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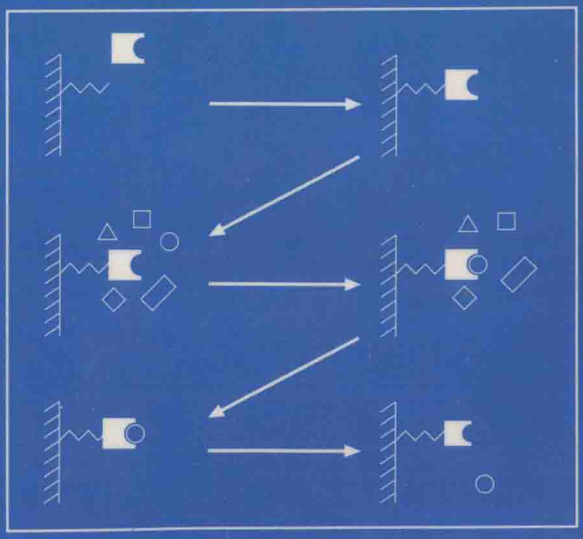
PROTEIN PURIFICATION PROTOCOLS

Edited by

Shawn Doonan



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Protein Purification Protocols

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Preface

Hans Neurath has written that this is the second golden era of enzymology (*Protein Science* [1994], vol. 3, pp. 1734–1739); he could with justice have been more general and referred to the second golden age of protein chemistry. The last two decades have seen enormous advances in our understanding of the structures and functions of proteins arising on the one hand from improvements and developments in analytical techniques (*see* the companion volume, *Basic Protein and Peptide Protocols,* in this series) and on the other hand from the technologies of molecular genetics. Far from turning the focus away from protein science, the ability to isolate, analyze, and express genes has increased interest in proteins as gene products. Hence, many laboratories are now getting involved in protein isolation for the first time, either as an essential adjunct to their work in molecular genetics or because of a curiosity to know more about the products of the genes that they have been studying.

Protein Purification Protocols is aimed mainly at these newcomers to protein purification, but it is hoped that it will also be of value to established practitioners who may find here techniques that they have not tried, but which might well be most applicable in their work. With the exception mainly of the first and last chapters, the format of the contributions to the present book conform to the established format of the *Methods in Molecular Biology* series. That is, they introduce the theoretical background of a method or group of related methods, provide a list of the reagents and equipment required for the procedure, follow with a detailed step-by-step description of how to carry out the protocol, and conclude with a set of Notes dealing with whatever problems are likely to arise and precisely how to deal with them. The aim has been to cover everything that is required to obtain a pure protein from initial extraction to drying and storage of the product. The success

of a purification schedule is critically dependent on obtaining a good initial extract with which to work; thus much of Chapters 2–12 is concerned with procedures for the extraction of proteins from various sources, including subcellular fractions of plant and animal tissues, for concentrating extracts, and for maintaining the integrity of proteins in those extracts. The remainder of the book then deals with individual techniques for fractionation and purification, including the special approaches required in the case of membrane proteins.

The first and last chapters of *Protein Purification Protocols* are somewhat different. Many of the individual methods described in the main section of the book are forms of column chromatography, but the chapters deal with particular techniques and not with the chromatographic method *per se*. Hence we have considered it worthwhile to conclude with a general chapter on practical column chromatography to fill in some of the experimental details that are common to all its applications. The first chapter deals with strategies for protein purification and is intended to put the rest of the book in context. Individual techniques used in protein purification are of limited value in isolation; the appropriate ones have to be used in the correct sequence to construct a complete purification schedule. It is hoped that this first chapter will help the newcomer to protein purification, as well as the seasoned investigator, to decide what has to be done and that the rest of *Protein Purification Protocols* will enable him or her to do it.

Shawn Doonan

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Contents

Chapter 1

General Strategies

Shawn Doonan

1. Defining the Problem

The chapters that follow in this volume give detailed instructions on how to use the various methods that are available for purification of proteins. The question arises, however, of which of these methods to use and in which order to use them to achieve purification in any particular case. That is, the purification problem must be clearly defined. What follows outlines the sorts of questions that need to be asked as part of that definition and how the answers affect the approach that might be taken to developing a purification schedule. It should be noted here that the discussion does not touch on the special cases of purification of proteins at industrial scale or for therapeutic applications; these raise very specific problems that are outside the scope of this chapter (*see* refs. *1* and *2*, respectively, for a coverage of these topics).

1.1. How Much Do I Need?

The answer to this question depends on the purpose for which the protein is required. For example, to carry out a full chemical and physical analysis of a protein may require several hundreds of milligrams of purified material while a kinetic analysis of the reaction catalyzed by an enzyme could perhaps be done with a few milligrams and <1 mg would be required to raise a polyclonal antibody. At the extreme end of the scale, if the objective is to obtain limited sequence information from the *N*-terminus of a protein as a preliminary to design of an oligonucleotide probe for clone screening, then using modern microsequencing techniques, a few micrograms will be sufficient. These different requirements for quantity

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may well dictate the source of the protein chosen (*see* Section 1.4.) and will certainly influence the approach to purification. Purification of large quantities of protein requires use of techniques, at least in the early stages, which have high capacity but low resolving power, such as fractional precipitation with salt or organic solvents (Chapter 13). Only when the volume and protein content of the extract has been reduced to manageable levels can methods of medium resolution and capacity, such as ion-exchange chromatography (Chapter 14) be used leading on, if necessary, to high-resolution but generally lower capacity techniques, such as affinity chromatography (Chapter 16) and isoelectric focusing (Chapter 23). On the other hand for isolation of small to medium amounts of proteins, it will usually be possible to move directly to the more refined methods of purification without the need for initial use of bulk methods. This is, of course, important because the fewer the steps that have to be used, the higher the final yield of the protein will be and the less time it will take to purify it.

1.2. Do I Want to Retain Biological Activity?

If the answer to this is positive then it restricts to some extent the range of techniques that can be employed and the conditions under which they can be performed. Most proteins retain activity when handled in neutral aqueous buffers at low temperature (although there are exceptions and these exceptions lend themselves to somewhat different approaches to purification). This consideration then rules out use of those techniques in which the conditions are likely to deviate substantially from the above. For example, immunoaffinity chromatography is a very powerful method but the conditions required to elute bound proteins are often rather severe, for example, the use of buffers of low pH, because of the tightness of binding between antibodies and antigens (*see* Chapters 16 and 19 for a discussion of this problem). Similarly, reversed-phase chromatography (Chapter 27) requires the use of organic solvents to elute proteins and rarely will be compatible with recovering an active species. Ion-exchange chromatography provides the most general method for isolation of proteins with retention of activity unless the protein has special characteristics that offer alternative strategies (*see* Section 2.4.). With labile molecules it is important to plan the purification schedule to contain as few steps as possible and with minimum requirement for changing buffers (Chapter 11), since this will reduce losses of activity.

In some cases, retention of biological activity is not required. This would be the case, for example, if the protein is needed for sequence analysis or perhaps for raising an antiserum. There is then no restriction on the methods that can be used and, indeed, the very powerful separation method of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) followed by blotting or elution from the gel can be used to isolate small amounts of pure protein either from partially purified extracts or even from crude extracts (Chapters 33–35). It is important in this context to differentiate between loss of biological activity arising from loss of three-dimensional structure, which will not be of concern in the applications outlined above, from loss of activity owing to modification of the chemical structure of the protein, which certainly would be a major concern. The most important route to chemical modification is proteolytic cleavage and ways in which this can be detected and avoided are discussed in Chapter 9.

1.3. Do I Need a Completely Pure Protein?

The concept of purity as applied to proteins is not entirely straightforward. It ought to mean that the protein sample contains, in addition to water and things like buffer ions that have been purposefully added, only one population of molecules all with identical covalent and three-dimensional structures. This is an unattainable goal and indeed an unnecessary one. What is required is a sample of protein that does not contain any species which will interfere with the experiments for which the protein is intended. This is not simply an academic point since it will usually become more and more difficult to remove residual contaminants from a protein sample as purification progresses. Extra purification steps will be required which take time (effectively an increase in cost of the product) and will inevitably lead to decreasing yields. What is required is an operational definition of purity for the particular project in hand because this will not only define the approach to the purification problem but may also govern its feasibility. It may not be possible to obtain a highly purified sample of a labile protein but it may be possible to obtain it in a sufficient state of purity for the purposes of a particular investigation.

The usual criterion of purity used for proteins is that a few micrograms of the sample produces a single band after electrophoresis on SDS-PAGE when stained with a reagent such as Coomasie blue or some similar non-specific stain (*see* ref. *3* for practical details of this procedure and other

chapters in the same volume for many other basic protein protocols). This simple criterion begs several questions. The most important of these is that SDS-PAGE separates proteins effectively on the basis of size and it may be that the sample contains two or more components that are sufficiently similar not to be resolved; the answer here is to subject the sample to an additional procedure, such as nondenaturing PAGE *(4)* since it is unlikely that two proteins will migrate identically in both systems. It must always be born in mind, however, that even if a single band is observed in two such systems, minor contaminants will inevitably become visible if the gel is more heavily loaded or if staining is carried out using a more sensitive procedure, such as silver staining *(5)*.

The major question is: Does it matter if the protein is 50, 90, or 99% pure? The answer is that it depends on the purpose of the purification. For example, a 50% pure protein may be entirely acceptable for use in raising a monoclonal antibody but a 95% pure protein may be entirely unacceptable for raising a monospecific polyclonal antibody particularly if the contaminants are highly immunogenic. Similarly, a relatively impure preparation of an enzyme may be acceptable for kinetic studies provided that it does not contain any competing activities; an affinity chromatography method might provide a rapid way of obtaining such a preparation. As a final example, a 95% pure protein sample is perfectly adequate for amino acid sequence analysis and indeed a lower state of purity is acceptable if proper quantitation is carried out to ensure that a particular sequence does not arise from a contaminant.

The message here is that preparation of a sample of protein approaching homogeneity is difficult and may not always be necessary so long as one knows what else is there. By taking account of the purpose for which the protein is required, it may be possible to decide on an acceptable level of contaminants and consideration of the nature of acceptable contaminants may suggest a purification strategy to be adopted.

1.4. What Source Should I Use?

The answer to this question may be partly or entirely dictated by the problem in hand. Clearly if the objective is to study the enzyme ribulose bisphosphate carboxylase, then there is no choice but to isolate it from a plant, but the plant can be chosen for its ready availability, high content of the enzyme, ease of extraction of proteins (Chapter 3), and low content of interfering polyphenolic compounds (Chapter 8). Of course, if

one is interested in, for example, comparative biochemistry or molecular evolution, then not only the desired protein but also its source may be completely constrained.

In general, however, plants will not be the source of choice for isolation of a protein of general occurrence and where species differences are not of interest. Microbial or fungal sources may be a better choice since they can usually be grown under defined conditions thus assuring the consistency of the starting material and, in some cases, allowing for manipulation of levels of desired proteins by control of growth media and conditions (Chapters 4 and 5). They have the disadvantage, however, of possesing tough cell walls that are difficult to break and, consequently, microorganisms are not ideal for large scale work unless the laboratory has specialized equipment needed for their disruption.

The most convenient source of proteins in most cases is animal tissue, such as heart and liver and, except for relatively small scale work, the tissues will normally be obtained from a commercial abattoir. Laboratory animals provide an alternative for smaller scale purifications. Content of a particular protein is likely to be tissue specific in which case the most abundant source will probably be the best choice. It is worth noting, however, that it is easier to isolate proteins from tissues, such as heart, than from liver (the reasons for which are outlined in Chapter 2) and hence the heart may be the better bet even if the levels of the protein are lower than in liver.

A different sort of question arises if the protein of interest exists in soluble form in a subcellular organelle, such as the mitochondrion or chloroplast. Once the source organism has been chosen, there remains the decision as to whether to carry out a total disruption of the tissue under conditions where the organelles will lyse or whether to homogenize under conditions where the organelles remain intact and can be isolated by methods such as those described in Chapters 6 and 7. The latter approach will, of course, result in a very significant initial enrichment of the protein and subsequent purification will be easier because the range and amount of contaminating proteins will be much decreased. In the case of animal tissues the decision will probably depend on the scale at which it is intended to work (assuming, of course, that access to the necessary preparative high-speed centrifuges is available). Subcellular fractionation of a few hundred grams of tissue is a realistic objective, but if it is intended to work with larger amounts, then the time required for organelle isolation probably will be prohibitive and is unlikely to

compensate for the extra work which will be involved in purification from a total cellular extract. Subcellular fractionation of plants is a much more difficult operation in most cases (*see* Chapter 7). Hence, except in the most favorable cases and for small scale work, purification from a total cellular extract will probably be the only realistic option.

In the case of membrane proteins, there again will be a considerable advantage in isolating as pure a sample of the membrane as possible before attempting purification. The ease with which this can be done depends on the organism and membrane system in question. Chapters 6 and 30 give some approaches to this problem for specific cases, but if it is intended to isolate a membrane protein from other sources, then a survey of the extensive literature on membrane purification is recommended (*see* ref. *6*).

For proteins which are present in only very small quantities or which are found only in inconvenient sources, gene cloning and expression in a suitable host now provide an alternative route to purification (for a review of methods *see* ref. *7*). This is, of course, a major undertaking and is likely to be used only when conventional methods are not successful. Sufffice it to say that once the protein is expressed and extracted from the host cell (*see* Chapter 4 for a method of extracting recombinant proteins from bacteria), the methods of purification are the same as those for proteins from conventional sources.

1.5. Has it Been Done Before?

It is quite common to need to purify a protein whose purification has been reported previously, perhaps to use it as an analytical tool or perhaps to carry out some novel investigations on it. In this case the first approach will be to repeat the previously described procedure. The chances are, however, that it will not work exactly as described since small variations in starting material, experimental conditions, and techniques—which are inevitable between different laboratories—can have a significant effect on the behavior of a protein during purification. This should not matter too much since adjustments to the procedures should be relatively easy to make once a little experience has been gained of the behavior of the protein. One pitfall to watch out for is the conviction that there ought to be a better way of doing it. It is possible to spend a great deal of time trying to improve on a published procedure often to little avail.

Even if the particular protein of interest has not been isolated previously, it may be that a related molecule has been, for example, the same