General Strategies

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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 question 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 concentrates mainly on laboratory-scale isolation of proteins. Special cases of purification of therapeutic proteins and isolation at industrial scale are covered in Chapters 43 and 44 (1–5).

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, whereas a kinetic analysis of the reaction catalyzed by an enzyme could perhaps be done with a few milligrams and less than 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 the design of an oligonucleotide probe for clone screening, then using modern microsequencing techniques, a few micrograms will be sufficient. In the field of proteomics, previously analytical techniques have become preparative with mass spectrometry commonplace for sensitive protein characterization from spots on gels. Chapters 36 and 40–42 describe these methodologies. These different requirements for quantity may well dictate the source of the protein chosen (see Subheading 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, that have a high capacity but low resolving power, such as fractional precipitation with salt or organic solvents (see Chapter 13). Process only when the volume and protein content of the extract has been reduced to manageable levels, methods of medium resolution and capacity, such as ion-exchange chromatography (see Chapter 14) can be used leading on, if necessary, to high-resolution
but generally lower-capacity techniques, such as affinity chromatography (see Chapter 16) and isoelectric focusing (see Chapter 24). 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. Often the decision as to whether or not to expose a costly matrix to the system early in the strategy will rest on issues related to the stability and/or the value of the target protein. This is, of course, important because the fewer 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 the 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 (e.g., 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 (see Chapter 28) 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 the isolation of proteins with retention of activity unless the protein has special characteristics that offer alternative strategies (see Subheading 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 (see Chapter 11), as this will reduce losses of activity. Most proteins retain their activity better at lower temperatures, although it should be remembered that this is not absolute because some proteins are cryoprecipitants and lose solubility at lower temperatures.

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 sulfate (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 (see Chapters 34 and 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 earlier, 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. Even therapeutic proteins will retain impurities all be it at the level of
parts per million (see Chapter 43). What is required is a sample of protein that does not
contain any species that will interfere with the experiments for which the protein is in-
tended. This is not simply an academic point because 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 in-
crease in cost of the product) and will inevitably lead to decreasing yields. What is re-
quired 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 pro-
tein, 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 sam-
ple produces a single band after electrophoresis on SDS-PAGE when stained with a
reagent such as Coomasie blue or some similar nonspecific stain (see ref. 6 for practi-
cal 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 whether 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 (7) because it is unlikely that two proteins will migrate
identically in both systems. It must always be kept in mind, however, that even if a sin-
gle 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 sensi-
tive procedure, such as silver staining (8).

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%
protein may be entirely unacceptable for raising a monospecific polyclonal anti-
body, 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 pro-
tein 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 partic-
ular sequence does not arise from a contaminant. The highest level of purity is needed
for therapeutic proteins. In this instance, other criteria need to observed such as com-
pliance with good laboratory practice (GLP) and good manufacturing practice (GMP),
which is beyond the scope of most standard research laboratories.

The message here is that preparation of a sample of protein approaching homogene-
ity is difficult and may not always be necessary so long as one knows what else there
is. By taking account of the purpose for which the protein is required, it may be possi-
bile 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 (see Chapter 3), and low content of interfering polyphenolic compounds (see 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 because 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 (see Chapters 4 and 5). They have the disadvantage, however, of possessing tough cell walls that are difficult to break and, consequently, micro-organisms 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. The 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 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 that 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 31 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. 9).

For proteins that are present in only very small quantities or 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. 10). This is, of course, a major undertaking and is likely to be used only when conventional methods are not successful. Suffice 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 Previously?

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 because 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 because 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 protein but from a different organism or a member of a closely related class of proteins. In the former case, particularly if the organisms are closely related, then the properties of the proteins should be quite similar and only minor variations in procedures (e.g., the pH used for an ion-exchange step) might be required. Even if the family relationships are more distant, significant clues might still be available, such as the fact that the target is a glycoprotein, which will provide valuable approaches to purification (see Subheading 2.4.). Much time and wasted effort can be saved by using information in the literature rather than trying to reinvent the wheel.

2. Exploiting Differences

Protein purification involves the separation of one species from perhaps 1000 or more species of essentially the same general characteristics (they are all proteins!) in a mixture of which it may constitute a small fraction of 1% of the total. It is, therefore, necessary to exploit to the full those properties in which proteins differ from one another in devising a purification schedule. The following lists the most important of those properties and outlines the techniques that make use of them with comments on their practical application. More details on each technique will be found in the chapters that follow.

2.1. Solubility

Proteins differ in the balance of charged, polar, and hydrophobic amino acids that they display on their surfaces and, hence, in their solubilities under a particular set of conditions. In particular, they tend to precipitate differentially from solution on the ad-
dition of species such as neutral salts or organic solvents and this provides a route to pu-
rification (see Chapter 13). It is, however, a rather gross procedure because precipitation
will occur over a range of solute concentrations and those ranges necessarily overlap for
different proteins. It is not to be expected, therefore, that a high degree of purification
can be achieved by such methods (perhaps twofold to threefold in most circumstances),
but the yield should be high and, most importantly, fractional precipitation can be car-
rried out easily on a large scale provided only that a suitable centrifuge is available. It is,
therefore, very common for this technique to be used at the stage immediately follow-
ing extraction when working on a moderate to large scale. An important added advan-
tage is that a substantial degree of concentration of the extract can be obtained at the
same time, which, considering that water is the major single contaminant in a protein
solution, is a considerable added benefit.

2.2. Charge

Proteins differ from one another in the proportions of the charged amino acids (as-
partic and glutamic acids, lysine, arginine, and histidine) that they contain. Hence, they
will differ in net charge at a particular pH or, another manifestation of them same differ-
ence, in the pH at which the net charge is zero (the isoelectric point). The first of these
differences is exploited in ion-exchange chromatography, which is perhaps the single
most powerful weapon in the protein purifier’s armory (see Chapter 14). This makes use
of the binding of proteins carrying a net charge of one sign onto a solid supporting ma-
terial bearing charged groups of the opposite sign; the strength of binding will depend on
the magnitude of the charge on the particular protein. Proteins may then be eluted from
the matrix in exchange for ions of the opposite charge, with the concentration of the ionic
species required being determined by the magnitude of the charge on the protein.

Ion-exchange chromatography is a technique of moderate to high resolution depend-
ning on the way in which it is implemented. For large-scale work (around 100 g of pro-
tein), use is generally made of fibrous cellulose-based resins that give good flow rates
with large bed volumes but not particularly high resolution; this would normally be done
at an early stage in a purification. Better resolution is available with the more advanced
Sepharose-based materials but generally on a smaller scale. For small quantities (<10
mg), the technique of fast protein liquid chromatography (see Chapter 27) is available,
which makes use of packing materials with very small diameters and correspondingly
high resolving power; this, however, requires specialized equipment that may not be
available in all laboratories. Because of the small scale, this method would usually be
used at a late stage for final cleanup of the product. It should be kept in mind that two
proteins that carry the same charge at a particular pH might well differ in charge at a
different pH. Hence, it is quite common for a purification procedure to contain two or
more ion-exchange steps either using the same resin at different pH values or perhaps
using two resins of opposite charge characteristics (e.g., one carrying the negatively
charged carboxymethyl [CM] group and the other the positively charged diethylamino-
ethyl [DEAE] group).

There are two main ways of exploiting differences in isoelectric points between pro-
teins. Chromatofocusing is essentially an ion-exchange technique in which the proteins
are bound to an anion exchanger and then eluted by a continuous decrease of the buffer
pH so that proteins elute in order of their isoelectric points (see Chapter 25). It is a method of moderately high resolving power and capacity and is hence best used to further separate partially purified mixtures. The other technique is isoelectric focusing (see Chapter 24), in which proteins are caused to migrate in an electric field through a system containing a stable pH gradient. At the pH at which a particular protein has no net charge (the isoelectric point), it will cease to move; if it diffuses away from that point, then it will regain a charge and migrate back again. This method, although of low capacity, is capable of very high resolution and is frequently used to separate mixtures of proteins that are otherwise difficult to fractionate.

2.3. Size

This property is exploited directly in the techniques of size-exclusion chromatography (see Chapter 26) and ultrafiltration (see Chapter 12). In the former, the protein solution is passed through a column of porous beads, the pore sizes being such that large proteins do not have access to the internal space, small proteins have free access to it, and intermediate-sized proteins have partial access; a range of these materials with different pore sizes is available. Clearly, large proteins will pass through the column most rapidly and small proteins will pass through most slowly with a range of behavior in between. The method is of limited resolving power but is useful in some circumstances, particularly when the protein of interest is at one of the extremes of size. The capacity is low because of the need to keep the volume of solution applied to the column as small as possible.

In ultrafiltration, liquid is forced through a membrane with pores of a controlled size such that small solutes can pass through but larger ones cannot. It, therefore, can be used to obtain a separation between large and small protein molecules and also has the advantage that it is not limited by scale. Use of the method for protein fractionation is, however, restricted to a few special cases (see Chapter 12) and the principal value of the technique is for concentration of protein solutions.

A completely different approach to the use of size differences to effect protein separation is SDS-PAGE. In this method, the protein molecules are denatured and coated with the detergent so that they carry a large negative charge (the inherent charge is swamped by the charge of the detergent). The proteins then migrate in gel electrophoresis on the basis of size; small proteins migrate most rapidly and large ones slowly because of the sieving effect of the gel. The method has enormously high resolving power and its use in various forms for analytical purposes is one of the most important techniques in analytical protein chemistry (6). The development of methods for recovery of the protein bands from the gel after electrophoresis (see Chapters 34 and 35) has enabled this resolving power to be exploited for purification purposes. Obviously, the scale of separation is small and the product is obtained in a denatured state, but a sufficient amount often can be obtained from very complex mixtures for the purposes of further investigation (see Subheading 1.2.). Combining isoelectric focusing and SDS-PAGE in two-dimensional gel electrophoresis also offers a very highly resolving preparatory technique (see Chapter 36) (11,12).

2.4. Specific Binding

Most proteins exert their biological functions by binding to some other component in the living system. For example, enzymes bind to substrates and sometimes to activators
or inhibitors, hormones bind to receptors, antibodies bind to antigens, and so on. These binding phenomena can be exploited to effect purification of proteins usually by attaching the ligand to a solid support and using this as a chromatographic medium. An extract or partially purified sample containing the target protein is then passed through this column to which the protein binds by virtue of its affinity for the ligand. Elution is achieved by varying the solvent conditions or introducing a solute that binds strongly either to the ligand or to the protein itself.

Various types of affinity chromatography, as the method is called, are described in detail in Chapters 16–20. Immunoaffinity chromatography, in particular, is capable of very high selectivity because of the extreme specificity of antibody–antigen interactions. As mentioned earlier and dealt with in more detail in Chapters 16 and 19, the most common problem with this technique is to effect elution of the target protein under conditions that retain biological activity (13). Lectin-affinity chromatography (see Chapter 18) exploits the selective binding between members of this class of plant proteins and particular carbohydrates. It has therefore found widespread use both in the isolation of glycoproteins and in removal of glycoprotein contaminants from other proteins, and it is also capable of high specificity.

Affinity methods that rely on interactions of the target protein with low-molecular-weight compounds (e.g., enzymes with substrates or substrate analogs) are frequently less specific because the ligand may bind to several proteins in a mixture. For example, immobilized NAD$^+$ will bind to many dehydrogenases, and benzamidine will bind to most serine proteases; thus, a group of related enzymes rather than individual species may be isolated using these ligands. A novel application of affinity methods is provided by the use of bifunctional NAD$^+$ derivatives to selectively precipitate dehydrogenases from solution (see Chapter 23).

The use of organic dyes as affinity ligands (see Chapter 17) is interesting because these molecules seem to bind fairly specifically to nucleotide-binding enzymes, although from their structures, it is not at all clear why they should do so; it is likely that hydrophobic interactions between the dye and protein also contribute to binding. Use of the latter interaction has led to development of a specific form of chromatography that uses hydrophobic stationary phases (see Chapter 15); this method has elements of biospecificity in that some proteins have binding sites for natural hydrophobic ligands, but in the general case, it relies on the fact that all proteins have hydrophobic surface regions to a greater or lesser extent (14).

Finally, many proteins are known that bind metal ions with varying degrees of specificity and this forms the basis of immobilized metal-ion affinity chromatography (see Chapter 20). Specific affinity of proteins for calcium ions may also be the basis, in part, for binding to hydroxyapatite but ion-exchange effects are probably also involved (see Chapter 21).

In summary, there are a variety of affinity methods available, ranging from medium to very high selectivity, and, in favorable cases, affinity chromatography can be used to obtain a single-step purification of a protein from an initial extract. Generally, however, the capacities of affinity media are not high and the materials can be very expensive, thus rendering their use on a large-scale unrealistic. For these reasons, affinity methods are usually used at a late stage in a purification schedule.
2.5. Special Properties

In a sense the specific binding properties discussed in the Subheading 2.4. are “special,” but that is not what is meant here. Some proteins have, for example, the property of greater than normal heat stability and in those circumstances it may be possible to obtain substantial purification by heating a crude extract at a temperature at which the target protein is stable, but contaminants are denatured and precipitate from solution (see ref. 15 for an example of the use of this method). It is not likely, of course, that this approach will be useful in purification of proteins from thermophilic organisms because all or most of the proteins present would be expected to share the property of thermostability. Another possibility is that the protein of interest may be particularly stable at one or other of the extremes of pH; in this case, incubation of an extract at low or high pH might well lead to selective precipitation of contaminants. It is always worthwhile carrying out some preliminary experiments with an unknown protein to see if it possesses special properties of this kind that would assist in its purification.

Finally, mention should be made of the fact that it is now feasible, if the need is sufficiently great, to engineer special properties into proteins to assist in their purification. Typical examples include the addition of polyarginine or polylysine tails to improve behavior on ion-exchange chromatography, or of polyhistidine tails to introduce affinity on immobilized metal affinity chromatography (16). It is, however, likely that these techniques would be used as a last resort if all other attempts to purify the protein failed unless recombinant DNA technology had been selected as the route to protein production and purification in the first place (see Subheading 1.4.).

3. Documenting the Purification

It is vitally important to keep an inventory at each stage of a purification of volumes of fractions, total protein content, and content of the protein of interest. The last of these is particularly important because otherwise it is very easy to end up with a vanishingly small yield of target protein and not to know at which step the protein was lost. If the protein has a measurable activity, then it is equally important to monitor this because it is also possible to end up with a protein sample that is inactive if one or more steps in the purification involves conditions under which the protein is unstable.

Measurement of the total protein content of fractions presents no problems. At early stages of a purification, it is usually sufficient to determine the absorbance of the solution at 280 nm (making sure that it is optically clear to avoid errors owing to light scattering) and to use the rough approximation that $A_{280} = 10$. At later stages, one of the more accurate methods, such as the Bradford procedure (17) or the bicinchoninic acid assay (18), should be used unless the absorbance/dry weight correlation for the target protein happens to be known.

Measurement of the amount and/or activity of the protein of interest may or may not be straightforward. For example, many enzymes can be assayed using simple and rapid spectrophotometric methods. For other proteins, the assay may be more difficult and time-consuming, such as bioassay or immunoassay. (It should also be recognized that these are not necessarily the same thing; immunoassay frequently will not distinguish between inactive and active molecules, so care must be taken in the interpretation of re-
sults using this method.) In other situations, the protein of interest may have no measurable biological activity; in such cases, immunoassay can be used or, more commonly, quantitation of the appropriate band after separation of the protein on polyacrylamide gels (19). Indeed, it may be that the target protein will only have been identified as a spot on two-dimensional polyacrylamide gels (20) and purification is being attempted as a preliminary to determining its biological activity.

Obviously, it is not possible to be prescriptive here about what methods of analysis and quantitation to use in any specific case. What must be said, however, is that it is very unwise to embark on an attempted purification without first devising a method for quantitation of the protein of interest. Not to do so is courting failure.

4. An Example

To give the newcomer to protein purification a “feel” for what the process might look like in practice, Table 1 shows the fully documented results of the isolation of a particular enzyme starting from 5 kg of pig liver. All techniques used are described in detail in subsequent chapters and are only summarized here.

The strategy was to start by totally homogenizing the tissue in 10 L of buffer and, after removal of cell debris by centrifugation, to carry out an initial crude purification by fractional precipitation with ammonium sulfate. This had the added advantages of removing residual insoluble material from the extract (this precipitated in the first ammonium sulfate fraction) and achieving a very large reduction in volume of the active fraction. Ammonium sulfate was removed from the active fraction by dialysis.

Because of the large amount of protein remaining in the active fraction, the next step was a relatively crude ion-exchange separation using a large column (7 × 50 cm) of CM–cellulose CM23 (this has a high capacity and good flow rates but is of only moderate resolving power). Conditions were chosen so that the enzyme was absorbed onto the column and then, after washing off unbound contaminants, it was eluted with a single stepwise increase in ionic strength to 0.1 M using sodium chloride.

Previous trial experiments had shown that the enzyme bound to an affinity matrix in a buffer at the same pH and salt content as that with which it was eluted from CM–cellulose, and so affinity chromatography was used for the next step without changing the buffer and without prior concentration. The enzyme was eluted by applying a linear salt gradient up to a concentration of 1 M.

At this stage, electrophoresis of the active fraction under nondenaturing conditions showed the presence of two major contaminants, both of them more basic than the protein of interest. Hence, the sample was applied to a column of DEAE–Sepharose under conditions where the target protein was absorbed, but the majority of the contaminating protein was not; the sample was equilibrated in starting buffer by dialysis before application to the column. The target protein was eluted from the column using a linear salt gradient and was found to be homogeneous by the usual techniques (see Subheading 1.3.).

The results in Table 1 show that the purification procedure was quite successful in that a high yield (50% overall) of enzyme activity was obtained; this was achieved by using a small number of steps each of which gave a good step yield. There will inevitably be losses on any purification step and the important point is that these and the number of steps should be kept as low as possible (a 5-step schedule in which the yield
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (mL)</th>
<th>Protein concentration (mg/mL)</th>
<th>Total protein (mg)</th>
<th>Activity&lt;sup&gt;a&lt;/sup&gt; (U/mL)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification factor&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Overall yield&lt;sup&gt;c&lt;/sup&gt; (%)</th>
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<tr>
<td>Homogenate</td>
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<td>40</td>
<td>340,000</td>
<td>1.8</td>
<td>15,300</td>
<td>0.045</td>
<td>1</td>
<td>100</td>
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<tr>
<td>45–70% (NH₄)₂SO₄</td>
<td>530</td>
<td>194</td>
<td>103,000</td>
<td>23.3</td>
<td>12,350</td>
<td>0.12</td>
<td>2.7</td>
<td>81</td>
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<tr>
<td>CM–cellulose</td>
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<td>19.5</td>
<td>8,190</td>
<td>25</td>
<td>10,500</td>
<td>1.28</td>
<td>28.4</td>
<td>69</td>
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<tr>
<td>Affinity chromatography</td>
<td>48</td>
<td>2.2</td>
<td>105.6</td>
<td>198</td>
<td>9,500</td>
<td>88.4</td>
<td>1,964</td>
<td>62</td>
</tr>
<tr>
<td>DEAE–Sepharose</td>
<td>12</td>
<td>2.3</td>
<td>27.6</td>
<td>633</td>
<td>7,600</td>
<td>275</td>
<td>6,110</td>
<td>50</td>
</tr>
</tbody>
</table>

<sup>a</sup>The unit of enzyme activity is defined as that amount which produces 1 µmol of product per min under standard assay conditions.

<sup>b</sup>Defined as follows: purification factor = specific activity of fraction/specific activity of homogenate.

<sup>c</sup>Defined as follows: overall yield = total activity of fraction/total activity of homogenate.
from each step is 50% will give an overall yield of 3%; a 10-step schedule with 80% step yield will give a final yield of 11%). It can also be seen from the final purification factor that the amount of this particular enzyme in the liver was low (about 0.016% of soluble protein) and, hence, a relatively large amount of tissue had to be used to obtain the required amount of product. This was an important factor in deciding the first two steps in the schedule (see Subheading 1.1).

The purification in its final form can be completed in 5–6 working days. It must be kept in mind, however, that each step has been optimized and that development of the procedure took several months of work. This is common when working out a new purification schedule and it is always necessary to be conscious of the time commitment when deciding to embark on purifying a protein.

References


Preparation of Extracts From Animal Tissues

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1. Introduction

The initial procedure in the isolation of an protein, a protein complex, or a subcellular organelle is the preparation of an extract that contains the required component in a soluble form. Indeed, when undertaking a proteomic study, the production of a suitable cellular extract is essential. Further isolation of subcellular fractions depends on the ability to rupture the animal tissues in such a manner that the organelle or macromolecule of interest can be purified in a high yield, free from contaminants and in an active form. The homogenization technique employed should, therefore, stress the cells sufficiently enough to cause the surface plasma membrane to rupture, thus releasing the cytosol; however, it should not cause extensive damage to the subcellular structures, organelles, and membrane vesicles. The extraction of proteins from animal tissues is relatively straightforward, as animal cells are enclosed only by a surface plasma membrane (also referred to as the limiting membrane or cell envelope) that is only weakly held by the cytoskeleton. They are relatively fragile compared to the rigid cell walls of many bacteria and all plants and are thus susceptible to shear forces. Animal tissues can be crudely divided into soft muscle (e.g., liver and kidney) or hard muscle (e.g., skeletal and cardiac). Reasonably gentle mechanical forces such as those produced by liquid shear may disrupt the soft tissues, whereas the hard tissues require strong mechanical shear forces provided by blenders and mincers. The homogenate produced by these disruptive methods is then centrifuged in order to remove the remaining cell debris.

The subcellular distribution of the protein or enzyme complex should be considered. If located in a specific cellular organelle such as the nuclei, mitochondria, lysosomes, or endoplasmic reticulum, then an initial subcellular fractionation to isolate the specific organelle can lead to a significant degree of purification in the first stages of the experiment (I). Subsequent purification steps may also be simplified, as contaminating proteins may be removed in the centrifugation steps. In addition, the deleterious affects of proteases released as a result of the disruption of lysosomes may also be avoided. Proteins may be released from organelles by treatment with detergents or by disruption resulting from osmotic shock or ultrasonication. Although there is clearly an ad-
vantage in producing a purer extract, yields of organelles are often low, so considera-
tion has to be made to the acceptability of a lower final yield of the desired protein.

Following production of the extract, some proteins will inevitably remain insoluble. For
animal tissues, these generally fall into two categories: membrane-bound proteins and
extracellular matrix proteins. Extracellular matrix proteins such as collagen and
elastin are rendered insoluble because of extensive covalent crosslinking between lysine
residues after oxidative deamination of one of the amino groups. These proteins can only
be solubilized following chemical hydrolysis or proteolytic cleavage.

Membrane-bound proteins can be subdivided into integral membrane proteins, where
the protein or proteins are integrated into the hydrophobic phospholipid bilayer, or ex-
trinsic membrane proteins, which are associated with the lipid membrane resulting from
interactions with other proteins or regions of the phospholipid bilayer. Extrinsic mem-
brane proteins can be extracted and purified by releasing them from their membrane an-
chors with a suitable protease. Integral membrane proteins, on the other hand, may be
extracted by disruption of the lipid bilayer with a detergent or, in some cases, an organic
solvent. In order to maintain the activity and solubility of an integral membrane pro-
tein during an entire purification strategy, the hydrophobic region of the protein must in-
teract with the detergent micelle. Isolation of integral membrane proteins is thought to
occur in four stages, where the detergent first binds to the membrane, membrane
lysis then occurs, followed by membrane solubilization by the detergent, forming a de-
tergent–lipid–protein complex. These complexes are then further solubilized to form
detergent–protein complexes and detergent–lipid complexes. The purification of mem-
brane proteins is, therefore, not generally as straightforward as that for soluble pro-
teins (2,3).

The principal aim of any extraction method must be that it be reproducible and dis-
rupt the tissue to the highest degree, using the minimum of force. In general, a cellular
disruption of up to 90% should be routinely achievable. The procedure described here
is a general method and can be applied, with suitable modifications, to the preparation
of tissue extracts from both laboratory animals and from slaughterhouse material (4,5).
In all cases tissues, should be kept on ice before processing. However, it is not gener-
ally recommended that tissues be stored frozen prior to the preparation of extracts.

2. Materials

The preparation of extracts from animal tissues requires normal laboratory glassware,
equipment, and reagents. All glassware should be thoroughly cleaned. If in doubt, clean
by immersion in a sulfuric–nitric acid bath. Apparatus should then be thoroughly rinsed
with deionized and distilled water. Reagents should be Analar grade or equivalent. In
addition, the following apparatuses are required:

1. Mixers and blenders: In general, laboratory apparatus of this type resemble their household
counterparts. The Waring blender is most often used. It is readily available from general
laboratory equipment suppliers and can be purchased in a variety of sizes, capable of han-
dling volumes from 10 mL to a few liters. Vessels made from stainless steel are preferable,
as they retain low temperatures when prechilled, thus counteracting the effects of any heat
produced during cell disruption.

2. Refrigerated centrifuge: Various types of centrifuge are available, manufacturers of which
are Beckman, Sorval-DuPont, and MSE. The particular centrifuge rotor used depends on
the scale of the preparation in hand. Generally, for the preparations of extracts, a six-position fixed-angle rotor capable of holding 250-mL tubes will be most useful. Where larger-scale preparations are undertaken, a six-position swing-out rotor capable of accommodating 1-L containers will be required.

3. Centrifuge tubes: Polypropylene tubes with screw caps are preferable, as they are more chemically resistant and withstand higher g forces than other materials such as polycarbonate. In all cases, the appropriate tubes for the centrifuge rotor should be used.

3. Methods

All equipment and reagents should be prechilled to 0–4°C. Centrifuges should be turned on ahead of time and allowed to cool down.

1. First, trim fat, connective tissue, and blood vessels from the fresh chilled tissue and dice into pieces of a few grams (see Note 1).

2. Place the tissue in the precooled blender vessel (see Note 2) and add cold extraction buffer using 2–2.5 vol of buffer by weight of tissue (see Note 3). Use a blender vessel that has a capacity approximately that of the volume of buffer plus tissue so that the air space is minimized; this will reduce aerosol formation.

3. Homogenize at full speed for 1–3 min depending on the toughness of the tissue. For long periods of homogenization, it is best to blend in 40-s to 1-min bursts with a few minutes in between to avoid excessive heating. This will also help reduce foaming.

4. Remove cell debris and other particulate matter from the homogenate by centrifugation at 4°C. For large-scale work, use a 6 × 1000-mL swing-out rotor operated at about 600–3000g for 30 min. For small-scale work (up to 3 L of homogenate), a 6 × 250-mL angle rotor operated at 5000g would be more appropriate (see Note 4).

5. Decant the supernatant carefully, avoiding disturbing the sedimented material, through a double layer of cheesecloth or muslin. This will remove any fatty material that has floated to the top. Alternatively, the supernatant may be filtered by passing it through a plug of glass wool placed in a filter funnel. The remaining pellet and intermediate fluffy layer may be re-extracted with more buffer to increase the yield (see Note 5) or discarded.

The crude extract obtained by the above procedure will vary in clarity depending on the tissue from which it was derived. Before further fractionation is undertaken, additional clarification steps may be required (see Note 6).

4. Notes

1. The fatty tissue surrounding the organ/tissue must be scrupulously removed prior to homogenization, as it can often interfere with subsequent protein isolation from the homogenate.

2. Where only small amounts of a soft tissue (1–5 g) such as liver, kidney, or brain are being homogenized, then it may be easier to use a hand-held Potter–Elvehjem homogenizer (6). This will release the major organelles; nuclei, lysosomes, peroxisomes, and mitochondria (7). The endoplasmic reticulum, smooth and rough, will vesiculate, as will the Golgi if homogenization conditions are too severe. On a larger scale, these soft tissues are easily disrupted/homogenized in a blender. However, tissues such as skeletal muscle, heart, and lung are too fibrous in nature to place directly in the blender and must first be passed through a meat mincer, equipped with rotating blades, to grind down the tissue before homogenization (8,9). As the minced tissue emerges from the apparatus, it is placed directly into an approximately equal volume by weight of a suitable buffer. This mixture is then squeezed...
through one thickness of cheesecloth, to remove the blood, before placing the minced tissue in the blender vessel.

3. Typically, a standard isotonic buffer used for homogenization of animal tissues is of moderate ionic strength and neutral pH. For instance, 0.25 M sucrose and 1 mM EDTA and buffered with a suitable organic buffer: Tris, MOPS, HEPES, and Tricine at pH 7.0–7.6 are commonly employed. The precise composition of the homogenization medium will depend on the aim of the experiment. If the desired outcome is the subsequent purification of nuclei, then EDTA should not be included in the buffer, but KCl and a divalent cation such as MgCl$_2$ should be present (10). MgCl$_2$ is preferred here when dealing with animal tissues, as Ca$^{2+}$ can activate certain proteases. The buffer used for the isolation of mitochondria varies depending on the tissue that is being fractionated. Buffers used in the preparation of mitochondria generally contain a nonelectrolyte such as sucrose (4,11). However, if mitochondria are being prepared from skeletal muscle, then the inclusion of sucrose leads to an inferior preparation, showing poor phosphorylating efficiency and a low yield of mitochondria. The poor quality is the result of the high content of Ca$^{2+}$ in muscle tissue, which absorbs to the mitochondria during homogenization; mitochondria are uncoupled by Ca$^{2+}$. The issue of yield arises from the fact that when skeletal muscle is homogenized in a sucrose medium, it forms a gelatinous consistency, which inhibits the disruption of the myofibrils. Here, the inclusion of salts such as KCl (100–150 mM) are preferred to the nonelectrolyte (8,12).

In order to protect organelles from the damaging effect of proteases, which may be released from lysosomes during homogenization, the inclusion of protease inhibitors to the homogenization buffer should also be considered. Again, their inclusion will depend on the nature of the extraction and the tissue being used. Certain proteins are more susceptible to degradation by proteases than others, and certain tissues such as liver contain higher protease levels than others. A suitable cocktail for animal tissues contains 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 µg/mL each of leupeptin, antipain, and aprotinin (see Table 1). These are normally added from concentrated stock solutions. Further additions to the homogenization media can be made in order to aid purification. A sulfhydryl reagent, 2-mercaptoethanol or dithiothreitol (0.1–0.5 mM), will protect enzymes and integral membrane proteins with reactive sulfhydryl groups, which are susceptible to oxidation. The addition of a cofactor to the media, to prevent dissociation of the cofactor from an enzyme or protein complex, can also assist in maintaining protein stability during purification.

4. Centrifugation is the application of radial acceleration by rotational motion. Particles that have a greater density than the medium in which they are suspended will move toward the outside of the centrifuge rotor, whereas particles lighter than the surrounding medium will move inward. The centrifugal force experienced by a particle will vary depending on its

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target proteases</th>
<th>Effective concentrations</th>
<th>Stock solutions</th>
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<tbody>
<tr>
<td>EDTA</td>
<td>Metalloproteases</td>
<td>0.5–2.0 mM</td>
<td>500 mM in water, pH 8.0</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Serine and thiolproteases</td>
<td>0.5–2 µg/mL</td>
<td>10 mg/mL in water</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>Acid proteases</td>
<td>1 µg/mL</td>
<td>1 mg/mL in methanol</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Serine proteases</td>
<td>0.1–2.0 µg/mL</td>
<td>10 mg/mL in phosphate-buffered saline</td>
</tr>
<tr>
<td>PMSF</td>
<td>Serine proteases</td>
<td>20–100 µg/mL</td>
<td>10 mg/mL in isopropanol</td>
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</tbody>
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distance from the center of rotation. Hence, values for centrifugation are always given in terms of \( g \) (usually the average centrifugal force) rather than as revolutions per minute (rpm), as this value will change according to the rotor used. Manufacturers provide tables that allow the relative centrifugal fields at a given run speed to be identified. The relative centrifugal field (RCF) is the ratio of the centrifugal acceleration at a certain radius and speed (rpm) to the standard acceleration of gravity (g) and can be described by the following equation:

\[
\text{RCF} = 1.118 (\text{rpm}/1000)^2
\]

where \( r \) is the radius in millimeters.

Centrifuges should always be used with care in order to prevent expensive damage to the centrifuge drive spindle and, in some instances, to the rotor itself. It is important that centrifuges and rotors are cleaned frequently. Essentially, this means rinsing with water and wiping dry after every use. Tubes must be balanced and placed opposite one another across the central axis of the rotor. Where small volumes are being centrifuged, the tubes can usually be balanced by eye to within 1 g. When the volumes are >200 mL, the most appropriate method of balancing is by weighing. Consideration should be given to the densities of the liquids being centrifuged, especially when balancing against water. A given volume of water will not weigh the same as an equal volume of homogenate. The volume of water used to balance the tubes can be increased, but it is better practice to divide the homogenate between two tubes. The tubes may well be of equal weight, but their centers of gravity will be different. As particles sediment, there will also be an increase in inertia and this should always be equal across the rotor. Care should also be taken not to over fill the screw-cap polypropylene tubes. Although they may appear sealed, under centrifugation the top of the tube can distort, leading to unwanted and potentially detrimental leakage of sample into the rotor. Fill tubes such that when they are placed in the angled rotor, the liquid level is just below the neck of the tube.

5. Following centrifugation of the homogenate, a large pellet occupying in the region of 25% of the tubes volume will remain. The pellet contains cells, tissue fragments, some organelles, and a significant amount of extraction buffer and, therefore, soluble proteins. If required, this pellet can be resuspended/washed in additional buffer. Disperse the pellet by using a glass stirring rod against the wall of the tube or, if desired, a hand-operated homogenizer. The resuspended material is centrifuged earlier and the supernatants combined. This washing will contribute to an increased yield but inevitably will also lead to a dilution of the extract. Therefore, the value of a repeat extraction needs to be assessed. For instance, when preparing liver or kidney mitochondria, washing the pellet in this way not only increases the yield, it also improves the integrity of the preparation, by allowing the recovery of the larger mitochondria.

6. The procedure outlined in this chapter is of general applicability and will, in some cases, produce extracts of sufficient clarity to proceed immediately to the next set of fractionation experiments. This is particularly true for cardiac muscle. However, for other tissues, the extract produced may require further steps to remove extraneous particulate matter before additional fractionations can be attempted. Colloidal particles made up of cell debris and fragments of cellular organelles are maintained as a suspension that will not readily sediment by increasing the run length and RCF applied. In these cases, it is often appropriate to bring about coagulation in order to clarify the extract. Coagulation may be induced in a number of ways, all of which alter the chemical environment of the suspended particles. The extract can be cooled or the pH may be adjusted to between pH 3.0 and 6.0. Indeed, rapidly altering the pH can be quite effective. Surfactants that alter the hydration of the particles