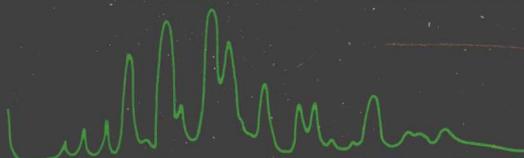
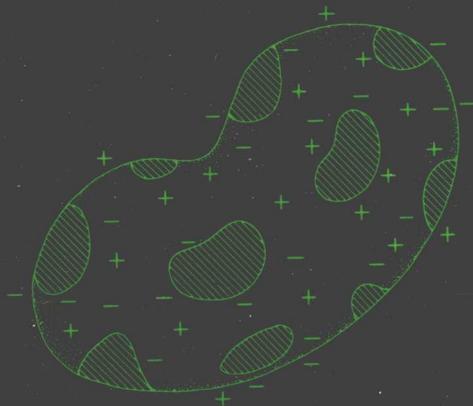
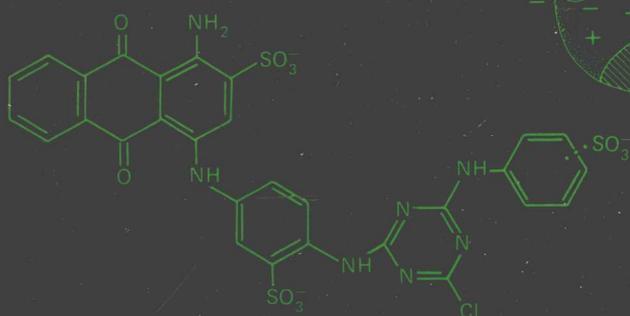


Robert K. Scopes

Protein Purification

Principles and Practice

Second Edition



Springer-Verlag

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Protein Purification

Principles and Practice

Second Edition

With 164 Figures

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Cover: (Top) Protein with charges and hydrophobic areas (see p. 42). *(Middle)* Structure of Cibacron Blue F36A and its reaction with hydroxyl groups (see p. 143). *(Bottom)* Scanning of stained gels for quantitation of protein zones (see p. 294).

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Series Preface

New textbooks at all levels of chemistry appear with great regularity. Some fields like basic biochemistry, organic reaction mechanisms, and chemical thermodynamics are well represented by many excellent texts, and new or revised editions are published sufficiently often to keep up with progress in research. However, some areas of chemistry, especially many of those taught at the graduate level, suffer from a real lack of up-to-date textbooks. The most serious needs occur in fields that are rapidly changing. Textbooks in these subjects usually have to be written by scientists actually involved in the research which is advancing the field. It is not often easy to persuade such individuals to set time aside to help spread the knowledge they have accumulated. Our goal, in this series, is to pinpoint areas of chemistry where recent progress has outpaced what is covered in any available textbooks, and then seek out and persuade experts in these fields to produce relatively concise but instructive introductions to their fields. These should serve the needs of one semester or one quarter graduate courses in chemistry and biochemistry. In some cases the availability of texts in active research areas should help stimulate the creation of new courses.

New York

CHARLES R. CANTOR

Preface to the Second Edition

The original plan for the first edition of this book was to title it *Enzyme Purification: Principles and Practice*. However, because all enzymes are proteins, and most methods applied also to nonenzymic proteins, I somewhat reluctantly agreed to the more general title. As a result, it has become increasingly clear during the past couple of years that the first edition was deficient in describing techniques that are in the main used for nonenzymic proteins. Indeed, there seems to be developing a separation between studies on enzymes and on nonenzymic proteins. With the advent of the new biotechnologies, a great amount of interest has been created in proteins such as hormones, hormone receptors, antigens, viral proteins, and others that for the most part are not enzymes and have structural properties that allow them to be treated quite differently. Most are extracellular, and as a result have a sturdier resistance to environmental factors. Many are very small proteins, they may have no subtle bioactivity to lose, and it is often not necessary to isolate them in a native, active state. Moreover, minute amounts, even a few micrograms, may be all that is needed to get a partial amino acid sequence to lead to gene isolation and expression in a host organism. Enzymes, on the other hand are generally less sturdy, larger molecules, whose native environment is usually within the protection of the cell, and they cannot always be handled in the same way as small, extracellular proteins. Also, the enzymologist likes to have at least several milligrams of pure product to carry out kinetic and structural studies.

Techniques less suited for enzymes include reverse-phase high-performance liquid chromatography (HPLC), sodium dodecylsulfate-polyacrylamide gel electrophoresis, and extremes of pH during extraction. In this second edition I have included a more extensive discussion on HPLC, but caution against automatic selection of this technique as being generally suitable for all enzyme

isolations. With large-scale enzyme production for commercial application becoming more widespread, the costs of the methodology (and HPLC certainly has a high capital cost factor) become important in assessing the usefulness of any method.

I have also taken the opportunity to give a detailed, and therefore disproportionate, description of techniques developed in our laboratory and in others for operating dye-ligand column chromatography. This is a technique that is complementary to HPLC, being mainly suited to large-scale work, having low capital costs, and in the crude sense a low resolution. But careful choice of dyes and operating conditions can make this a very successful procedure, which can revolutionize large-scale protein (and especially enzyme) purification as much as HPLC has revolutionized small-scale operations.

Once again I acknowledge the advice and help of numerous colleagues throughout the world who have made suggestions for improvements on the first edition.

Bundoora, Victoria
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ROBERT K. SCOPES

Preface to the First Edition

This book marks twenty years of research involving enzyme purifications, initiated partly because at the time of my higher degree (I needed some creatine kinase for ATP regeneration) we could not afford to buy commercial purified enzymes; there were hardly any available at that time anyway. The fascination of isolating a reasonably pure enzyme from a complex natural soup of proteins remains with me; as a challenge and an academic exercise I still spend much time on enzyme purification even when I have no real use for the final product! In those days everything was empirical: try an ammonium sulfate cut; try organic solvents; will it adsorb on calcium phosphate gel? There was not much else available. Now there is such a vast range of methods, of materials, and of approaches to the problem of enzyme purification that the difficulties lie not in the subtleties of manipulating a few available techniques, but in trying to decide which of the plethora of possibilities are most suitable.

It is the purpose of this book to guide the newcomer through the range of protein fractionation methods, while pointing out the advantages and disadvantages of each, so that a choice can be made to suit the problem at hand. Thus, traditional procedures such as salt fractionation are presented, along with the many modern developments in affinity chromatography and related techniques, which have been so successful in many cases, but have not proved to be the answer to all problems. During the two years of preparation of the manuscript, many new techniques and, equally important, new commercial products have been reported; preliminary drafts were updated as a new product came on the market, but by the time this is published there will undoubtedly be still more. In some cases the new product will be so superior that it will quickly supersede the older variety. Increasingly often, however, the question of cost-effectiveness is raised: this new system may be the best, but will it ruin our budget? For this reason I feel that the older, cheaper

products (and more classical techniques) will be with us for many years to come.

A few sentences on what this book does, and does not attempt to do: It is not a comprehensive presentation of all the methods ever used in enzyme purification, nor does it give detailed examples or precise instructions; if it did, it would occupy far more shelf space and would only duplicate excellent multi-volume treatises already available—in particular the series *Methods in Enzymology* and *Laboratory Techniques in Biochemistry and Molecular Biology*, and *The Proteins* (3rd edn., Vol. 1, eds. Newarth and Hill). Instead, it gives a brief account of the main procedures available, with some simple (even simplistic) theoretical and thermodynamic explanations of the events occurring. A basic background in biochemistry and protein chemistry is assumed: I expect the reader already to have on his shelves textbooks describing protein structure, simple enzyme kinetics, and thermodynamics. It is aimed to assist all students and researchers involved in the process of isolating an enzyme, from whatever source, whether it be a new project or simply following a published procedure.

In most places in this book the words “enzyme” and “protein” can be interchanged. All enzymes are proteins, but the reverse is not so. For the most part, I have adopted the system of using “enzyme” when referring to the particular protein being purified (even though it may not be an enzyme), and “protein” when referring either to the complete mixture at hand, or else to the proteins *other than* the particular one being purified.

There are many audiences to satisfy in a book such as this. Three, not necessarily exclusive, categories are: (i) those purifying a protein that has never successfully been purified before from any source; (ii) those purifying a protein from a new source, there being an adequate method available using some other source; and (iii) those who are simply following a recipe in an attempt to obtain a pure protein equivalent to that reported previously. As to the first category: it is becoming less common now for anyone to be purifying an enzyme for the first time, simply because there are fewer enzymes being discovered and, one presumes, few to be discovered. But in the second category, it is more common than it used to be for people to be purifying enzymes from new sources: no longer do biochemists restrict themselves to *E. coli*, yeast, rat liver, rabbit muscle, pig heart and spinach! Even a small shift in evolutionary terms (e.g., from pig to bovine heart) results in different behaviors not only of the enzyme being isolated but, equally importantly, of the other proteins present. Many standard methods are so critically dependent on precise conditions that a shift in source material may result in complete failure because of a minor variation in protein properties. Nevertheless, it is rare for the one enzyme to vary much in molecular weight even over large evolutionary distances; within phyla other properties such as ion exchange behaviors (dependent on isoelectric points) are not often widely at variance, and solubility behavior may well be similar.

The third category contains those who wish only to duplicate a preparation

that someone else has reported. If sufficient important details have been published, and the raw material and laboratory operating conditions are essentially identical, then there is no need for further help; unfortunately these criteria are not always met. Without experience and general knowledge of the principles, most beginners are unable to reproduce a method the first time—or even at all. Also, if it does not work they are afraid to depart one iota from the written word in case things get worse. For these people my advice will be to make use of the information given but, if things are not going well, not to be afraid to change the conditions (they may have been reported incorrectly anyway, and a misprint not spotted in proof-reading could waste months); also, not to worry if they do not have a PX28 rotor for an SS26 model D centrifuge, if all that is needed is the pellet. If things are still going wrong, then it is time to introduce greater variations, using new materials or methods that have been developed since the original publication. Because enzyme purification is essentially a methodology-oriented practice, it is usually worth manipulating conditions repeatedly until the ideal combinations are found. This can be time-consuming, but is worth the effort in the end, as you can be sure of reproducibility if you are confident of the limitations on each step.

Finally I wish to acknowledge the contributions over the years of many students and the staff at La Trobe University. I should also like to thank Prof. K. Mosbach, Dr. C. R. Lowe, Dr. I. P. Trayer, Dr. C.-Y. Lee and Dr. F. von der Haar for making available reprints and preprints of their work on enzyme purification. Financial support from the Australian Research Grants Committee is gratefully acknowledged.

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ROBERT K. SCOPES

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Chapter 1

The Enzyme Purification Laboratory

This chapter introduces the basic methods and equipment used in enzyme purification. It is entirely possible to purify enzymes using relatively simple equipment; the bare essentials might be considered a centrifuge and a UV spectrophotometer. Of course, access to a wider range of equipment and automated systems can make life much easier. But more elaborate equipment and procedures have their cost. It will be stressed many times in this book that *delays during isolation of any enzyme must be avoided*. Any procedure that relies on using equipment that is slow, unreliable, or sometimes unavailable is dangerous. By relying only on relatively simple and common apparatus, delays can usually be avoided. The message is clear: always use the simplest of procedures first, and use specialized methods only when all else has failed.

1.1 Apparatus, Special Materials, and Reagents

There are many complex, sophisticated pieces of equipment designed for protein separations, and as in every other walk of life, these are becoming more automated for convenience and simplicity of operation. Yet as the apparatus becomes more enclosed in black boxes, controlled by microprocessors, we become further removed from the realities of what is happening, and in some ways have less control over what we want to do. Most of the more complex equipment is designed for repeating routine operations reliably, and as such is not always the most appropriate for developing new methods. But apart from these considerations, there is the one of cost effectiveness; duplication of cheap equipment is often more useful than getting one of the most

expensive on the market; money spent on the simplest things, such as plenty of pipettes, test tubes, and beakers, can be a better investment than purchasing an esoteric apparatus for carrying out one particular type of protein separation process that may not be used often (and may break down).

Having said that, there remains a baseline of minimal apparatus that any enzyme purification laboratory must have available. Measures of enzyme activity will usually be based on either spectrophotometry or radiochemistry. Although one can do without a scintillation counter if all assays can be done spectrophotometrically, a spectrophotometer is indispensable, since it is required for protein concentration measurements (cf. section 9.5). A spectrophotometer with UV lamp, preferably with an attached recorder for timed reactions, is regarded as a prime requirement.

Centrifuging can rarely be avoided. On an industrial scale filter systems are commonly used for separating precipitates, but in the laboratory a centrifuge is more convenient. The principles and practices of centrifuging are described below (cf. section 1.2). For most purposes the standard workhorse centrifuge is a refrigerated instrument capable of maintaining temperatures at or somewhat below 0°C, while centrifuging ability, expressed as relative g force \times capacity in liters, should be of the order of 10,000–15,000. Many other types of centrifuge can be useful, from the benchtop small-scale machine to preparative ultracentrifuges. But these are not generally necessary and would rarely get as much use in enzyme purification work as the basic machine described above.

Column chromatography is so generally employed that equipment for this is essential. Although column work can be carried out with a home-made glass tube or a syringe (cf. section 1.3), with manual fraction-collecting and subsequent spectrophotometric measurements on each tube, much more work can be done more easily with the basic automated setup, which costs about the same as the spectrophotometer and less than the centrifuge. This would consist of a variety of sizes of columns, a fraction collector with UV monitor, a peristaltic pump, and a magnetic stirrer (for gradient formation). A wide variety of column sizes and shapes should be available so that the optimum amount of column packing material can be used for the sample available; columns with adjustable plungers are advisable. A range of sizes suitable for “desalting” by gel filtration is described in section 1.4. For adsorption chromatography still other sizes may be required. A given amount of money may be better spent on two or more columns of simple design rather than one made to optimum specifications.

Homogenizers for disrupting cells are needed, and are described in section 2.2. Routine laboratory equipment such as balance, pH meter, stirrers, and ice machines are as necessary in enzyme purification as in other biochemical methodology. Volumetric measurements can be made with graduated cylinders (rarely is the accuracy of a volumetric flask necessary), pipettes, and, for small amounts, microliter syringes. Enzyme assays frequently demand the addition of a very small sample of the test solution, perhaps less than 1 μ l.

Modern syringes are capable of delivering such volumes with sufficient accuracy. A range of syringes from 0.5 up to 250 μl is highly desirable. In addition, continuously adjustable “Eppendorf”-type pipettes, using disposable tips, are useful for volume ranges from 5 μl up to 5 ml.

Analysis of protein mixtures by electrophoresis is carried out by gel electrophoresis or isoelectric focusing, most often on polyacrylamide gel which may or may not contain dodecyl sulfate as denaturant. Methodology is described in section 10.1. Many excellent equipment designs are now available commercially; starting from scratch a newcomer would be advised to obtain a thin-slab apparatus. There can be few biochemical laboratories in the world that do not have any equipment for analytical gel electrophoresis.

There are a number of special reagents that the protein purifier requires. These are mainly packing materials for column chromatography and chemicals that are frequently used. Ion exchangers (section 5.1), gel filtration materials (section 6.1), and affinity adsorbents (section 5.3) should be on hand. More mundane, but vitally important, are such things as dialysis tubing (a range of sizes), a range of buffers (section 8.1), bulk ammonium sulfate of adequate purity (section 3.3), thiol compounds such as 2-mercaptoethanol and dithiothreitol, EDTA (ethylene diamine tetraacetic acid), sodium dodecyl sulfate, protamine sulfate, phenylmethylsulfonyl fluoride, Folin–Ciocalteu reagent, Coomassie blue, etc., the uses for which will be described in various places throughout this book.

1.2 Separation of Precipitates and Particulate Material

Filtration

The development of efficient refrigerated centrifuges provided a cleaner, more efficient method than filtration for the separation of precipitated proteins and other matter, especially on a small scale. However, there are still occasions when filtration is preferable. On a very large scale, handling of large volumes by centrifugation may require nonstandard equipment such as continuous-flow adaptors (Figure 1.1), and in any case the process will take a long time, perhaps comparable with large-scale filtration. On the other hand, the size and softness of most biological precipitates lead to rapid clogging of filtration materials so that after an initial burst of clear filtrate, the flow reduces to a trickle, even under suction, and the filtration material must be replaced. On an industrial scale it is possible to use special rigid filters with automatic scraping equipment for removing the slimelike precipitate as it collects, but such systems are not normally available in a laboratory.

Filtration can be greatly improved using filter aids such as Celite, a diatomaceous earth consisting mainly of SiO_2 (see Figure 1.2) (as used for

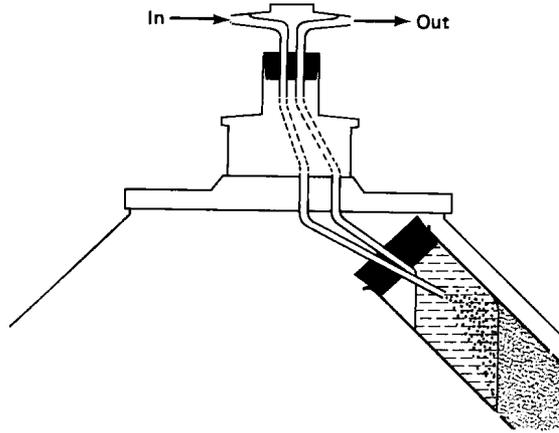


Figure 1.1. Simplified diagram of the continuous-flow adaptor for Sorvall SS-34 centrifuge rotor. Liquid can be passed through at several hundred milliliters per minute, collecting particulate matter up to 300 cm³ in volume.

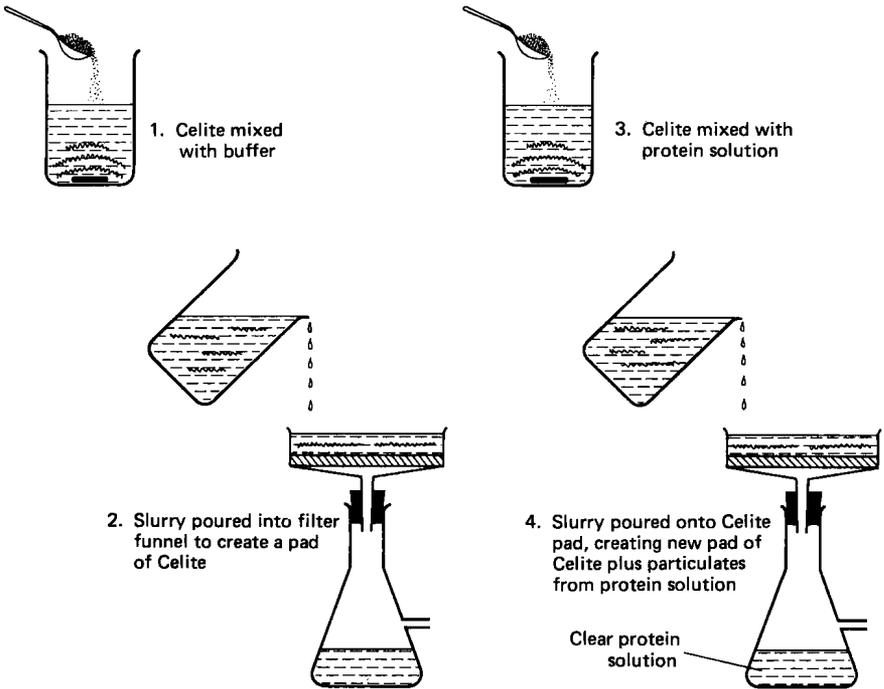


Figure 1.2. Use of Celite as a filter aid for clarifying protein solutions.

swimming pools). The best results are obtained by mixing in the Celite with the sample to be filtered, then pouring the suspension onto a Büchner funnel under slight suction. By creating a very large surface to trap the gelatinous precipitate, the filter aid allows much more filtrate to be obtained before eventually clogging up. The process is mainly used for removing small amounts of unwanted particulate material; it cannot deal with large quantities of precipitate successfully.

For removing obviously lumpy precipitates, hair, dirt, insoluble salt residues, etc., filtration through a no. 54 (Whatman) filter paper can be carried out; this is particularly appropriate immediately prior to a column procedure where the precipitate would otherwise be "filtered" on the packing material. Centrifugation is usually equally satisfactory.

A recent development in large-scale "ultrafiltration" (applying pressure to force liquid through a membrane while retaining particles too large to pass through it) is the Pellicon Cassette System from Millipore Corporation. Using a total filter area of up to 2 m², small particles are retained, removing filtrate at rates of up to 1 liter/min. This system is particularly useful for concentrating microorganisms, before centrifuging to obtain a proper precipitate; it does not itself produce a "paste", just a more concentrated suspension.

Centrifugation

Centrifugation relies on the sedimentation of particles in an increased gravitational field. The forces acting on a particle are illustrated in Figure 1.3. For rigid spherical particles the time required for a particle to sediment in a given

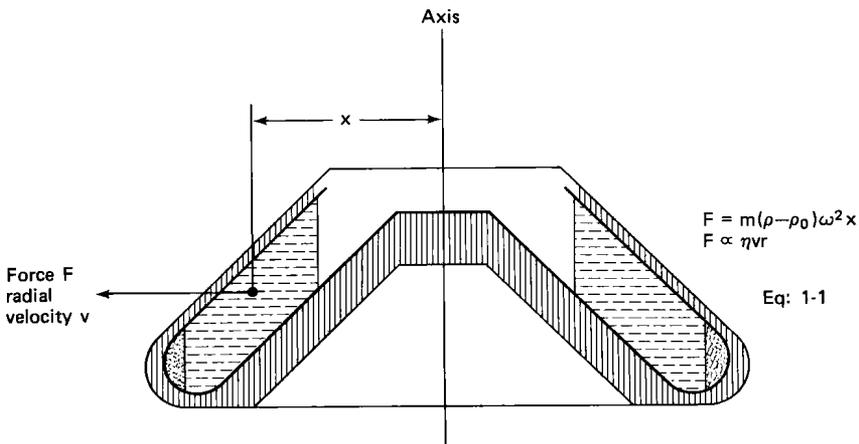


Figure 1.3. Forces acting on a particle in a centrifuge rotor. Mass of particle = m , density of particle = ρ , density of solution = ρ_0 , angular velocity of rotor = ω , viscosity of solution = η , and Stokes's radius of particle = r .