Example 1 PROTEIN ASSAY

Question A series of dilutions of bovine serum albumin (BSA) was prepared and 0.1 cm^3 of each solution subjected to a Bradford assay. The increase in absorbance at 595 nm relative to an appropriate blank was determined in each case, and the results are shown in the table.

Concentration of BSA ($mg\,cm^{-3}$)	A_{595}
1.5	1.40
1.0	0.97
0.8	0.79
0.6	0.59
0.4	0.37
0.2	0.17

A sample (0.1 cm^3) of a protein extract from *E. coli* gave an A_{595} of 0.84 in the same assay. What was the concentration of protein in the *E. coli* extract?

Answer If a graph of BSA concentration against A_{595} is plotted it is seen to be linear. From the graph, at an A_{595} of 0.84 it can be seen that the protein concentration of the *E. coli* extracted is 0.85 $mg\,cm^{-3}$.

with concentrated sulphuric acid in the presence of sodium sulphate (to raise the boiling point) and a copper and/or selenium catalyst. The digestion converts all the organic nitrogen to ammonia, which is trapped as ammonium sulphate. Completion of the digestion stage is generally recognised by the formation of a clear solution. The ammonia is released by the addition of excess sodium hydroxide and removed by steam distillation in a Markham still. It is collected in boric acid and titrated with standard hydrochloric acid using methyl red–methylene blue as indicator. It is possible to carry out the analysis automatically in an autokjeldahl apparatus. Alternatively, a selective ammonium ion electrode may be used to directly determine the content of ammonium ion in the digest. Although Kjeldahl analysis is a precise and reproducible method for the determination of nitrogen, the determination of the protein content of the original sample is complicated by the variation of the nitrogen content of individual proteins and by the presence of nitrogen in contaminants such as DNA. In practice, the nitrogen content of proteins is generally assumed to be 16% by weight.

8.3.3 Cell disruption and production of initial crude extract

The initial step of any purification procedure must, of course, be to disrupt the starting tissue to release proteins from within the cell. The means of disrupting the tissue will depend on the cell type (see Cell disruption, below), but thought must first be given to the composition of the buffer used to extract the proteins.

Extraction buffer

Normally extraction buffers are at an ionic strength (0.1–0.2 M) and pH (7.0–8.0) that is considered to be compatible with that found inside the cell. Tris or phosphate buffers are most commonly used. However, in addition a range of other reagents may be included in the buffer for specific purposes. These include:

- *An anti-oxidant*: Within the cell the protein is in a highly reducing environment, but when released into the buffer it is exposed to a more oxidising environment. Since most proteins contain a number of free sulphydryl groups (from the amino acid cysteine) these can undergo oxidation to give inter- and intramolecular disulphide bridges. To prevent this, reducing agents such as dithiothreitol, β-mercaptoethanol, cysteine or reduced glutathione are often included in the buffer.
- *Enzyme inhibitors*: Once the cell is disrupted the organisational integrity of the cell is lost, and proteolytic enzymes that were carefully packaged and controlled within the intact cells are released, for example from lysosomes. Such enzymes will of course start to degrade proteins in the extract, including the protein of interest. To slow down unwanted proteolysis, all extraction and purification steps are carried out at 4 °C, and in addition a range of protease inhibitors is included in the buffer. Each inhibitor is specific for a particular type of protease, for example serine proteases, thiol proteases, aspartic proteases and metalloproteases. Common examples of inhibitors include: di-isopropylphosphofluoridate (DFP), phenylmethyl sulphonylfluoride (PMSF) and tosylphenylalanyl-chloromethylketone (TPCK) (all serine protease inhibitors); iodoacetate and cystatin (thiol protease inhibitors); pepstatin (aspartic protease inhibitor); EDTA and 1,10-phenanthroline (metalloprotease inhibitors); and amastatin and bestatin (exopeptidase inhibitors).
- *Enzyme substrate and cofactors*: Low levels of substrate are often included in extraction buffers when an enzyme is purified, since binding of substrate to the enzyme active site can stabilise the enzyme during purification processes. Where relevant, cofactors that otherwise might be lost during purification are also included to maintain enzyme activity so that activity can be detected when column fractions, etc. are screened.
- *EDTA*: This can be present to remove divalent metal ions that can react with thiol groups in proteins giving *mercaptids*.

$$R - SH + Me^{2+} \rightarrow R - S - Me^{+} + H^{+}$$

- *Polyvinylpyrrolidone (PVP)*: This is often added to extraction buffers for plant tissue. Plant tissues contain considerable amounts of phenolic compounds (both monomeric, such as *p*-hydroxybenzoic acid, and polymeric, such as tannins) that can bind to enzymes and other proteins by non-covalent forces, including hydrophobic, ionic and hydrogen bonds, causing protein precipitation. These phenolic compounds are also easily oxidised, predominantly by endogenous phenol oxidases, to form quinones, which are highly reactive and can combine with reactive groups in proteins causing cross-linking, and further aggregation and precipitation. Insoluble PVP (which mimics the polypeptide backbone) is therefore added to adsorb the phenolic compounds which

can then be removed by centrifugation. Thiol compounds (reducing agents) are also added to minimise the activity of phenol oxidases, and thus prevent the formation of quinones.

- *Sodium azide*: For buffers that are going to be stored for long periods of time, antibacterial and/or antifungal agents are sometimes added at low concentrations. Sodium azide is frequently used as a bacteriostatic agent.

Membrane proteins

Membrane-bound proteins (normally glycoproteins) require special conditions for extraction as they are not released by simple cell disruption procedures alone. Two classes of membrane proteins are identified. Extrinsic (or peripheral) membrane proteins are bound only to the surface of the cell, normally via electrostatic and hydrogen bonds. These proteins are predominantly hydrophilic in nature and are relatively easily extracted either by raising the ionic concentration of the extraction buffer (e.g. to 1 M NaCl) or by changes of pH (e.g. to pH 3–5 or pH 9–12). Once extracted, they can be purified by conventional chromatographic procedures. Intrinsic membrane proteins are those that are embedded in the membrane (integrated membrane proteins). These invariably have significant regions of hydrophobic amino acids (those regions of the protein that are embedded in the membrane, and associated with lipids) and have low solubility in aqueous buffer systems. Hence, once extracted into an aqueous polar environment, appropriate conditions must be used to retain their solubility. Intrinsic proteins are usually extracted with buffer containing detergents. The choice of detergent is mainly one of trial and error but can include ionic detergents such as sodium dodecyl sulphate (SDS), sodium deoxycholate, cetyl trimethylammonium bromide (CTAB) and CHAPS, and non-ionic detergents such as Triton X-100 and Nonidet P-40.

Once extracted, intrinsic membrane proteins can be purified using conventional chromatographic techniques such as gel filtration, ion-exchange chromatography or affinity chromatography (using lectins). However, in each case it is necessary to include detergent in all buffers to maintain protein solubility. The level of detergent used is normally 10- to 100-fold less than that used to extract the protein, in order to minimise any interference of the detergent with the chromatographic process.

Cell disruption

Unless one is isolating proteins from extracellular fluids such as blood, protein purification procedures necessarily start with the disruption of cells or tissue to release the protein content of the cells into an appropriate buffer. This initial extract is therefore the starting point for protein purification. Clearly one chooses, where possible, a starting material that has a high level of the protein of interest. Depending on the protein being isolated one might therefore start with a microbial culture, plant tissue, or mammalian tissue. The last of these has generally been the tissue of choice where possible, owing to the relatively large amounts of starting material available. However, the ability to clone and overexpress genes for proteins from any source, in both bacteria and yeast, means that nowadays more and more protein purification protocols are starting with a microbial lysate. The different methods available for

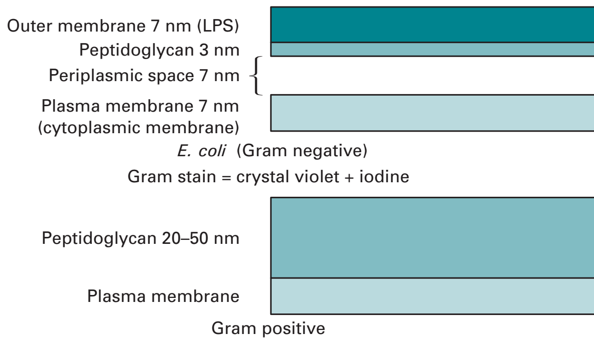

Fig. 8.2 The structure of the cell wall of Gram-positive and of Gram-negative bacteria. LPS, lipopolysaccharide.

disrupting cells are described below. Which method one uses depends on the nature of the cell wall/membrane being disrupted.

Mammalian cells

Mammalian cells are of the order of 10 μm in diameter and enclosed by a plasma membrane, weakly supported by a cytoskeleton. These cells therefore lack any great rigidity and are easy to disrupt by shear forces.

Plant cells

Plant cells are of the order of 100 μm in diameter and have a fairly rigid cell wall, comprising carbohydrate complexes and lignin or wax that surround the plasma membrane. Although the plasma membrane is protected by this outer layer, the large size of the cell still makes it susceptible to shear forces.

Bacteria

Bacteria have cell diameters of the order of 1 to 4 μm and generally have extremely rigid cell walls. Bacteria can be classified as either Gram positive or Gram negative depending on whether or not they are stained by the Gram stain (crystal violet and iodine). In Gram-positive bacteria (Fig. 8.2) the plasma membrane is surrounded by a thick shell of peptidoglycan (20–50 nm), which stains with the Gram stain. In Gram-negative bacteria (e.g. *Escherichia coli*) the plasma membrane is surrounded by a thin (2–3 nm) layer of peptidoglycan but this is compensated for by having a second outer membrane of lipopolysaccharide. The negatively charged lipopolysaccharide polymers interact laterally, being linked by divalent cations such as Mg^{2+}. A number of Gram-negative bacteria secrete proteins into the periplasmic space.

Fungi and yeast

Filamentous fungi and yeasts have a rigid cell wall that is composed mainly of polysaccharide (80–90%). In lower fungi and yeast the polysaccharides are mannan and glucan. In filamentous fungi it is chitin cross-linked with glucans. Yeasts also have a small percentage of glycoprotein in the cell wall, and there is a periplasmic space between the cell wall and cell membrane. If the cell wall is removed the cell content, surrounded by a membrane, is referred to as a spheroplast.

Cell disruption methods

Blenders

These are commercially available, although a typical domestic kitchen blender will suffice. This method is ideal for disrupting mammalian or plant tissue by shear force. Tissue is cut into small pieces and blended, in the presence of buffer, for about 1 min to disrupt the tissue, and then centrifuged to remove debris. This method is inappropriate for bacteria and yeast, but a blender can be used for these microorganisms if small glass beads are introduced to produce a bead mill. Cells are trapped between colliding beads and physically disrupted by shear forces.

Grinding with abrasives

Grinding in a pestle and mortar, in the presence of sand or alumina and a small amount of buffer, is a useful method for disrupting bacterial or plant cells; cell walls are physically ripped off by the abrasive. However, the method is appropriate for handling only relatively small samples. The Dynomill is a large-scale mechanical version of this approach. The Dynomill comprises a chamber containing glass beads and a number of rotating impeller discs. Cells are ruptured when caught between colliding beads. A 600 cm^3 laboratory scale model can process 5 kg of bacteria per hour.

Presses

The use of a press such as a French Press, or the Manton–Gaulin Press, which is a larger-scale version, is an excellent means for disrupting microbial cells. A cell suspension ($\sim$50 cm^3) is forced by a piston-type pump, under high pressure (10 000 PSI = $lbf\,in.^{-2} \approx$ 1450 kPa) through a small orifice. Breakage occurs due to shear forces as the cells are forced through the small orifice, and also by the rapid drop in pressure as the cells emerge from the orifice, which allows the previously compressed cells to expand rapidly and effectively burst. Multiple passes are usually needed to lyse all the cells, but under carefully controlled conditions it can be possible to selectively release proteins from the periplasmic space. The X-Press and Hughes Press are variations on this method; the cells are forced through the orifice as a frozen paste, often mixed with an abrasive. Both the ice crystal and abrasive aid in disrupting the cell walls.

Enzymatic methods

The enzyme lysozyme, isolated from hen egg whites, cleaves peptidoglycan. The peptidoglycan cell wall can therefore be removed from Gram-positive bacteria (see Fig. 8.2) by treatment with lysozyme, and if carried out in a suitable buffer, once the cell wall has been digested the cell membrane will rupture owing to the osmotic effect of the suspending buffer.

Gram-negative bacteria can similarly be disrupted by lysozyme but treatment with EDTA (to remove Ca^{2+}, thus destabilising the outer lipopolysaccharide layer) and the inclusion of a non-ionic detergent to solubilise the cell membrane are also needed. This effectively permeabilises the outer membrane, allowing access of the lysozyme to the peptidoglycan layer. If carried out in an isotonic medium so that the cell membrane is not ruptured, it is possible to selectively release proteins from the periplasmic space.

Yeast can be similarly disrupted using enzymes to degrade the cell wall and either osmotic shock or mild physical force to disrupt the cell membrane. Enzyme digestion alone allows the selective release of proteins from the periplasmic space. The two most commonly used enzyme preparations for yeast are zymolyase or lyticase, both of which have β-1, 3-glucanase activity as their major activity, together with a proteolytic activity specific for the yeast cell wall. Chitinase is commonly used to disrupt filamentous fungi. Enzymic methods tend to be used for laboratory-scale work, since for large-scale work their use is limited by cost.

Sonication

This method is ideal for a suspension of cultured cells or microbial cells. A sonicator probe is lowered into the suspension of cells and high frequency sound waves (<20 kHz) generated for 30–60 s. These sound waves cause disruption of cells by shear force and cavitation. Cavitation refers to areas where there is alternate compression and rarefaction, which rapidly interchange. The gas bubbles in the buffer are initially under pressure but, as they decompress, shock waves are released and disrupt the cells. This method is suitable for relatively small volumes (50–100 cm^3). Since considerable heat is generated by this method, samples must be kept on ice during treatment.

8.3.4 Fractionation methods

Monitoring protein purification

As will be seen below, the purification of a protein invariably involves the application of one or more column chromatographic steps, each of which generates a relatively large number of test tubes (fractions) containing buffer and protein eluted from the column. It is necessary to determine how much protein is present in each tube so that an elution profile (a plot of protein concentration versus tube number) can be produced. Appropriate methods for detecting and quantifying protein in solution are described in Section 8.3.2. A method is also required for determining which tubes contain the protein of interest so that their contents can be pooled and the pooled sample progressed to the next purification step. If one is purifying an enzyme, this is relatively easy as each tube simply has to be assayed for the presence of enzyme activity.

For proteins that have no easily measured biological activity, other approaches have to be used. If an antibody to the protein of interest is available then samples from each tube can be dried onto nitrocellulose and the antibody used to detect the protein-containing fractions using the dot blot method (Section 5.9.2). Alternatively, an immunoassay such as ELISA or radioimmunoassay (Section 7.3.1) can be used to detect the protein. If an antibody is not available, then portions from each fraction can be run on a sodium dodecyl sulphate–polyacrylamide gel and the protein-containing fraction identified from the appearance of the protein band of interest on the gel (Section 10.3.1).

An alternative approach that can be used for cloned genes that are expressed in cells is to express the protein as a fusion protein, i.e. one that is linked via a short peptide sequence to a second protein. This can have advantages for protein purification (see Section 8.3.5). However, it can also prove extremely useful for monitoring the purification of a protein that has no easily measurable activity. If the second protein is an enzyme that can be easily assayed (e.g. using a simple colorimetric