

# Protocol list

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# Chapter 2

## Getting started

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This chapter provides a more detailed overview of the equipment requirements for protein purification and the practical aspects of developing a purification strategy, including the ordering of unit operations, buffer preparation, and approaches to minimizing yield losses.

### 1 Overview of lab equipment

A well-equipped laboratory is an essential prerequisite to successful protein purification and time spent in deciding needs and purchasing essential items will help to avoid panics mid-way through a protocol when a key piece of equipment is lacking. This said, one should avoid expensive purchases (unless budget is no object) as the majority of purifications can be achieved with fairly routine equipment. The few essential pieces of expensive equipment are a spectrophotometer, a centrifuge, and a chromatography set-up. In general, money is best spent in purchasing plenty of the cheaper items such as tubes, beakers, measuring cylinders, salts, and buffers.

Chromatography equipment is an essential item for any purification laboratory. There are plenty of expensive chromatography set-ups available which can provide a remarkable saving in time for process development purposes. If you are not familiar with such equipment and are new to the world of protein purification, I suggest you make do with a simple chromatography set-up to start with, until you become familiar with the technique and learn more about your exact requirements and how alternative tailor-made process development kits differ.

A protein purification laboratory should be equipped with supplies of tap-water, de-mineralized water, and distilled water. Electricity and sinks are taken for granted. Required equipment can be roughly grouped into three categories:

- those for ancillary purposes
- those for detection
- those for separation

*Table 1* lists the essential items for the protein purification laboratory.

**Table 1** Recommended equipment and materials for the protein purification laboratory

Spectrophotometer with chart recorder
Gel electrophoresis, isoelectric focusing equipment
Refrigerated centrifuge
Bench-top centrifuge
Homogenizers
Chromatography set-up (pumps, gradient mixer, columns, UV detector, chart recorder)
Balances, pH meters, magnetic stirrers
Ice machine
Graduated cylinders
Pipettes (adjustable with disposable tips 5 $\mu$ l to 5 ml)
Beakers
Chromatographic media
Ammonium sulfate
Buffers
Dialysis tubing
Salt
Stabilizing reagents

### 1.1 Ancillary equipment

Time and money spent wisely on buying adequate supplies of support materials will pay dividends. There is nothing more irritating than having to rush around in search of a clock or some salt half-way through a delicate purification with your enzyme degrading in front of your eyes! Key requirements are tubes, beakers, pipettes, stirrers, and timers. In addition essential chemicals include salts and buffers. Adjustable pipettes (e.g. Gilson) are essential and those suitable for sample volumes from 10  $\mu$ l to 5000  $\mu$ l are recommended. A freezer and fridge are also essential, while an ice machine is desirable. Freezers should chill to  $-20^{\circ}\text{C}$ , although a deep freeze providing around  $-70^{\circ}\text{C}$  may also be needed. Magnetic stirrers with a built-in hot-plate are preferred.

### 1.2 Detection and analysis equipment

While analytical techniques are discussed in detail in Chapter 3, the broad requirements will be covered briefly here. The spectrophotometer is perhaps the single most important piece of analytical equipment in the purification laboratory. This should allow measurements of absorption in the ultraviolet (UV) and visible wavelength range, preferably between around 190 and 800 nm. Common uses are in total protein determination (e.g. absorption at 280 nm) and in specific assays such as enzyme kinetics measurements. Many spectrophotometers can be linked up to a chart recorder for recording enzyme kinetics assays. An ample supply of disposable cuvettes for visible wavelength measurements, and a small supply of quartz cuvettes for UV wavelength measurements are needed.

The second essential item is a gel electrophoresis set-up. Electrophoresis is the principal method for generating information on composition of a protein sample, including the approximate molecular weight and isoelectric point of the target protein and main contaminants. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focusing (IEF) are the two commonly used techniques for determining approximate molecular weight and isoelectric point respectively. Complete gel electrophoresis systems are typically purchased from laboratory suppliers and normally use vertical gel slabs between two glass plates, although ready-made horizontal gels are also available.

### 1.3 Separations equipment

The exact equipment requirements for separation will depend on the particular application. However the most likely requirements are a centrifuge, equipment for cell lysis, and a chromatography set-up.

Centrifuges are commonly used for separating cells and cell debris from supernatant and for settling precipitates. Common models are refrigerated and spin at up to 20 000 r.p.m. A selection of rotors is essential to accommodate a variety of sample sizes and centrifuge tubes for sample volumes of between 5–500 ml are recommended. Should fractionation of subcellular components such as organelles be anticipated then an ultracentrifuge capable of spinning at up to 80 000 r.p.m. will be required. Bench-top centrifuges are very useful and have a fixed or swing-arm rotor which typically takes 10 ml centrifuge tubes. Microcentrifuges are also useful for sample volumes of 1 ml and less.

The type of cell lysis equipment required will depend on the source material being used for protein purification. Blenders are commonly used for animal and plant tissue while bead mills are more suitable for microbial cell lysis. Ultrasonication equipment is also useful at a laboratory scale.

Chromatography is so extensively used in modern protein purification that money spent in purchasing reliable equipment will pay dividends. The essential requirements are a variety of column dimensions, a pump, fraction collector, UV monitor, chart recorder, tubing, valves, and a selection of gels and resins. Chromatography columns come in varying degrees of complexity and ideally several columns of varying bed volume from 1 ml up to around 200 ml should suit most applications. It is important to be able to vary the column volume using an adjustable plunger.

A peristaltic pump will suffice for most applications, although dual piston pumps are available with many automated chromatography set-ups, providing a more pulse-free delivery of mobile phase. A reliable fraction collector is essential and extra money spent on this piece of equipment is definitely worthwhile since it is the one piece of equipment which seems to go wrong during overnight purifications—a pool of sample on the bench top being the evidence which is found the following morning. Should extensive protein purification be anticipated, it is worthwhile considering a tailor-made automated chromatography set-up (e.g. Bio-Rad, Pharmacia). When using elution gradients, two peristaltic

pumps and a simple stirrer for gradient formation will be required and automated chromatography apparatus is normally fitted with a mixer for gradient formation. Ion exchange is the single most common chromatography medium used in protein purification, although additional media such as size exclusion and hydrophobic interaction are also likely to be needed.

## 2 Control of pH—buffers—principles, selection, and use

Proteins are pH-sensitive molecules and their stability, and possibly activity, may be dependent on a narrow pH window. Many enzyme-catalysed reactions use up or release protons, so causing a shift in pH. In addition biochemical reactions are generally sensitive to quite small changes in pH. Similarly, the behaviour of proteins during purification is often highly pH-dependent and requires that the pH of a protein sample is known at all times, and, if necessary, adjusted. Buffers are essential to control pH and the protein purification scientist should be familiar with the common types of buffers used in purification, their characteristics, and the range of pH in which they can be used. The important criteria for choosing a buffer are shown in *Table 2*.

**Table 2** Key criteria in buffer selection

pK <sub>a</sub>
Variation in the pK <sub>a</sub> with temperature
Ionic strength required
Form of the buffer is anionic or cationic
Multiple charges
Solubility
Interaction with other components
Cost
UV absorption

Buffers maintain the pH by absorbing and releasing protons and are essential in protein purification to ensure that proteins are not denatured due to a shift in pH, and that purifications can be carried out reproducibly under optimum conditions of pH and ionic strength. Buffers consist of an acid and its conjugate base (e.g. acetic acid and acetate) and are formed by mixing the appropriate amount of each form of the buffer to achieve the required pH. It is recommended to keep a concentrated stock of buffer (e.g. a 100-fold concentrate) which is diluted when required and then the pH fine-adjusted prior to use. The concentrate should contain a bactericidal agent such as sodium azide to prevent microbial growth and which will be diluted out when the buffer is prepared for use.

A list of commonly used buffers and their pH windows is shown in *Table 3*. Buffers stabilize pH most effectively at their pK value, but can generally be used within 1 pH unit on either side. In selecting the most suitable buffer it is im-

**Table 3** Useful buffers and  $pK_a$  values at 25°C

<b>Buffer name</b>	<b><math>pK_a</math> value</b>
Lactic acid	3.86
Acetic acid	4.76
Pyridine	5.23
Succinic acid	5.64
Histidine	6.0
<i>N</i> -morpholinoethane sulfonic acid (Mes)	6.15
Bis-(2-hydroxyethyl)imino-Tris-(hydroxyethyl) methane (Bis-tris)	6.5
Imidazole	6.95
Phosphate	7.2
<i>N</i> -morpholinopropane sulfonic acid (Mops)	7.2
<i>N</i> -2-hydroxyethylpiperazine- <i>N</i> -2-ethane sulfonic acid (Hepes)	7.48
Triethanolamine	7.75
Tris(hydroxymethyl) aminomethane (Tris)	8.06
<i>N</i> -Tris(hydroxymethyl)methyl glycine (tricine)	8.15
Diethanolamine	8.9
Ammonia	9.25
Boric acid	9.23
Ethanolamine	9.5
Glycine	9.8
Carbonate	10.3
Piperidine	11.12

portant that it does not interfere with enzyme function—phosphate buffers, for example, inhibit many enzymes, such as urease, kinases, and dehydrogenases. In addition, Tris does not buffer well below pH 7.5 and its buffering capacity is very temperature-dependent.

Buffer pH is temperature-sensitive and the pH should always be checked at the temperature at which the buffer will be used. In addition, after addition of a buffer to a protein solution, the pH should always be re-checked since proteins are also buffers and some alteration in pH may occur. Buffers are typically used at a 10–50 mM concentration, with Tris, phosphate, and acetate being most commonly used.

A buffer is only as good as the pH meter which is used for its preparation. Consequently, all pH meters should be regularly calibrated using commercially available buffer standards, commonly of pH 5, 7, and 10. It is good practice to ensure that all staff working in a purification laboratory follow the correct, and the same, procedure for pH meter calibration and note down in an equipment log when a meter has been calibrated. It is useful to nominate a member of staff to carry out such calibrations on all pH meters at a regular basis.

Since Tris and phosphate are two of the most commonly used buffers in protein purification, recipes for the preparation of these buffers at varying pH are given in *Protocol 1* (1).

## Protocol 1

### Preparation of phosphate and Tris buffers—standard recipes

#### Reagents

- Sodium dihydrogen phosphate
- Disodium hydrogen phosphate ( $7\text{H}_2\text{O}$ )
- Tris(hydroxymethyl)aminomethane
- HCl

#### A Phosphate buffer

- 1 Preparation of 0.2 M stock solutions:
  - (a) Stock A: add 27.8 g of sodium dihydrogen phosphate in 1 litre of water.
  - (b) Stock B: add 53.65 g of disodium hydrogen phosphate ( $7\text{H}_2\text{O}$ ) in 1 litre of water.
- 2 Mix  $x$  ml of stock A with  $y$  ml of stock B, then dilute to a total of 200 ml for the following pH of buffer:

$x$	$y$	pH	$x$	$y$	pH
87.7	12.3	6.0	39.0	61.0	7.0
85.0	15.0	6.1	33.0	67.0	7.1
81.5	18.5	6.2	28.0	72.0	7.2
77.5	22.5	6.3	23.0	77.0	7.3
73.5	26.5	6.4	19.0	81.0	7.4
68.5	31.5	6.5	16.0	84.0	7.5
62.5	37.5	6.6	13.0	87.0	7.6
56.5	43.5	6.7	10.5	90.5	7.7
56.5	43.5	6.7	8.5	91.5	7.8
51.0	49.0	6.8	7.0	93.0	7.9
45.0	55.0	6.9	5.3	94.7	8.0

#### B Tris buffer

- 1 Preparation of 0.2 M stock solutions:
  - (a) Stock A: add 24.2 g of Tris(hydroxymethyl)aminomethane to 1 litre of water.
  - (b) Stock B: 0.2 M HCl.
- 2 Mix 50 ml of stock A with  $x$  ml of stock B and dilute to a total of 200 ml for the following pH of buffer:

$x$	pH
16.5	8.4
12.2	8.6
8.1	8.8
5.0	9.0

## 3 Purification strategy

### 3.1 Ordering of steps

The following section provides an overview of techniques used in protein purification. There is now a bewildering range of available techniques and separations products to choose from such that there are potentially many pitfalls awaiting those new to the science. The key requirements of the majority of purifications are:

- (a) Release of product from source material—making an extract.
- (b) Removal of solids—separation of cells and cell debris from soluble protein.
- (c) Removal of water—concentration to simplify subsequent handling.
- (d) Removal of contaminants—to a degree necessitated by purpose of purification.
- (e) Stabilization of the product—to a degree dependent on lability and time before designated use.

As a general rule, initial steps in protein purification aim to generate a crude extract of the target protein along with other contaminants in a supernatant which is free of particulate matter. It is clearly important to start with a quantity of material (whether a volume of fermentation broth or a sample of animal tissue) which will yield the desired quantity of target protein following purification. Normally this is then followed by a number of adsorption and chromatography steps to generate the target protein at the required degree of purity. Chromatographic media vary in their capacity to bind protein and their cost. In general, high capacity, low cost media such as ion exchangers and group affinity media are used for initial purification of a protein from gross contaminants. Low capacity, high cost media such as affinity and HPLC matrices are used for a final step purification where a small volume column can be used to selectively remove any contaminants which remain. If the product is valuable (as with many therapeutic proteins) then yield following each purification step is most important. Speed of processing is often also important (even at the expense of resolution from contaminants) since it will limit the extent of protein degradation.

### 3.2 Nature of starting material

You may have little choice in the nature of the starting material for a purification. A fermentation which has previously been optimized to produce a high titre of a particular enzyme is a classic example. However it is advisable, where appropriate, to review the options for the starting material, and if following a recipe from a scientific paper, not to assume that the material used in the reference is necessarily the best to use for the job in hand.

The starting material for a protein purification may come from a wide variety of sources—animal tissue, plant material, milk, blood, and fermentation culture being typical examples. It is always important to maximize the total amount of

product in the starting material and, if possible, minimize the amount of contaminants present. In this way the purification of the target protein will be simplified. For non-fermentation-based products this is often difficult since to a large extent one has to accept the starting material one is given. It is important to calculate that the starting material has a sufficient amount of the target product for the purification to be completed successfully.

Proteins are the products of ribosomal activity and are therefore associated with cellular metabolism. Consequently, regardless of the source, proteins are located either within or form part of the structure of, a cell or are secreted from a cell. Cellular-located proteins include insoluble inclusion bodies, soluble enzymes, or structural proteins which form part of a cellular structure such as a membrane. Proteins which are secreted from cells or localized in the periplasmic space may also provide simplified purification since the level of other protein contaminants is reduced compared to intracellular proteins. In addition, extracellular proteins (such as lysozyme and amylase) are generally more robust since they have to operate in a harsh environment. Intracellular proteins are generally less stable and present a greater challenge to the purification scientist due to their increased lability and presence of other proteins as contaminants. Where proteins are associated with cellular material it is advisable to generate a clarified (i.e. non-particulate) extract of the starting material as soon as possible through cell disruption, solubilization of the target protein in an appropriate buffer and removal of cells and cell debris through filtration or centrifugation. Where the protein is extracellular to cellular material, an initial separation to remove cells and leave a clarified supernatant containing the target protein is required. Thereafter the clarified preparation may be used immediately for purification or frozen in aliquots for future use.

### 3.3 Storage of material

Freshness of starting material is important since natural degradation processes and contamination from micro-organisms take place rapidly and result in a reduced level of the target protein and a non-representative starting material. Therefore unless purifications can be started within hours of delivery of the starting material, degradation should be slowed through reducing the temperature, to 5°C for a period of a few hours, or through freezing to -70°C for periods of days. Freezing can use the initial material or the product of an initial extraction. During the freezing process certain changes take place in the starting material which, if not checked, may reduce subsequent purification efficiency. Any starting material will probably have a high water content. As this water freezes ice crystals will grow which will disrupt cellular organelles and membranes. Secondly, salts will crystallize out after the water, with the less soluble salt in a pair coming out of solution first, causing a shift in the pH. Finally, proteases which are released from disrupted cells can cause a slow but irreversible degradation of a target protein.

To counteract these degradative processes and ensure that the target protein

is preserved until a purification can be started it is advisable to freeze as soon as possible, preferably to a temperature of  $-70^{\circ}\text{C}$  so as to minimize any deterioration. In addition, protease inhibitors may be added to an extract and the pH stabilized through addition of a buffer. Where appropriate the starting material, or an initial extract, may be split into identical aliquots and frozen separately for subsequent repeat purifications. On recovery of the material from freezing rapid thawing should be used, with immersion of the material in warm water where possible.

### 3.4 Fermentation products

In fermentation there is considerable scope for increasing product titre and reducing contaminants through strain selection, genetic manipulation, and fermentation optimization. Contaminants may be derived from the ingredients used in the fermentation, added as it proceeds, or produced as a result of the fermentation along with the target protein. In addition certain contaminants such as albumin may protect the protein product and minimize degradation. Recombinant organisms such as *E. coli* strains frequently produce proteins as insoluble inclusion bodies which will require solubilization and refolding during the purification. Fermentations which use defined nutrients (as opposed to crude ingredients such as corn steep liquor and malt extract) may produce a feedstock which contains fewer contaminants, while minimizing antifoam addition may also reduce fouling problems in purification caused by antifoam blinding of membranes or adsorbents. Attention should be given to checking the optimum time for fermentation harvesting (often in log phase) and such evaluations should take into consideration the level of contaminants which may be problematical during purification as well as the concentration of the target protein. Clearly, this approach may require an interactive process in which initial purification work will identify the most problematical contaminants present in the fermentation.

### 3.5 Other sources of proteins

Other sources of proteins will include animal tissue such as muscle, heart, brain, or liver, plant tissue, blood, and milk. Animal tissue should be as fresh as possible and where possible it is recommended to use the local abattoir. Common starting materials include rat liver, rabbit muscle, beef and pig heart, liver, and kidney. Skeletal muscle should be allowed to go into rigor mortis so that ATP is reduced and actin and myosin do not partially solubilize during formation of the extract. Providing it is fresh, plant material can be obtained from the local shop. Yeast is a readily available source of proteins in fresh or dried form. Fresh baker's yeast contains active enzymes and should be frozen or chilled until use. Drying of yeast may inactivate some labile enzymes.

### 3.6 Making an extract

Many proteins such as enzymes are intracellular and necessitate preparation of an extract prior to further processing. Extraction technique should preferentially

release the required protein, leave as many contaminants behind as possible, and minimize degradation of the protein product while further protein is being released. Extraction medium conditions must therefore be selected in which the target protein is stable and efficiently extracted. It should be noted that the pH for stability is not always the same as the pH which ensures maximum activity. Initial preparation steps should be carried out as fast as possible and, if the protein is particularly labile, at a low temperature. Many intracellular enzymes become unstable when removed from the stabilizing internal environment of the cell. Freezing (ideally to  $-70^{\circ}\text{C}$ ) of cellular material in batch sizes appropriate to the purification scale provides a very convenient source of a consistent starting material.

A variety of disruption techniques exist, the most popular being freeze-thawing, bead milling, and homogenization. The various options for disruption permit selection of a technique appropriate to the nature of the cellular material. The disruption technique selected should never be more vigorous than required to lyse the cells since over-disruption may cause inactivation of enzymes once released. When isolating organelles such as mitochondria and chloroplasts the use of gentle disruption technique such as cell wall lysis with proteolytic enzymes may be evaluated to allow effective release of organelles without damage. However such lysis techniques may result in reduced yields such that a preferable option is to use a harsh disruption technique which while causing release of all cell contents, provides higher yields of target protein as a result. Sufficient extraction buffer (at least two volumes is recommended) must be used to ensure release of the remaining protein still trapped with the lysed cell material so as to ensure efficient recovery. At this stage it is wise to evaluate the optimum extraction time since prolonged exposure to the extraction method can lead to reduced product yields through denaturation. In addition it may be necessary to cool the cell slurry when using certain techniques such as homogenization or bead milling since heat is generated during the extraction process. Cells contain salts and other charged materials such as proteins and nucleic acids, giving an intracellular ionic strength typically between 0.15–0.2 M. When the intracellular contents are released into a lower ionic strength extraction buffer, charged particulates can act as ion exchangers and adsorb the target protein, so reducing yields. A buffer of ionic strength and pH similar to the intracellular conditions should therefore be used, with the addition of stabilizing agents to minimize denaturation. As an example I recommend 20–50 mM phosphate pH 7–7.5, with optional addition of EDTA, 2-mercaptoethanol, cysteine, and metal ions such as  $\text{Zn}^{2+}$ .

Plant material only contains a small volume of cytoplasm retaining intracellular enzymes since the majority of the intracellular volume is occupied by the vacuole. Consequently much liquid is released on lysing plant cells and although additional extracting liquid is not usually needed it is essential to control the pH and minimize protein inactivation since acidification and oxidation are likely after lysis. In addition phenolics are released which oxidize to form dark pigments which may interfere with the extraction process by attaching to

proteins and inactivating enzymes. Thiol compounds such as 2-mercaptoethanol minimize the action of phenol oxidases and powdered polyvinylpyrrolidone may be used to adsorb the coloured phenolics during the extraction process. A typical protocol is to use 0.5–1.0 volumes of cold extracting buffer containing 20–30 mM 2-mercaptoethanol prior to disruption using a Waring blender for 30 sec followed by centrifugation. Polyvinylpyrrolidone may be added if needed.

Animal tissue is typically diced and trimmed to remove unwanted fat before shredding in a Waring blender for 30 sec with 2–3 volumes of extraction buffer per gram of tissue. The pH should then be checked before centrifugation at 5000–10 000 g for up to 60 min. The lysate should then be coarse filtered to remove fats in suspension.

Bacteria may be disrupted using ultrasonication, bead milling, or homogenization using a Manton-Gaulin homogenizer. Gram positive strains will easily disrupt in lysozyme, typically using 0.2 mg/ml egg white lysozyme at 37°C for 15 min. Deoxyribonuclease I may be added at a concentration of 10 µg/ml to reduce the viscosity caused by DNA release. Gram negative strains may be lysed using a combination of detergent, osmotic shock, and lysozyme. After extraction the pH should always be checked and the suspension centrifuged to remove particulates. Cell debris will settle with spinning at 10 000 g for 15 min. Any remaining cloudiness will usually be removed during subsequent purification steps and fat globules may be removed using a coarse filter or glass wool. Inclusion bodies are commonly produced in bacteria with intracellularly expressed recombinant proteins and accumulate as insoluble aggregates which need to be solubilized and then refolded to recover the native state. At a laboratory scale, low speed fractional centrifugation allows for preferential separation of particles prior to treatment.

Yeast are typically disrupted using a Manton-Gaulin homogenizer or bead mill with use of two volumes of buffer per gram wet weight of yeast.

### 3.7 Separation of particulates

Purification techniques such as chromatography require that a protein sample is free of particulate material so as to prevent bed clogging, poor purification, build-up of back-pressure, and equipment break-down. Such particulate material is usually derived from cells, cells debris, and aggregated cell components such as proteins.

Consequently, particulate removal techniques such as centrifugation and filtration are commonly used following generation of an extract and before subsequent purification using chromatography. Centrifugation is the sedimentation of particles in an increased gravitational field. At a laboratory scale, batch centrifugation, in which samples are clarified in bottles or tubes in a rotor, is typically used. At a larger scale, continuous centrifugation is adopted to process the larger volumes. Differential centrifugation is also used to fractionate organelles. Filtration with filter papers can also be used, with suction applied using a Buchner funnel. This approach is less common since the filter papers tend to

clog with fine particulate material and so reduce the flow of clarified supernatant. The flow-rate through the filter may be improved by adding filter aids such as celite with the material to be filtered prior to pouring into the Buchner funnel. Membrane processing using microfiltration and ultrafiltration are increasingly used, particularly to produce a more concentrated suspension of cells and cross-flow microfiltration is particularly useful for large scale purification.

### **3.8 Protein concentration**

Following separation of particulates from the supernatant, a prime requirement is the removal of water to concentrate the target protein. This reduction in volume simplifies subsequent handling and reduces the time required for the purification steps to follow. Commonly used techniques include precipitation, ultrafiltration, adsorption, dialysis, and addition of dry gel permeation media.

Precipitation is commonly used early on in a purification strategy to concentrate and partially purify proteins. Following addition of ammonium sulfate the precipitate is separated from the supernatant (typically using batch centrifugation) and the precipitate redissolved in a smaller volume of an appropriate buffer. This technique is usually applied if the protein concentration is above 1 mg/ml, since lower concentrations may lead to denaturation or poor aggregation to form a precipitate.

Ultrafiltration using membranes with a 1000–300 000 Da cut-off separates low molecular weight molecules and water from proteins. In ultrafiltration, water and low molecular weight solutes are forced through a membrane to leave the target protein in a concentrated form in the retentate. Concentration through addition of dry gel filtration media is appropriate at a laboratory scale. Simple adsorption–desorption steps using a packed bed or batch adsorption achieve a similar result. Adsorption to concentrate usually uses ion exchange, with elution in a small volume using a stepwise increase in salt concentration.

Addition of a dry media of appropriate pore size will lead to the rapid uptake of water to swell the gel, with the exclusion of protein. The swollen media is then washed to remove protein from outside the particles prior to filtration under suction. Since the washing stage causes dilution, adequate concentration may only be achieved if the washing stage is avoided and this may lead to unacceptable yield losses.

Dialysis may be used to concentrate the protein sample and provide buffer exchange. For concentration purposes, water-attracting polymers such as carboxymethyl cellulose and polyethylene glycol are commonly used. The protein sample is placed inside a bag formed from a strip of dialysis tubing which is tied and immersed in a solution containing the polymer which will then attract water from the bag and concentrate the protein sample.

### **3.9 Adjusting sample composition between steps**

Optimum integration of the processing steps used in protein purification will lead to a minimum need to adjust sample composition between each technique.

However it is rare that an entire purification will be carried out without a need for sample adjustment, typically involving alteration of pH and/or ionic strength. Such adjustment is necessary so that the protein sample has the required properties for the next purification step to work satisfactorily. Sample composition should always be checked prior to the next step and diafiltration, dialysis, and gel filtration can all be used to change the sample buffer. Wrong pH can be adjusted simply by adding acid or alkali while an excessively high ionic strength can be remedied through dilution. An appearance of turbidity is a common problem during purification, particularly as protein concentration increases, and centrifugation or filtration may be useful to reduce such haziness before continuing with a purification. However, the cause of such turbidity should be examined in some detail since although it may be easily removed at a small scale it may be more noticeable, and cause more serious problems as a process is scaled-up.

Dialysis is typically used for the removal of unwanted low molecular weight solutes from a sample and for their replacement with the buffer in the dialysate. A high concentration of salt in the dialysis bag causes water to enter while generally molecules larger than 15 000–20 000 Da do not pass out of the bag from the interior. Eventually, the buffer composition on each side of the dialysis membrane will equalize. It should be pointed out that dialysis does not remove unwanted solutes but just dilutes them with the dialysis buffer since the concentration reaches an equilibrium on either side of the dialysis membrane. Stirring the dialysis buffer and bag will help the process to speed-up reaching equilibrium which typically reaches 90% completion in 2–3 h. Dialysis is a convenient step to carry out overnight if no loss of protein activity is likely and the tubing is normally used from a roll, wetted, and a knot tied in one end. The protein sample is poured through a filter funnel into the open end of the tubing which is then knotted to seal the tube and placed in a beaker of the dialysis buffer. It is wise to leave an air space in the tube to allow for entry of dialysis buffer and expansion of the internal liquid volume.

Although dialysis is very simple and can be used overnight, since it can be a slow process, gel filtration is often used in preference to perform a buffer exchange. In gel filtration all the previous buffer is removed in a single quick step using a sample volume of less than 1/5 of the bed volume. The bed must be equilibrated in the desired buffer and the protein concentration kept to less than 30 mg/ml.

## **4 Protein lability and structure—implications for purification**

### **4.1 Introduction**

Proteins, and in particular, enzymes, are often sensitive molecules and the working environment of a protein is often indicative of the robustness of the molecule itself. As an example, intracellular enzymes are protected in a natural

environment which is highly stabilizing—high in protein concentration, low in oxygen tension, and with reducing compounds present. As a consequence enzymes which are located within cells are often vulnerable to denaturation or loss of activity when exposed to the harsh extracellular environment following cell lysis. In contrast, extracellular enzymes are more robust since they are designed to work in an environment which may be highly oxygenated, exposed to proteolytic enzymes, and in which the protein concentration may be low.

During purification the target protein is taken from its natural working environment and exposed to conditions which, if not carefully controlled, may cause a loss in yield and a resultant poor purification efficiency.

There are three primary causes of loss of enzyme activity—denaturation, inactivation, and proteolysis.

## 4.2 Denaturation

This is caused by extremes of pH, temperature, and denaturants such as organic solvents. The interior of a cell commonly has a pH of between 6–8 and so it is normal practice to use buffers of similar pH during purification. When working outside this pH range, for example, during chromatographic separations, the stability of the protein should be closely monitored.

## 4.3 Catalytic site inactivation

This is a common cause of loss of enzyme activity. If inactivation is due to loss of cofactors, then simple addition will often restore enzyme activity. However inactivation may also be the result of covalent modification of the active site. Here cysteine residues are particularly prone to oxidation, leading to disulfide bond formation, partial oxidation to sulfinic acid, or irreversible oxidation to sulfonic acid. Disulfide bond formation is accelerated by the presence of divalent ions such as calcium. Consequently a common means of prevention of covalent modification is through the removal of heavy metal ions by addition of chelating agents such as EDTA at 10–25 mM. In addition, a sulfhydryl-containing reagent such as 2-mercaptoethanol (5–20 mM) or dithiothreitol may be added. Alternative additives include glutathione thioglycolate and EGTA, which is more specific for calcium. When adding EDTA to a buffer, final pH adjustment should be carried out after addition of the chelating agent. It should also be noted that some proteins are actually stabilized by the presence of ions such as calcium and magnesium such that addition of metal ions may be necessary. Very dilute enzymes (e.g. 1  $\mu\text{g/ml}$ ) lose activity quickly and so it is recommended to keep the protein concentration high or add a stabilizing protein such as BSA (up to 10 mg/ml).

## 4.4 Proteolysis

This is commonly caused by the exposure of proteins to the digestive enzymes within cells. This often occurs as a result of cell lysis in which intracellular proteins which are normally separated through compartmentalization are rapidly

mixed. Proteases are enzymes which cause the digestion of other proteins through hydrolysis and play an important role in cellular digestive processes. They are found in lysosomes in mammalian cells, in vacuoles in plants, and between the plasma membrane and cell wall in micro-organisms. Protein purifications which involve a cell lysis step in order to isolate intracellular enzymes will therefore potentially expose the target protein to such degradative enzymes, and steps may be necessary to minimize the activity of proteases so as to minimize yield losses. A common approach is to add chemicals to the initial extract which will slow or prevent the hydrolytic action of proteases. PMSF is the most commonly used additive, inhibiting serine, thiol, and some carboxypeptidase protease activity using a final concentration of 0.5–1.0 mM. In addition, pepstatin may be used to inhibit acid proteases. As a general rule, if protease activity is causing yield losses of the target protein, it is preferable to work as quickly as possible and, if possible, to carry out the purification at reduced temperatures (e.g. 5 °C).

A common approach to minimizing loss of activity is to use a standard cocktails of reagents which can be stored until used and added to an extract to ensure protein inactivation is slowed. A good combination is 2–5 mM EDTA, 0.5–1 mM PMSF, and  $10^{-7}$  M pepstatin A.

#### 4.5 Other causes of yield loss

In addition to the need to control pH and temperature, certain other precautions may be needed to prevent loss of target protein. These include minimizing bacterial growth, reducing water activity, and prevention of aggregation. Protein extracts which are produced during a purification, and buffers which are used during the process, are potentially suitable substrates for microbial growth, causing a hydrolysis of proteins through action of secreted enzymes and consequential loss of protein yields, in addition to potential microbial fouling of purification equipment. Consequently all extracts should be stored at 5 °C overnight, or frozen if left for longer periods. Similarly, all buffers should be freshly prepared and sterile filtered if used for chromatography.

Maintenance of a low water activity in protein preparations may also help to minimize yield losses. Glycerol, which forms strong hydrogen bonds to slow down the motion of water in buffers, may be used at up to 50% (w/v), although the increase in viscosity which results usually means that a 20–30% (w/v) solution is preferred. Sucrose and glucose are also used for a similar purpose. Any reagent added to protein extracts or buffers must be compatible with the purification techniques being used. Ammonium sulfate, for example, may be used to reduce water activity and is compatible with hydrophobic interaction chromatography, but is not suitable for addition to extracts prior to ion exchange since it will increase the salt concentration and prevent binding.

Certain proteins may be bound to insoluble cellular components such as membranes, or aggregated into insoluble particles, such as inclusion bodies. In these cases it is necessary to reduce hydrophobic interaction so as to solubilize

the target protein and addition of detergents or chaotropic agents is common. Mild non-denaturing detergents such as Triton X-100 are preferred to strong detergents such as SDS which will interfere with protein structure and cause denaturation. Detergent levels added to protein preparations must also be kept below the critical micelle concentration. Chaotropic agents such as urea and guanidine hydrochloride are also useful, particularly for solubilization of inclusion bodies. Reducing agents are useful for use with bacterial enzymes derived from a reducing environment while mammalian cell enzymes are often best stabilized with surfactants and protease inhibitors.

## References

1. Stoll, V. E. and Blanchard, J. S. (1990). In *Methods in enzymology* (ed. M. P. Deutscher), Vol. 182, pp. 34-5. Academic Press.