Protein Extraction and Purification Methods-2

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Reference: Protein Purification techniques Second edition
edited by Simone Roe

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:

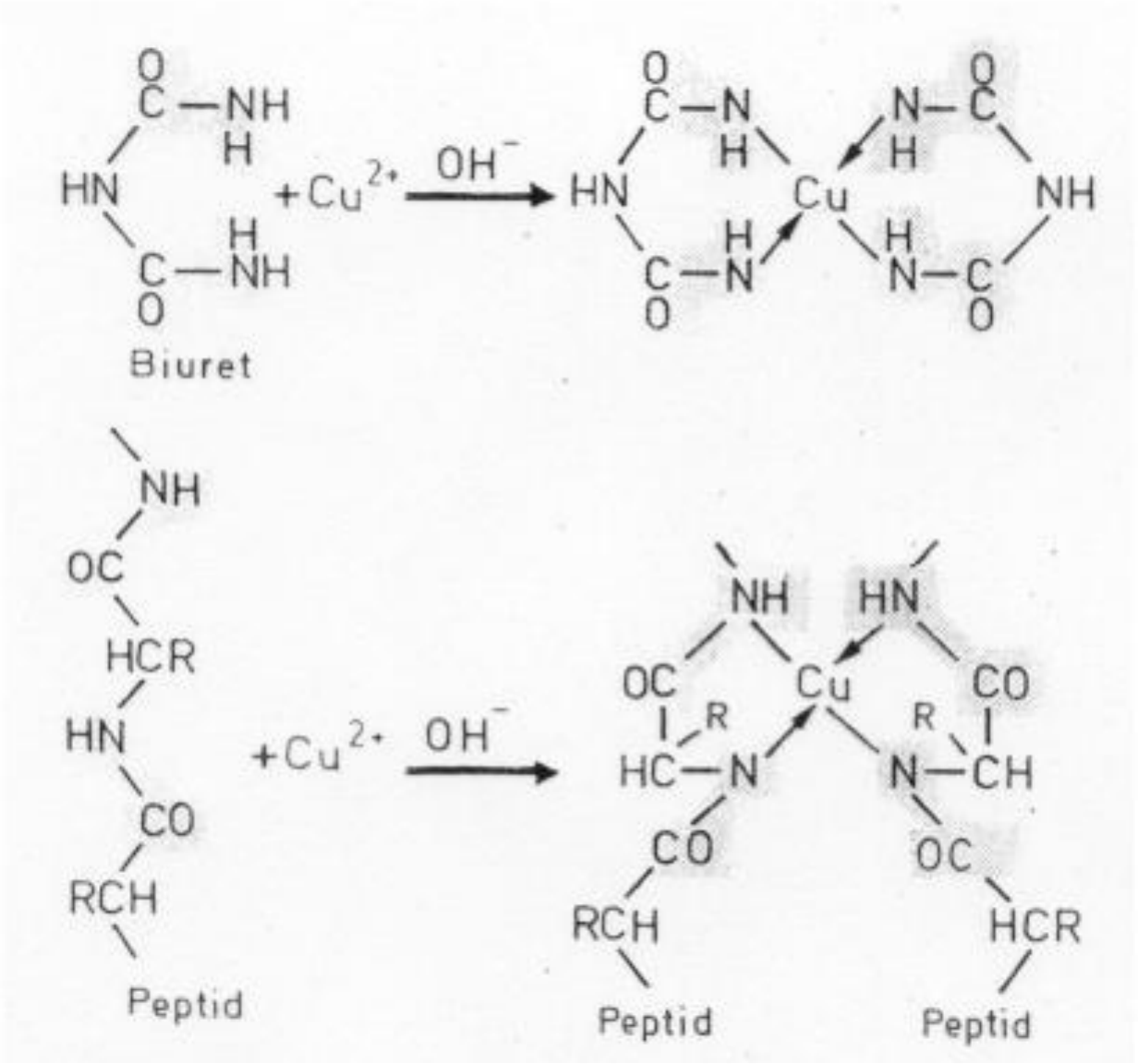
$$\varepsilon_{280}\,[M^{-1}cm^{-1}] = 5500 \times \eta_{Trp} + 1490 \times \eta_{Tyr} + 125 \times \eta_{ss}$$

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

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.

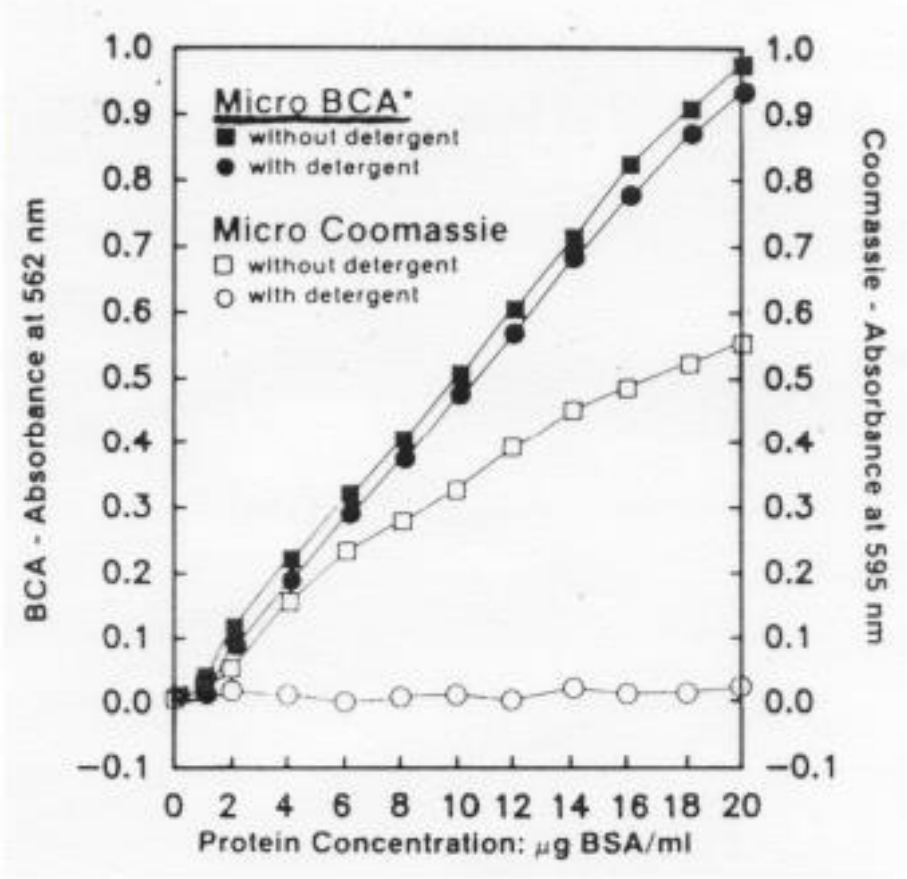
Bradford (Coomassie Brilliant Blue) dye-binding protein assay

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.