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

assay), such as β-galactosidase, then the presence of the protein of interest can be detected by the presence of the linked β-galactosidase activity.

A successful fractionation step is recognised by an increase in the specific activity of the sample, where the specific activity of the enzyme relates its total activity to the total amount of protein present in the preparation:

$$\text{specific activity} = \frac{\text{total units of enzyme in fraction}}{\text{total amount of protein in fraction}}$$

The measurement of units of an enzyme relies on an appreciation of certain basic kinetic concepts and upon the availability of a suitable analytical procedure. These are discussed in Section 15.2.2.

The amount of enzyme present in a particular fraction is expressed conventionally not in terms of units of mass or moles but in terms of units based upon the rate of the reaction that the enzyme promotes. The international unit (IU) of an enzyme is defined as the amount of enzyme that will convert 1 μmole of substrate to product in 1 minute under defined conditions (generally 25 or 30 °C at the optimum pH). The SI unit of enzyme activity is defined as the amount of enzyme that will convert 1 mole of substrate to product in 1 second. It has units of katal (kat) such that $1\ \text{kat} = 6 \times 10^7$ IU and $1\ \text{IU} = 1.7 \times 10^{-8}$ kat. For some enzymes, especially those where the substrate is a macromolecule of unknown relative molecular mass (e.g. amylase, pepsin, RNase, DNase), it is not possible to define either of these units. In such cases arbitrary units are used generally that are based upon some observable change in a chemical or physical property of the substrate.

For a purification step to be successful, therefore, the specific activity of the protein must be greater after the purification step than it was before. This increase is best represented as the fold purification:

$$\text{fold purification} = \frac{\text{specific activity of fraction}}{\text{original specific activity}}$$

A significant increase in specific activity is clearly necessary for a successful purification step. However, another important factor is the yield of the step. It is no use having an increased specific activity if you lose 95% of the protein you are trying to purify. Yield is defined as follows:

$$\text{yield} = \frac{\text{units of enzyme in fraction}}{\text{units of enzyme in original preparation}}$$

A yield of 70% or more in any purification step would normally be considered as acceptable. Table 8.3 shows how yield and specific activity vary during a purification schedule.

Preliminary purification steps

The initial extract, produced by the disruption of cells and tissue, and referred to at this stage as a homogenate, will invariably contain insoluble matter. For example, for mammalian tissue there will be incompletely homogenised connective and/or vascular tissue, and small fragments of non-homogenised tissue. This is most easily removed by filtering through a double layer of cheesecloth or by low speed (5 000 *g*)

Table 8.3 **Example of a protein purification schedule**

Fraction	Volume (cm^3)	Protein concentration (mg U cm^{-3})	Total protein (mg)	Activity[a] (mg U cm^{-3})	Total activity (U)	Specific activity (U mg^{-1})	Purification factor[b]	Overall yield[c] (%)
Homogenate	8 500	40	340 000	1.8	15 300	0.045	1	100
45%–70%$(NH_4)_2SO_4$	530	194	103 000	23.3	12 350	0.12	2.7	81
CM-cellulose	420	19.5	8 190	25	10 500	1.28	28.4	69
Affinity chromotography	48	2.2	105.6	198	9 500	88.4	1 964	62
DEAE-Sepharose	12	2.3	27.6	633	7 600	275	6 110	50

Notes: [a]The unit of enzyme activity (U) is defined as that amount which produces 1 μmole of product per minute under standard assay conditions.
[b]Defined as: purification factor = (specific activity of fraction/specific activity of homogenate).
[c]Defined as: overall yield = (total activity of fraction/total activity of homogenate).
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proteins denature can be a very useful preliminary step. The temperature at which the protein being purified is denatured is first determined by a small-scale experiment. Once this temperature is known, it is possible to remove more thermolabile contaminating proteins by heating the mixture to a temperature 5–10 °C below this critical temperature for a period of 15–30 min. The denatured, unwanted protein is then removed by centrifugation. The presence of the substrate, product or a competitive inhibitor of an enzyme often stabilises it and allows an even higher heat denaturation temperature to be employed. In a similar way, proteins differ in the ease with which they are denatured by extremes of pH (< 3 and > 10). The sensitivity of the protein under investigation to extreme pH is determined by a small-scale trial. The whole protein extract is then adjusted to a pH not less than 1 pH unit within that at which the test protein is precipitated. More sensitive proteins will precipitate and are removed by centrifugation.

Solubility

Proteins differ in the balance of charged, polar and hydrophobic amino acids that they display on their surfaces. Charged and polar groups on the surface are solvated by water molecules, thus making the protein molecule soluble, whereas hydrophobic residues are masked by water molecules that are necessarily found adjacent to these regions. Since solubility is a consequence of solvation of charged and polar groups on the surfaces of the protein, it follows that, under a particular set of conditions, proteins will differ in their solubilities. In particular, one exploits the fact that proteins precipitate differentially from solution on the addition of species such as neutral salts or organic solvents. It should be stressed here that these methods precipitate native (i.e. active) protein that has become insoluble by aggregation; we have not denatured the protein.

Salt fractionation is frequently carried out using ammonium sulphate. As increasing salt is added to a protein solution, so the salt ions are solvated by water molecules in the solution. As the salt concentration increases, freely available water molecules that can solvate the ions become scarce. At this stage those water molecules that have been forced into contact with hydrophobic groups on the surface of the protein are the next most freely available water molecules (rather than those involved in solvating polar groups on the protein surface, which are bound by electrostatic interactions and are far less easily given up) and these are therefore removed to solvate the salt molecules, thus leaving the hydrophobic patches exposed. As the ammonium sulphate concentration increases, the hydrophobic surfaces on the protein are progressively exposed. Thus revealed, these hydrophobic patches cause proteins to aggregate by hydrophobic interaction, resulting in precipitation. The first proteins to aggregate are therefore those with the most hydrophobic residues on the surface, followed by those with less hydrophobic residues. Clearly the aggregates formed are made of mixtures of more than one protein. Individual identical molecules do not seek out each other, but simply bind to another adjacent molecule with an exposed hydrophobic patch. However, many proteins are precipitated from solution over a narrow range of salt concentrations, making this a suitably simple procedure for enriching the proteins of interest.

Organic solvent fractionation is based on differences in the solubility of proteins in aqueous solutions containing water-miscible organic solvents such as ethanol, acetone and butanol. The addition of organic solvent effectively ‘dilutes out’ the water present

(reduces the dielectric constant) and at the same time water molecules are used up in hydrating the organic solvent molecules. Water of solvation is therefore removed from the charged and polar groups on the surface of proteins, thus exposing their charged groups. Aggregation of proteins therefore occurs by charge (ionic) interactions between molecules. Proteins consequently precipitate in decreasing order of the number of charged groups on their surface as the organic solvent concentration is increased.

Organic polymers can also be used for the fractional precipitation of proteins. This method resembles organic solvent fractionation in its mechanism of action but requires lower concentrations to cause protein precipitation and is less likely to cause protein denaturation. The most commonly used polymer is polyethylene glycol (PEG), with a relative molecular mass in the range 6000–20 000.

The fractionation of a protein mixture using ammonium sulphate is given here as a practical example of fractional precipitation. As explained above, as increasing amounts of ammonium sulphate are dissolved in a protein solution, certain proteins start to aggregate and precipitate out of solution. Increasing the salt strength results in further, different proteins precipitating out. By carrying out a controlled pilot experiment where the percentage of ammonium sulphate is increased stepwise say from 10% to 20% to 30% etc., the resultant precipitate at each step being recovered by centrifugation, redissolved in buffer and analysed for the protein of interest, it is possible to determine a fractionation procedure that will give a significantly purified sample. In the example shown in Table 8.3, the original homogenate was made in 45% ammonium sulphate and the precipitate recovered and discarded. The supernatant was then made in 70% ammonium sulphate, the precipitate collected, redissolved in buffer, and kept, with the supernatant being discarded. This produced a purification factor of 2.7. As can be seen, a significant amount of protein has been removed at this step (237 000 mg of protein) while 81% of the total enzyme present was recovered, i.e. the yield was good. This step has clearly produced an enrichment of the protein of interest from a large volume of extract and at the same time has concentrated the sample.

Isoelectric precipitation fractionation is based upon the observations that proteins have their minimum solubility at their isoelectric point. At this pH there are equal numbers of positive and negative charges on the protein molecule; intermolecular repulsions are therefore minimised and protein molecules can approach each other. This therefore allows opposite charges on different molecules to interact, resulting in the formation of insoluble aggregates. The principle can be exploited either to remove unwanted protein, by adjusting the pH of the protein extract so as to cause the precipitation of these proteins but not that of the test protein, or to remove the test protein, by adjusting the pH of the extract to its pI. In practice, the former alternative is preferable, since some denaturation of the precipitation protein inevitably occurs.

Finally, an unusual solubility phenomenon can be utilised in some cases for protein purification from *E. coli*. Early workers who were overexpressing heterologous proteins in *E. coli* at high levels were alarmed to discover that, although their protein was expressed in high yield (up to 40% of the total cell protein), the protein aggregated to form insoluble particles that became known as inclusion bodies. Initially this was seen as a major impediment to the production of proteins in *E. coli*, the inclusion bodies effectively being a mixture of monomeric and polymeric denatured proteins formed