Preface

The first edition of *Protein Purification Protocols* (1996), edited by Professor Shawn Doonan, rapidly became very successful. Professor Doonan achieved his aims of producing a list of protocols that were invaluable to newcomers in protein purification and of significant benefit to established practitioners. Each chapter was written by an experienced expert in the field. In the intervening time, a number of advances have warranted a second edition. However, in attempting to encompass the recent developments in several areas, the intention has been to expand on the original format, retaining the concepts that made the initial edition so successful. This is reflected in the structure of this second edition. I am indebted to Professor Doonan for his involvement in this new edition and the continuity that this brings.

Each chapter that appeared in the original volume has been reviewed and updated to reflect advances and bring the topic into the 21st century. In many cases, this reflects new applications or new matrices available from vendors. Many of these have increased the performance and/or scope of the given method. Several new chapters have been introduced, including chapters on all the currently used protein fractionation and chromatographic techniques. They introduce the theory and background for each method, providing lists of the equipment and reagents required for their successful execution, as well as a detailed description of how each is performed. The Notes section constitutes a reference guide on the issues and pitfalls that may be encountered and provides the means for circumventing or overcoming them effectively.

Around the time of the first edition, the concept of proteomics was being forged and has subsequently led to a rapid growth in a new and exciting area of protein isolation and analysis. Techniques such as two-dimensional gel electrophoresis have now entered the mainstream, not only in analysis, but also as a preparative technique for protein characterization. Even newer techniques combine with analytical chromatography as multidimensional separations of proteins and peptides. In combination with mass spectrometric techniques, these are now the most powerful methods for isolating proteins. *Protein Purification Protocols* reflects these developments, with chapters encompassing all the current thinking. In addition, since the advance of technology means that simple spectrometric detection is no longer the only option for separating proteins, the various methods for detecting proteins are covered.

Each chapter is designed to allow a particular step of a purification to be performed in isolation; however, it is understood that a number of steps may need to be run in sequence from initial sample fractionation (e.g., tissue homogenization) to chromatography and final polishing steps (e.g., buffer exchange). Our book's format allows for this, and the initial chapter addresses strategies that should place the various methodologies in context. At the end of the book it was also felt timely to include brief descriptions of how to scale-up purification methods and evaluate the purification of proteins for therapeutic use. These do not rigidly follow the regular pattern for the main body of protocols, but should give an insight into the strategies needed for different final applications.

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Preparation of Extracts From Animal Tissues

J. Mark Skehel

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 ***(1)***. 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-

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vantage in producing a purer extract, yields of organelles are often low, so consideration 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 extrinsic membrane proteins, which are associated with the lipid membrane resulting from interactions with other proteins or regions of the phospholipid bilayer. Extrinsic membrane proteins can be extracted and purified by releasing them from their membrane anchors 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 protein during an entire purification strategy, the hydrophobic region of the protein must interact 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 detergent–lipid–protein complex. These complexes are then further solubilized to form detergent–protein complexes and detergent–lipid complexes. The purification of membrane proteins is, therefore, not generally as straightforward as that for soluble proteins ***(2,3)***.

The principal aim of any extraction method must be that it be reproducible and disrupt 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 generally 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 handling 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 600–3000*g* for 30 min. For smal-scale work (up to 3 L of homogenate), a 6 × 250-mL angle rotor operated at 5000*g* 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

Table 1
Protease Inhibitors

Inhibitor	Target proteases	Effective concentrations	Stock solutions
EDTA	Metalloproteases	0.5–2.0 m*M*	500 m*M* in water, pH 8.0
Leupeptin	Serine and thiolproteases	0.5–2 μg/mL	10 mg/mL in water
Pepstatin	Acid proteases	1 μg/mL	1 mg/mL in methanol
Aprotinin	Serine proteases	0.1–2.0 μg/mL	10 mg/mL in phosphate-buffered saline
PMSF	Serine proteases	20–100 μg/mL	10 mg/mL in isopropanol

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 m*M* 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 m*M*) 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 m*M* 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 m*M*), 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, wheras particles lighter than the surrounding medium will move inward. The centrifugal force experienced by a particle will vary depending on its

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:

$$\text{RCF} = 1.118r\ (\text{rpm}/1000)^2 \quad (1)$$

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

may also be used. In some situations, the presence of excessive amounts of nucleic acid can cause turbidity and increased viscosity of the extract. In these situations, it may be appropriate to precipitate with a polycationic macromolecule such as protamine sulfate in order to cause aggregation of the nucleic acid (addition to a final concentration of 0.1% w/v). The agglutinated particles will now sediment more easily when the mixture is recentrifuged.

Conditions for the clarification of an extract by coagulation should be arrived at through a series of small-scale tests, such that coagulation is optimized, whereas any detrimental effects such as denaturation are minimized. The coagulant should be added to the extract that is being stirred at high speed, thus maximizing particle interactions. Reducing the speed at which the mixture is stirred will then aid coagulation.

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