Protein Extraction and Purification Methods-1

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Reference: Protein Purification techniques Second edition
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Key considerations

1. Keep the purification simple—minimize the number of steps and avoid difficult manipulations which will not reproduce.
2. Keep it cheap—avoid expensive techniques where a cheaper one will do.
3. Adopt a step approach—and optimize each step as you go.
4. Speed is important—avoid delays and slow equipment.
5. Use reliable techniques and apparatus.
6. Spend money on simple bits and pieces—e.g. test-tubes, pipettes.

7. Write out your methods before you start and record what you have done accurately.

8. Ensure your assays are developed to monitor the purification.

9. Keep notes on yields and activity throughout.

10. Bear in mind your objectives—be it high yield, high purity, final scale of operation, reproducibility, economical use of reagents/apparatus, convenience, throughput.

Aims of purification—why?

Typical reasons for purifying a protein may be:

(a) To identify the function of the protein (e.g. an enzyme).

(b) To identify the structure of the protein.

(c) To use the enzyme to generate a desired product as part of a research project.

(d) To produce a commercial product such as a diagnostic or therapeutic.

The target protein and contaminants

Information on molecular weight, isoelectric point, hydrophobicity, presence of carbohydrate, affinity for substrates, and sensitivity to metal ions can help in establishing the key steps in a purification.

What is known of the stability of the protein at varying pH and temperature, and following exposure to organic solvents, heavy metal ions, shear, and proteases?

If no information can be found in the literature, then some simple laboratory tests can help in establishing this data.

- The types of contaminants will depend very much on the source of the target protein (e.g. fermentation, animal tissue, plant material) and its location within that material (e.g. Extracellular versus intracellular).

Table 1 Typical classes of contaminants found in protein purification

Particulates	Include cells and cell debris. Usually removed initially using centrifugation or filtration.
Proteins	Include general host cell protein and proteins of similar properties to the target protein. Gross protein contaminants removed using precipitation while adsorption and chromatography techniques are widely applicable.
Modified target protein	Target protein modified through altered amino acid sequence, glycosylation, denaturation, etc. Removed using chromatography.
Lipids, lipoproteins	May be derived from host cells (e.g. membranes) or added to a fermentation (e.g. antifoams).
Small molecules	Include salts, sugars and reagents added to a purification. Typically removed using gel permeation chromatography or diafiltration.
Polyphenols	Coloured compounds often derived from plant sources, and often included in crude fermentation ingredients such as corn steep liquor. Removed by precipitation or chromatography.
Nucleic acids	Released during cell lysis and may increase sample viscosity. Removed using ion exchange, precipitation techniques such as protamine sulfate, or through hydrolysis with nucleases.
Pyrogens	Usually lipopolysaccharides derived from Gram negative bacterial cell walls.
Aggregates	Include inclusion bodies which are solubilized using chaotropic agents such as 6 M urea. Aggregate contaminants may be removed using gel permeation chromatography.

Protein structure

All proteins are composed of amino acids which are linked together into a peptide chain via peptide bonds.

There are 20 amino acids which form the basic building blocks of proteins, each with a characteristic side group which determines the properties of the amino acid.

Proteins are composed of one or more peptide chain in a three-dimensional (tertiary) structure which may also include linkages with non-protein molecules such as lipids and carbohydrates.

The combination of number and type of amino acids and the non-protein components of the tertiary structure determine the properties of the protein.

The properties of a protein (Table 2) are determined by the chemical groups which are exposed on the surface and the overall molecular weight of the molecule, which determines the Stokes radius

Table 2 Important properties of proteins and their relevance to purification

Charge	A measure of the surface ionic properties of a protein, giving the molecule a net negative or positive charge. Charge varies with pH and the isoelectric point is the pH at which the net charge is zero.
Biological activity	The presence of sites on a protein which can interact with other biological molecules with a high degree of affinity. Such interactions can be used to purify the target protein.
Hydrophobicity	A measure of the water-hating character of a protein. Highly hydrophobic molecules such as membrane proteins may be insoluble without addition of solvents or detergents. Highly hydrophobic proteins also precipitate at low concentrations of ammonium sulfate.
Size	Molecular weight is usually measured by gel electrophoresis (SDS–PAGE). The Stokes' radius, which gives an indication of shape.
Solubility/stability	The ability of a protein molecule to remain in solution is influenced by pH, salt concentration, presence of solvents and detergents, hydrophobicity, and biological affinity.

Source of the product

Proteins are purified from a wide variety of sources, including blood, animal tissue (e.g. heart, muscle, and liver), plant tissue (spinach, horseradish), and fermentation.

A common source is fermentation and cell culture with yeast, fungi, bacteria, animal, and plant cells all commonly used.

Any starting material such as plant and animal tissue must readily be available in a fresh stock—old material may have lost the required protein through degradation processes

Key steps in purification

While no two purifications are the same, and the separation scientist is faced with a bewildering variety of techniques available to achieve their goal, the majority of purifications aim to achieve the same result. These are:

- release of target protein from the starting material
- removal of solids to leave the protein in a supernatant
- removal of water to concentrate the protein
- removal of contaminants to achieve the desired purity
- stabilization of the target protein

Apparatus, special materials and reagents

There are many complex sophisticated pieces of equipment designed for protein seperations and as in every other walk of life, these are becoming more automated for convenience and simplicity of operation.

There are number of special reagents that the protein purifier requires. These are mainly packing materials for column chromatography and chemicals that are frequently used.

These five goals form the basis of the stages used in protein purification as follows:

(a) Stage 1: Initial fractionation. Here the goal is to prepare the protein as a clarified solution. This typically involves techniques such as centrifugation, microfiltration, and cell lysis.

(b) Stage 2: Purification. Initial fractionation is followed by a sequence of operations designed to remove contaminants and concentrate the product further.

(c) Stage 3: Polishing.. The removal of aggregated or degraded protein is also important at this stage, using size exclusion chromatography as a key step.

Overview of lab equipment

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 μl to 5 ml
Beakers
Chromatographic media
Ammonium sulfate
Buffers
Dialysis tubing
Salt
Stabilizing reagents

- 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.

- 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.

Considering yield and purity

- In most cases, analyses will involve measurement of the mass of the protein in the sample and quantitation of specific property of the target molecule (e.g. activity) to provide values for the yield. Thus, the calculation of specific activity of given fractions through the purification process provides a valuable indication of the level of the purity attained.

Total protein quantitation

1. Ultraviolet absorption protein assay

Proteins show maximal ultraviolet (UV) absorption at about 280 nm arising from the contribution made by aromatic residues (of tryptophan, tyrosine) and to some extent from cystine groups. Absorption at 280 nm can, therefore, be used to provide an approximate protein assay since, the intensity of absorption bears some relationship to the number of these residues present in the protein.

Protocol 1

Calculation of the absorbance coefficient of protein

1. Count the number of tryptophan residues (η_{Trp}) and tyrosine residues (η_{Tyr}) in the sequence.
2. Count the number of disulfide bonds (η_{ss}) and if unknown assume that for intracellular located proteins the value is zero and for secreted proteins $\eta_{ss} = \eta_{Cys}/2$.
3. Use the following equation to calculate absorbance coefficient:

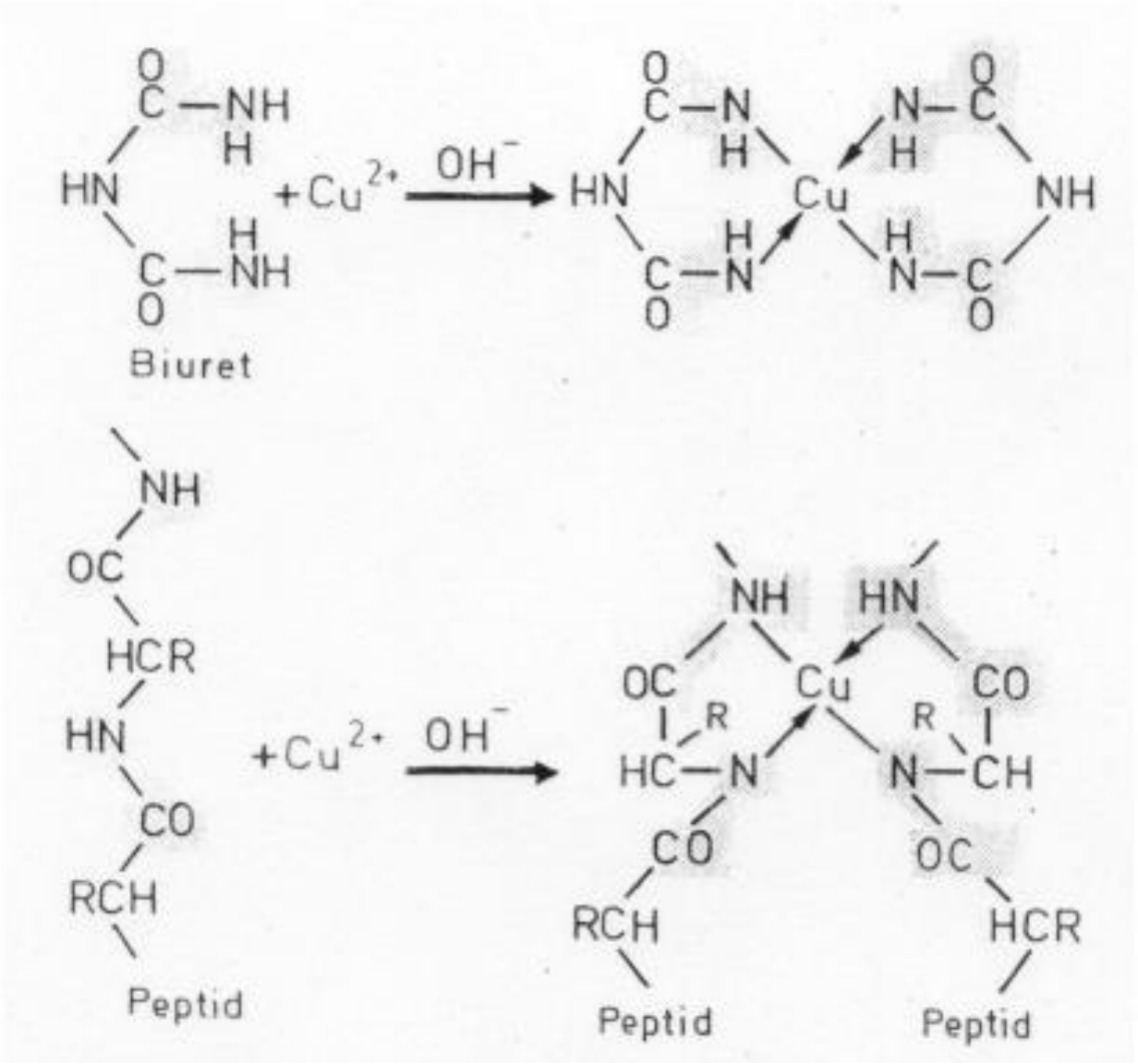
$$\varepsilon_{280}\,[M^{-1}cm^{-1}] = 5500 \times \eta_{Trp} + 1490 \times \eta_{Tyr} + 125 \times \eta_{ss}$$

$$\text{Protein (mg/ml)} = 1.55\,A_{280\,\text{nm}} - 0.76\,A_{260\,\text{nm}}$$

Lowry (Folin-Ciocalteau) protein assay

This is the most widely-used method for quantitative determination of protein concentration. Reaction of the phenolic moiety of tyrosinc in protein with Folin-Ciocalteau reagent, which contains phosphomolybdic/tungstic acid mixture produces a blue/purple colour with absorption maximum around 660 nm. Additionally, the use of a copper reagent enhances the colour formation by chelating with the peptide bonds and allowing for efficient electron transfer to the chromophore formed. This method is sensitive down to 10 ug protein per ml. The Folin-Ciocalteau reagent is commercially available.

Note that many of the commonly used reagents, e.g. Tris. Pipes, Hepes, EDTA, and detergents
are known to interfere with this assay.


Biuret
+Cu2+ OH−
Peptid
+Cu2+ OH−
Peptid
Peptid

Lowry method:

The assay is a colorimetric assay based on reduction of the Cupric Cu2+ to cuprous ions Cu+ in alkaline pH when reacting with peptide. Cuprous ion and the phenolic group of Tyr; indole of Trp; -SH of Cys then react with Folin-Ciocalteau reagent to produce an unstable “molydenum blue”-type product (A=650nm).

Lowry-Folin-Ciocalteau reagent consits of phosphomolybdate and phosphotungstate which create the color when reduced. Most proteins contain little Trp or Cys. Therefore, the colour here is largely due to Tyr content.

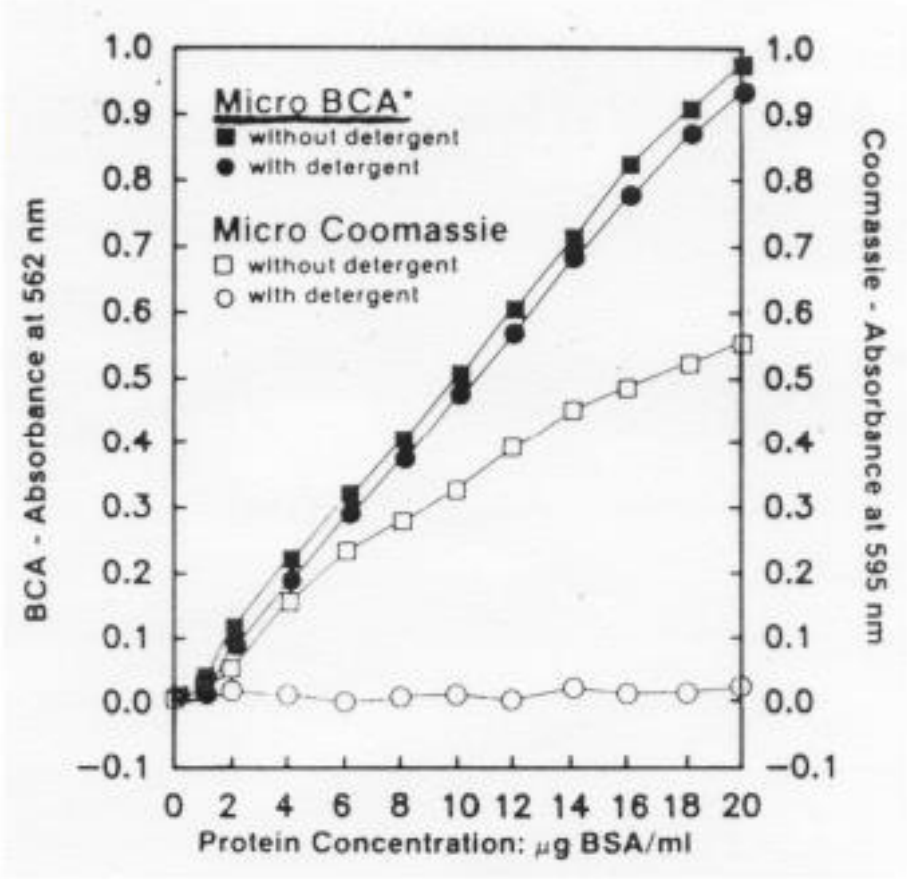
Bradford (Coomassie Brilliant Blue) dye-binding protein assay

Coomassie Brilliant Blue complexes with proteins to give an absorption maximum at 595 nm. It is a simple method with the colour developing rapidly to produce a stable complex and is sensitive down to 20 ug protein per ml, but the amount of dye binding to the different protein molecules is variable and does require a careful selection of a protein standard for generating the calibration curve.

Bradford method:

This assay is based on binding of Coomassie dye by protein (Brilliant blue G250).
Binds to aromatic, K,R,H
Shifts A465 max to A595 nm

→ Easy to use compared with Biuret, Lowry, and BCA but more sensitive for other substances, eg detergents



Bicinchoninic acid protein assay

Bicinchoninic acid (BCA) reagent provides a more convenient and reproducible cuprous ion base assay and has a sensitivity similar to the Lowry method, although it is subject to interference from DTT concentrations above 1 mM (7). The sensitivity range for this assay is 0.2-50 ug.

Colloidal gold protein assay

Metal-binding assays are, particularly, useful for measuring low concentrations (20-640 ng) of proteins. Commercially formulated colloidal gold protein assay reagent used in the original description of the assay has been changed and cannot be used for the protein assay.

Detergents

- The attraction of chemical methods of cell disruption is that the cell will be left substantially intact after release of its contents. This facilitates separation of the cell debris from the supernatant. However, the chemicals used must be compatible with further downstream processes. It will, probably, be necessary to remove the DNA from the lysate, since it is likely to be released in a high molecular weight form, and therefore, greatly increases the viscosity of the lysate.

- (a) Chelating agents such as EDTA sequest divalent cations. Loss of Mg^{2+} and Ca^{2+} from Gram negative cell walls results in the loss of their permeability barrier. In fact, the inner membrane of E. colt remains intact so only periplasmic enzymes are released (35). Chelating agents are not usually used on their own but in combination with another lysis method. Thus, they could be viewed as pre-treatments.

- (b) Chaotropic agents such as guanidine, ethanol, and urea weaken interaction between hydrophobic molecules. They generally have to be used at high concentrations, which does not make them suitable for large scale extraction processes. Some details are shown in Table 5.

Triton X-100 has often been used on its own or in combination with other disruption methods. The 0.2 M guanidine releases periplasmic proteins, treatment with 0.2 M guanidine and 0,5% Triton X-100 releases cytoplasmic protein as well.

A method for using Triton X-100 and details of other systems are given in Table 5.

Anionic detergents (e.g. SDS at low concentrations, 0.05%) released 24% of intracellular protein (and similar amounts of nucleic acids).

Examples for SDS lysis of bacterial cells and animal cells are given in Protocols 11 and 12.

Table 5 Examples of chemical and enzymic extraction of cells

Cell type (and lysis method)	Details	Ref
Bacillus subtilis with lysozyme and surface active agent	Cells suspended in buffer with sucrose, dithiothreitol, lysozyme (300 μg/ml, EDTA, and Brij 58). Incubated for 1 h on ice. Supernatant obtained after 30 min at 40 000 *g*.	36
β-Galactosidase release from the yeast: *Kluyveromyces lactis*	2% chloroform, with 10% ethanol. 5–37 °C, toluene also tested.	37
Protein and β-lactamase release from *E. coli*	Cell suspension mixed with lysis buffer to give 0.4 M guanidine and 0.5% Triton X-100. Treatment time about 4 h. Clarification by centrifugation.	38

Protocol 11

SDS lysis of E. coli cells[a]

Equipment and reagents

- Suspension of *E. coli* cells in 0.5 M glycerol, 1 mM sodium phosphate at 4 °C, made up to an OD (660 nm) of between 10–15
- Ice bath
- Lysis buffer: 2% SDS (v/v) in 0.25 M NaOH with 0.2 M Na_2EDTA, stored at 4 °C

Method

1. Add 1 ml of lysis buffer to 4.0 ml cell suspension.
2. Mix by inversion once and store on ice.
3. Lysis should occur in about 5 min. The lysate will be very viscous due to the release of DNA, which should remain intact since minimum agitation has been used.

[a] Stephenson *et al.* (46).

Protocol 12

Lysis of animal cells (from cell lines) with SDS[a]

Reagents

- Chloroform
- Cell suspension
- 0.1% SDS

Method

1. Add 15 µl of chloroform and 15 µl of 0.1% SDS to 1 ml of cell suspension.
2. Agitate the mixture for 10 sec.
3. Centrifuge at 12 000 *g* for 15 min at 4 °C.
4. Assay of protein can be carried out but there is interference from SDS. SDS of the same concentration needs to be included in blanks and standards.

[a] Shin *et al.* (25).

Concentration of the extract

Concentration is achieved by removal of water and other small molecules:

(a) By addition of a dry matrix polymer with pores that are too small to allow entry of the large protein molecules (Section 2).

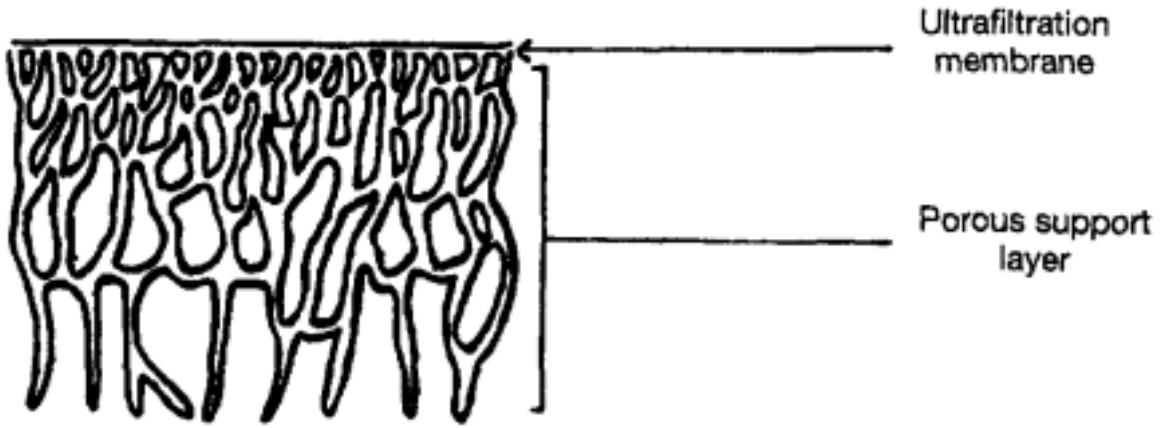
(b) By removal of the small molecules through a semi-permeable membrane which will not allow the large molecules through (i.e. ultrafiltration, Section 3).

(c) By removal of water in vacua

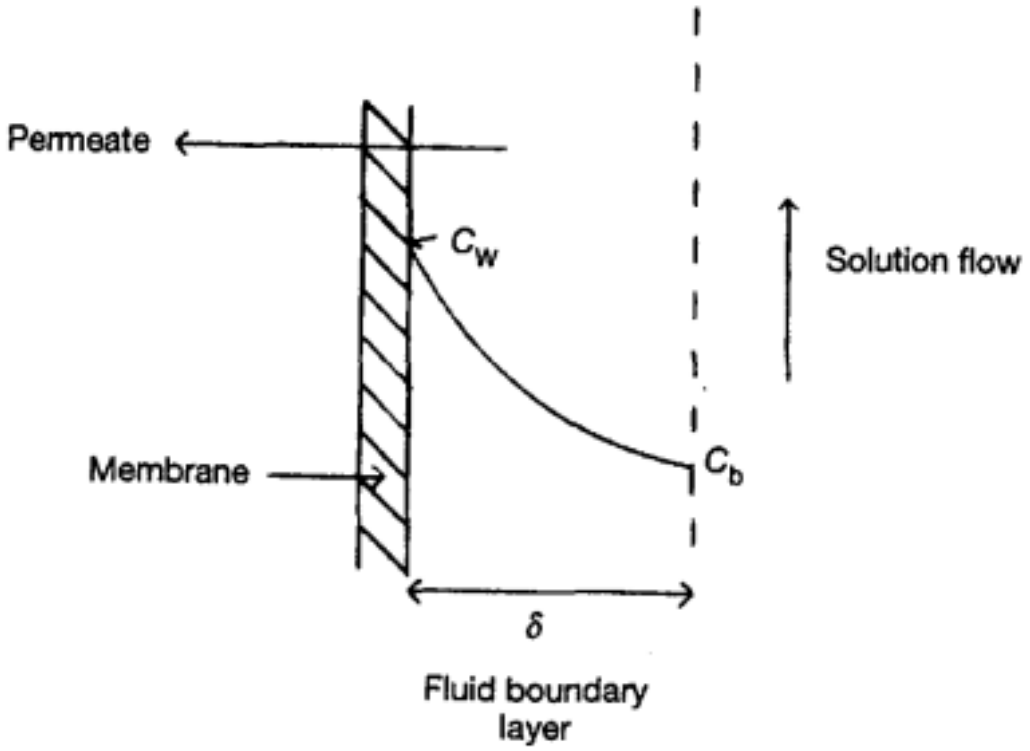
Precipitation can also be used to concentrate proteins if the pellet is redissolved in a smaller volume, and in addition often results in some degree of purification of the protein of interest. However, as mentioned above precipitation is more effective if the total protein concentration is above 100 ug/ml

Ultrafiltration

- In ultrafiltration, water and other small molecules are driven through a semipermeable membrane by a transmembrane force such as centrifugation or high pressure.
- For ultrafiltration, the membrane pores range in diameter from 1-20 nm; the diameter is chosen such that the protein of interest is too large to pass through. Pore sizes of microfiltration membranes range from 0.1-10 µm diameter and allow proteins and other macromolecules to pass through, whilst retaining larger particles such as cells.


Ultrafiltration membrane
Porous support layer

(a)


Permeate
C_w
Solution flow
Membrane
C_b
δ
Fluid boundary layer

(b)

Rather than quote the pore size of the ultrafiltration membranes it is more common to quote a nominal molecular weight cut-off (NMWC) for the membrane. The NMWC is defined as the minimum molecular weight globular molecule which will not pass through the membrane. Concentration by ultrafiltration offers several advantages over alternative methods.