examples are provided. Most methods have many variations, and the one that is

detailed has proven to work successfully. There is often a great deal of flexibility in the application of protein methods. Therefore, it should be possible, and it is encouraged, to alter or adapt a technique to your specific needs. Be flexible in finding the optimal conditions and then stick to them.

The evolution of a biomedical research project goes from a foundation of solid understanding of a particular process to a point where novel hypotheses need to be tested. As so often happens in scientific experimentation, the results of an experiment will dictate the follow-up experiment. For instance, one is given an antibody, a cDNA clone, or perhaps a biochemical assay to be used as a means of identifying and characterizing a specific protein that may play a role in a physiological function or disease process. The goal at this stage of the project is to produce enough of the specific protein in a purified form so that functional and biochemical questions can be asked without worrying that the effect might be attributable to a minor contaminant. The first step is to make use of analytical techniques that will give some basic information regarding the protein of interest.

In studying a biological system using a biochemical approach, researchers have traditionally attempted to purify each of the system's components. Using a reductionist approach, each component is then studied in detail with the ultimate aim being to reconstitute the system *in vitro* from the isolated components. The availability of completely sequenced genomes has triggered a shift towards studying the biological system as a whole. Experimental systems are being used that are designed to study all the concurrent physiological processes in a cell.

New methodologies have made brute force protein purification nearly obsolete. Protein science is being transformed by molecular biology. Innovations, the commercialization of reagent production, and simple modern conveniences such as the microwave oven have transformed labs into safer and more efficient places to work. Technology and capitalism have together provided instruments and kits that have replaced laboratory chores that in the 60s and 70s would have taken weeks to months to perform. Most labs no longer distill their own phenol or cast their own polyacrylamide gels. With each breakthrough and technological advance, researchers discover new challenges as they strive to do more in less time with smaller amounts of material. New protein sequences, as they are discovered, are entered into databases, as exemplified by the EMBL Nucleotide Sequence Database, which have grown nearly exponentially. Even with this ever-expanding electronic database, the techniques employed by the protein chemists of the 1960s and 1970s are still worth mastering in the 2000s. Often times, a preliminary step is needed to remove a bothersome contaminant prior to using an affinity method, high performance liquid chromatography (HPLC) or performing polyacrylamide gel electrophoresis (PAGE) to isolate the target protein for microsequencing. Therefore, many basic techniques will be described.

Kits, Cores, and Computers

Most institutions have at least one major resource core. The notion of shared instrumentation, a cost sharing measure and time saver, has evolved into a "core", laboratories that were set up to provide services for analyzing and synthesizing biomolecules. The cores of the late 80s and early 90s such as HPLC and amino acid analysis have expanded into a new generation of cores such as chip technology for genomics, proteomics, mass spectrometry, and real-time PCR reflecting the evolving needs and technologies of the day.

The increased use of pre-made kits is skyrocketing. In some cases it's less expensive to purchase the ready-made reagent than to buy the raw materials and make it in house! Kits make a technique or even a whole field accessible to researchers outside their area of expertise. One does not have to be a molecular biologist to sequence a gene. The downside of the kit generation is that it deprives beginners of the value and the understanding of assembling things from scratch. Young scientists should still know what goes into the test tube and why (DeFrancesco, 2002).

Now, performing scientific research without computers is unimaginable. Computers have become an integral part of lab life, collecting, analyzing and plotting data, writing papers and grants, searching and reading the literature, and emailing, surfing and playing games during long incubations. In fact, with the number and breadth of databases that are available online, in silico scientists can make discoveries without ever going into the lab.

How to Use This Book

This manual is aimed at first-time users and designed to shorten the time to a reliable result for those who are more experienced with a technique. It is comprised of a collection of selected traditional techniques and novel approaches to protein purification and analysis. In addition, newer techniques for purifying recombinant proteins are presented. The analytical chapters contain methods that examine the structure of proteins with emphasis on identifying the target protein in a complex mixture and on the recognition and analysis of posttranslational modifications and how to take advantage of them. In-depth theoretical explanations are not provided. Whenever possible, user-friendly step-by-step protocols for the at-the-bench scientist are provided in a simplified, clear form.

It is hoped that each protocol will be self-contained. Therefore, there will be a fair amount of repetition as many reagents and procedures are referred to repeatedly throughout the manual. Although the protocols have been designed to be complete, information from other protocols may need to be cross-checked. A list of reagents and equipment necessary to successfully complete the protocol will appear immediately prior to the step-by-step instructions.

Names of some solutions might be the same from protocol to protocol, while the composition of the solutions may differ. Be sure that the solution described in a particular protocol is prepared from the correct recipe. Recipes may appear cross-referenced to avoid potential confusion. Use the highest quality water available for the preparation of solutions and reagents. Centrifuge speeds are provided as $\times g$ or rpm (revolutions per minute). Consult the nomogram in Appendix F to convert these speeds for your centrifuge and rotor.

Throughout the manual suppliers of certain reagents and equipment are noted where pertinent. You may experiment with substituting your own favorite brand. Useful tips are included as part of the method or directly following it in a "*Comment*."

The Appendices are an essential feature of this manual. They are an eclectic collection of tables and charts, as well as trivial but useful information designed to provide bench-top access to diversified general information found scattered in various catalogs, brochures, manuals, handbooks, and other Methods books.

If you own this book for more than a few weeks and do not bend or smear any of its pages, then you are probably not using it correctly.

Basic Laboratory Equipment

At your work-station you should have access to the following items:

3 pipettes of each of the volumes, 1–20 µl, 20–200 µl, 200–1000 µl
Vortex mixer
Magnetic stirrer
Hot plate (Available for your routine use)
Comfortable chair
Centrifuge, low speed (20,000 RPM)
Refrigerated centrifuge
Ultra-centrifuge (20,000–80,000 RPM)
Microcentrifuge that holds standard 1.5 ml microfuge tubes
Geiger counter
Radiation shielding
Refrigerators and freezers
X-ray film
Cassettes and intensifying screens
Spectrophotometer
pH meter
Scintillation counter
Tissue culture equipment
Balance
Cell disrupter/sonicator
SDS-PAGE supplies
Power supply

Fume hood
Lyophilizer
Microscope
Water purification facility with the ability to produce ultrapure water (double-distilled, deionized, >18 Ω) should be used for all reagent preparations
Ice-maker
Computer terminal with internet access and printer
Glassware
Plastic containers of various dimensions

Laboratory Automation

Robotics technologies have revolutionized the way scientific research is being performed in the twenty-first century. Advances from the Human Genome Project have emphasized the concept of high-throughput technologies. To keep pace with the new reality in the lab, tools and resources have changed which change the way that scientific questions are asked and the strategies for answering them. The overall effect of laboratory automation is the increase in the speed at which data is generated. Scientists who once spent a lot of time performing repetitive tasks now spend more time analyzing data and planning experiments.

A computer is a necessity for accessing databases and to store, view and analyze one's own data. The desktop computer has become an integral piece of laboratory equipment. It is routinely used for word processing, storing experimental records and keeping laboratory databases. Computers are used as lab notebook by some investigators. Most importantly, computers allow access to the internet where one can scan catalogs, get technical assistance and order lab supplies. The web can replace lab clutter by cutting down on the volume of catalogs. Electronic journals are readily accessible from the comfort of one's computer, making trips to the library a special event. One can now download that article from an obscure journal found only in a library on the other side of town.

All of the major biological databases including GenBank®, European Molecular Biology Laboratory (EMBL), SWISS-PROT, Protein Information Resource (PIR), MEDLINE, and all of the ACDB species databases managed by the USDA have made information available on the Web. Simple direct access to these databases has changed the way that research is performed. Use frustrating experiences with computers as a chance to learn general computer problem solving skills. Try to know where the best tools for the specific task are located (Brown, 2000).

Automated liquid handling continues to be a major focus of lab automation. Hand-held electronic devices and sophisticated robots will both rapidly and tirelessly perform a range of tedious liquid handling tasks on 96, 384, and 1536 well plates. There are many liquid-handling options available that enable researchers to address their specific needs.

Clearly, not all labs need or require automatic pipetting capabilities or can afford a $75,000 system.

Imaging technology is playing an increasing role in experimental biology. Automation of experiments will require visible results to be recorded and interpreted by computerized imaging systems.

Beyond Protein Analysis and Purification

Much of the methodology presented in this manual is designed to put the researcher in position to characterize the target protein. The ultimate goal is to provide for every protein a database that would include a description of its sequence, its domain structure, sub-cellular localization, posttranslational modifications, variants, homologies to other proteins on the whole protein level and at the domain level, its interacting partners, its cellular function and knock-out and mutant information.

Once a partial amino acid sequence has been obtained, an in-depth computer search for homology with previously characterized proteins is usually performed. Expanding electronic protein databases offer the researcher a growing opportunity to gain clues to the uniqueness and function of a potentially new and important molecule. Eventually, the cDNA will be cloned and antibodies will be produced. The usefulness of this manual does not stop at this point. It provides the tools for answering some of the following biological and biochemically relevant questions pertaining to the target protein and its purification. (1) What are the options? (2) How does a particular method work and what are its strengths and weaknesses? (3) How can the method be exploited while minimizing its limitations? How does it fit with other methods? (4) What is the amount of target protein and the level of synthesis in a particular cell type? (5) What is the subcellular localization (nuclear, cytoskeletal, membrane, membrane receptors, specific organelles) of the target protein? Does the protein translocate in response to specific stimuli? (6) Is the protein posttranslationally modified, and how do these modifications affect its function? (7) Is the protein expressed in a cell-cycle-specific manner? Are there variations in the level of synthesis? (8) Is the level of target protein regulated by hormones, growth factors, heat shock etc.? (9) What is the rate of synthesis in normal and transformed cells? Is the target protein proliferation-sensitive, an oncoprotein, a component of a pathway that controls cell proliferation? (10) What is the function of the target protein? Does the protein belong to a family i.e., phosphoprotein, cytoskeletal protein, kinase or a protein with cyclin-like properties? (11) Is the target protein differentially expressed in various tissues? (12) When performing immunoprecipitation, do other proteins coprecipitate with the target protein? (13) Is the target protein specific to a given disease? How does the protein compare when isolated from healthy and diseased individuals? (14) What is the effect of transfected cDNA in cells that do not express the target protein? In brief, the structural and biochemical information that is obtained will allow the

investigator to propose hypotheses and design experiments to test their validity.

References

Brown SM (2000): *Bioinformatics: A Biologist's Guide to Biocomputing and the Internet*. Natick, MA: Eaton Pub

DeFrancesco L (2002): Today's lab. *The Scientist* 16:14–16

2

Protein Structure

Introduction

Proteus, a Greek sea god and keeper of all knowledge, would not give up information easily. Even while being held down, he would struggle mightily, assuming different forms before revealing any of his secrets. Although proteins were not named after Proteus, the description could not be more appropriate.

Proteins are complex macromolecules made up of successive amino acids that are covalently bonded together in a head-to-tail arrangement through substituted amide linkages called peptide bonds. Each protein molecule is composed of an exact sequence of amino acids arranged in a linear, unbranched fashion. Protein molecules have the property of acquiring a distinctive 3-dimensional configuration.

The amino acid backbone may have many posttranslational modifications contributing to the size, charge and function of the mature protein. A functionally active protein, being the product of posttranslational structural modifications, cannot be determined by reference to any single gene. Rather, the active form is often the result of complex biochemical reactions performed on the target protein that are not all controlled by any one gene product.

A. The Amino Acids

A representative amino acid is shown in Figure 2.1. The α-amino acids contain an α-carbon to which an amino group ($—NH_3^+$) and a carboxylate group ($—COO^-$) are attached. The 20 amino acids that make up the building blocks of proteins differ in the structure of their R groups, which may be hydrophilic or hydrophobic, acidic, basic, or neutral. Of the 20 amino acids normally used to build proteins, 19 have the general structure shown in Figure 2.1. Proline, the 20th natural amino acid, is similar but has the side chain bonded to the nitrogen atom to give the amino acid. The chemical composition of the unique R groups of the amino acids is responsible for the most important