Enzyme Assay Methods:-

1**-Absorption Photometry :-(Spectrophotometric Method)**

Because of its **simple technique** and **dependable**, nearly **priced instruments,** photometry is today one of the ideal methods of enzyme assay. It can be carried out most **quickly** and **easily** when the substrate or the product is **colored Visible or absorbs light in the ultraviolet region,** the rate of appearance or disappearance of a light-absorbing product or substrate can be followed with a spectrophotometer. **(Measure the change in absorption of light – Visible range (color change colorimetric**)

According to the Lambert–**Beer law,** which is suitable for very dilute solutions, the following relationship exists between absorbance A and concentration:

$$A = \varepsilon . c . d. \qquad C = \text{----------}$$

where **c** is the concentration in mill moles per liter

ε the mill molar absorption coefficient in liters per mole and per millimeter

d the path length

The **catalytic activity** (z) then corresponds to the absorbance change per minute.

The unit of z is then **micromoles per minute** and corresponds to the definition of the International Unit U.

Example: Assay of Lactate Dehydrogenase (LDH). In the reaction catalyzed by LDH

(E.C. 1.1.1.27), hydrogen is transferred from NADH to pyruvate, to yield L-lactate and NAD

Pyruvate +NADH----------> Lactate + NAD

The reduced coenzyme NADH absorbs at 340 nm (UV range), whereas the oxidized form NAD, lactate, and pyruvate do not. Thus, the progress of reaction can be followed by measuring the decrease in light absorption at 340 nm

Coupled assay

When a spectrophotometer can't use to measure directly the product or substrate,

the product of the reaction of interest can be used as the substrate of a coupled react ion that can be measure in some manner in the Vis/UV range .

Example: Assay of Phosphatase. colorless 4-nitrophenyl compounds are incubated, and 4-nitrophenolate is formed under alkaline conditions with a characteristic maximum between 400 and 420 nm. In this way, the catalytic activity of such enzymes can be measured easily at this wavelength:

4-Nitrophenyl phosphate + H_2O ----------> 4-Nitrophenol + Phosphate

4-Nitrophenol + OH----------> **4-Nitrophenolate**

in the assay of such enzymes as glycerol phosphate oxidase (E.C.1.1.3.21)

The catalytic activity of the first is determined according to the following reaction

sequence:

2L-α-Glycerolphosphate+$2O_2$ ----------->2Dihydroxyacetonephosphate+$2H_2O_2$

$2H_2O_2$ +4-Aminoantipyrine+Phenol----------->Quinonimine dye+4H2O

The broad absorption maximum of the quinonimine dye is around 500 nm. The increase in **absorbance per minute** at 500 nm is measured to determine the activity of glycerol phosphate oxidase.

2-Fluorometry: -

The fluorometric method is not often used for determining the catalytic activity of raw or purified enzyme preparations. Because of its high sensitivity, it permits the assay of small amounts of enzymes in organs or tissue sections.

For example, systems that depend on NAD and NADP can be measured by fluorometry. The overall sensitivity of this method is a thousand times that of absorption photometry.

Fluorescence is

Emission of light at a specific wavelength after absorption of light at a different wavelength.

Fluorometric assays use a difference in the fluorescence of substrate from product to measure the enzyme reaction. These assays are in general much more sensitive than spectrophotometric assays,

An example of these assays is again the use of the nucleotide coenzymes **NADH** and **NADPH.** Here, the reduced forms are fluorescent and the oxidized forms non-fluorescent. Oxidation reactions can therefore be followed by a decrease in fluorescence and reduction reactions by an increase. Synthetic substrates that

release a fluorescent dye in an enzyme-catalyzed reaction are also available, such as 4-methylumbelliferyl-β-D-galactoside for assaying β-galactosidase.

3-Potentiometry: -

A pH-sensitive glass electrode can be used to measure reactions in which protons are produced or consumed. For this purpose, the pH is kept constant by counter titration, and the consumption of acid or base required to do this is measured.

A typical example is the determination of the catalytic activity of lipase (E.C. 3.1.1.3). A fat (triglyceride) is hydrolyzed by this enzyme, and the fatty acid formed is neutralized by counter titration with NaOH in a pH-stat mode:

Triglyceride + H_2O ----------> Diglyceride + Free Fatty acid

4-Conductometry:-

all enzymatic reactions that lead to a change in overall **ionic mobili**ty can be measured by conductometry. In this way, the elastolytic activity of elastase (E.C.3.4.21.36) by using unmodified elastin as substrate has been determined. In this reaction, protons are liberated by cleavage of peptide bonds.

6-Calorimetry:-

Many enzymatic reactions evolve heat; therefore, some interest in calorimetric (enthalpimetric) methods has developed. In a suitable experimental arrangement, a temperature sensor serves as a device form ensuring the catalytic activity of the enzyme.

The method is used mainly in research.

7-Viscosimetry

Viscosimetry has been practically discarded for enzyme assays. before, e.g., cellulase (E.C. 3.2.1.4) activity has been determined by the change of Catalytic Activity of Enzymes viscosity per unit time. Nowadays the cellulase assay is performed with a colorimetric reaction:

Cellulose ----------> Oligosaccharide + nGlucose---------->Red dye

8-Turbidimetry

The turbidimetric method can be adapted for different enzyme assays. As an example, lysozyme (muramidase, E.C. 3.2.1.17) cleaves bacterial cell walls. A standard substrate suspension (dried germs of Micrococcus) is used. The decrease of absorbency is measured at 450 nm and 25.8C°.

Controlled factors in assays:-

1-Salt Concentration-Ions interfere with ionic bonds of proteins.

Effects of temperature-Increases in temperature generally lead to increases in reacti on rates (until denaturation occurs).

3-Effects of pH-Most enzymes are sensitive to pH and have an optimum pH for functionality. pH denatures enzymes by breaking ionic, and hydrog en bonds.

4- Substrate Saturation
Increasing the substrate concentration increases the rate of reaction but enzyme satu ration limits reaction rates. At the saturation point, the reaction will not speed up an y further.

5-

Level of crowding-Large amounts of macromolecules in a solution will alter the rat es and equilibrium constants of enzyme reactions.

Protein Determination: -

protein content is the most important reference point for determination of the specific activity of an enzyme preparation, There are many methods of protein determination . All of these methods are based on different principles and depend on the amino acid composition of the enzyme proteins. They will, therefore, yield different values.

1-Ultraviolet Absorption: - Because of their content of aromatic amino acids, proteins show an absorption maximum at 270–280 nm. **For many pure protein**s, reference values have been established for the 280-nm absorbance of a solution containing 10 mg/mL.

2-Ultraviolet Absorbance of Aromatic Amino Acids

How does it work?

- Monitors the absorbance of aromatic amino acids, **tyrosine and tryptophan** or if the wavelength is lowered, the absorbance of the **peptide bond**. Higher order structure in the proteins will influence the absorption

Detection Limitations

- 20 µg to 3 mg
-

Advantages

- Quick
- Sample can be recovered
- Well suited for identifying protein in column fractions

Disadvantages

- Highly disposed to contamination by buffers, biological materials and salts
- Protein amino acid composition is very important, thus the choice of a standard is very difficult, especially for purified proteins.
- Absorbance is greatly influence by pH and ionic strength of the solution.

Quantitative Procedure

- Zero the spectrophotometer with a buffer blank
- Make a standard curve using your standard of choice in the expected concentration range, using the same buffer that your unknown sample is in.
- Take the absorbance values at 280 nm in a quartz cuvette
- Place sample into quartz cuvette (make sure concentration is in the range of 20 µg to 3 mg
- Take absorbance at 280 nm

Estimation Procedure

- Zero spectrophotometer to water (or buffer)
- Take the absorbance at 280 nm in a **quartz cuvette**
- Change wavelength to 260 nm and zero with water (or buffer)

- Take absorption at 260 nm in a **quartz cuvette**
- Use the following equation to estimate the protein concentration

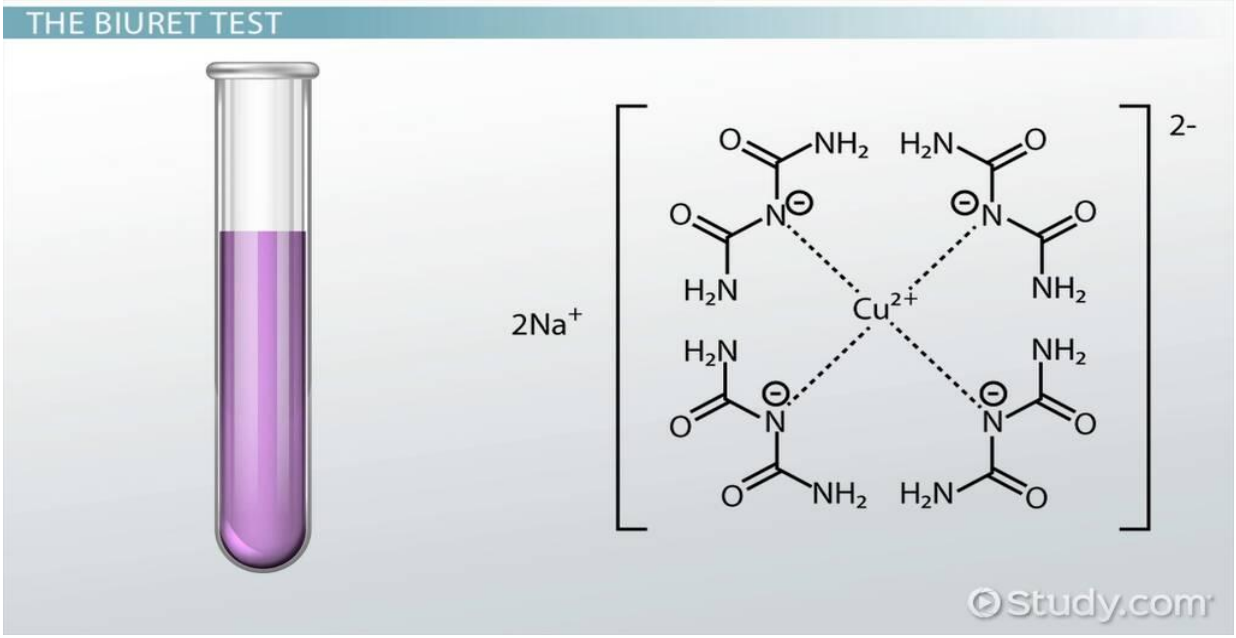
[Protein] (mg/mL) = 1.55*A280 - 0.76*A260

3-Biuret Protein Assay

The principle of the biuret assay is similar to that of the Lowry; however it involves a single incubation of 20 min. The reaction of peptide bonds with copper ions in an alkaline solution yields a purple complex which can be determined photometrically. The intensity is a linear function of protein concentration. There are very few interfering agents (ammonium salts being one such agent),. However, the biuret assay consumes much more material. The biuret is a good general protein assay for batches of material for which yield is not a problem. The Bradford, UV, Lowry assays are more sensitive.

Principle

Under alkaline conditions substances containing two or more peptide bonds form a purple complex with copper salts in the reagent.



Equipment

In addition to standard liquid handling supplies a visible light spectrophotometer is needed, with maximum transmission in the region of 450 nm. Glass or polystyrene (cheap) cuvettes may be used.

Procedure

Reagent

A formula for biuret reagent is (per liter final volume) 9 gm Sodium potassium tartrate (f.w. 282.22), 3 gm Copper sulfate x 5 H2O (f.w. 249.68), 5 gm Potassium iodide (166.0), all dissolved in order in 400 ml 0.2 M NaOH (f.w. 40.0) bringing to final volume.

Assay

Prepare standards from bovine serum albumin.

Prepare a reference tube with 1 ml buffer.

Use 1 ml sample per assay tube

Add 9 ml Biuret reagent to each tube, vortex immediately, and let stand 20 min.

Read at 550 nm.

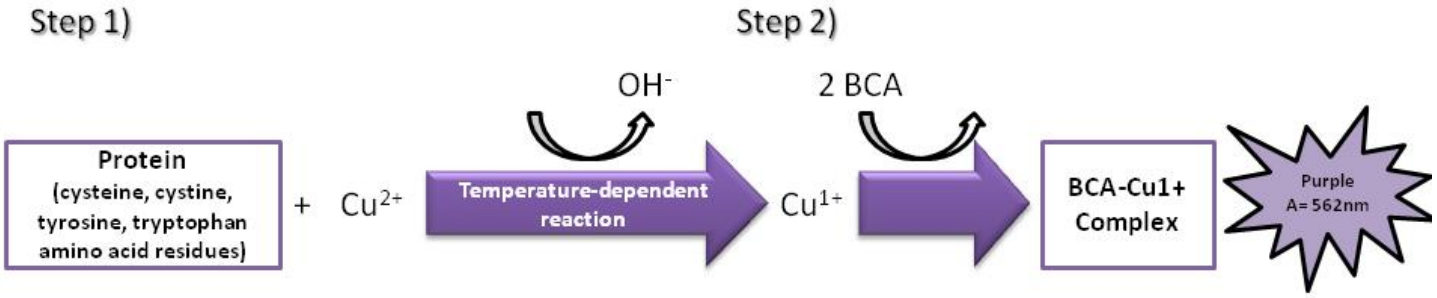
4- Bicinchoninic acid (**BCA) Method:-**

It combines the biuret method with the BCA:

Protein + Cu + Bicinchoninic acid ----------> Red dye (Cu complex)

The complex allows the spectrophotometric quantitation of the protein in aqueous

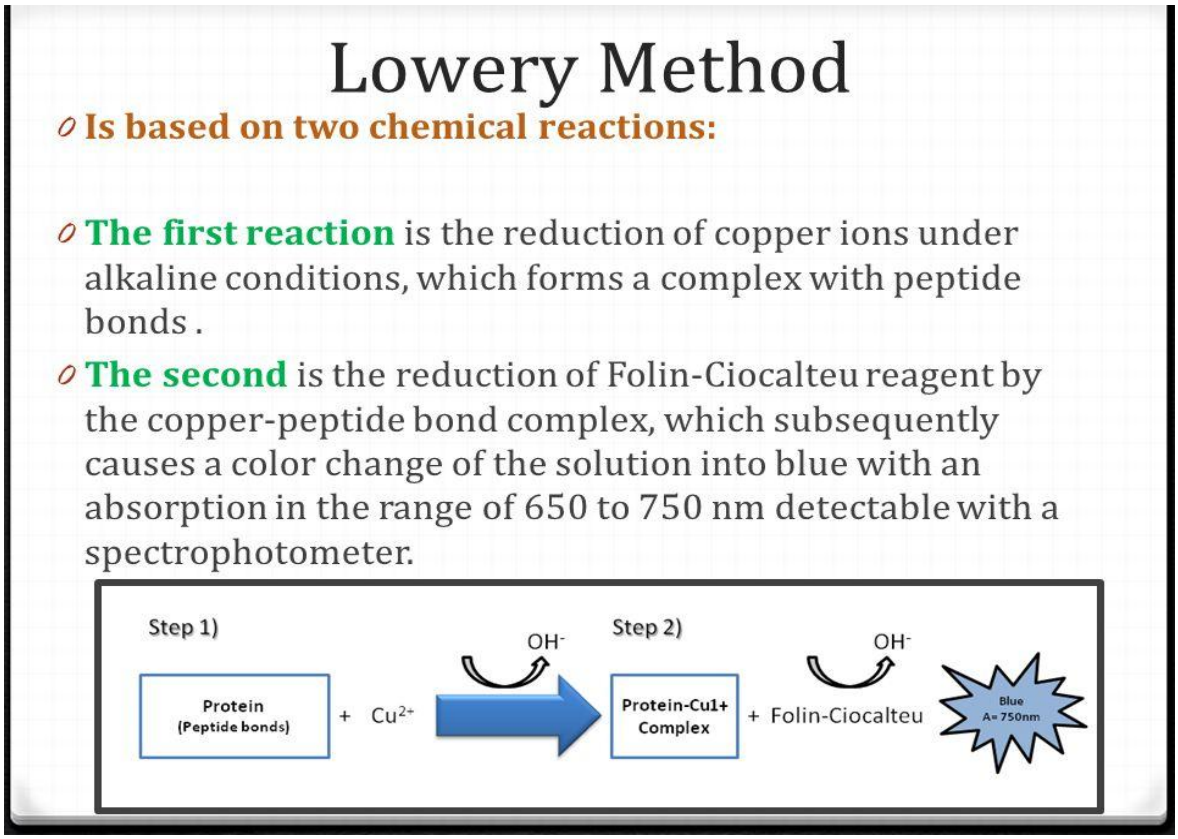
Solutions



5-Protein Determination - Lowry Procedure

Introduction

The principle behind the Lowry method of determining protein concentrations depend in the reactivity of the peptide nitrogen[s] (Peptide bonds) with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids .The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10 - 10.5.



Similar to other colorimetric assays, the absorbance of an unknown solution is related to the concentration by using a standard curve. For Lowry assay, any protein could act as a standard but the most commonly used protein is BSA (bovine serum albumin).

The Lowry method is sensitive to low concentrations of protein. Dunn [1992] suggests concentrations ranging from 0.10 - 2 mg of protein per mL while Price [1996] suggests concentrations of 0.005 - 0.10 mg of protein per mL. The major disadvantage of the Lowry method is the narrow pH range within which it is accurate. However, we will be using very small volumes of sample which will have little or no effect on pH of the reaction mixture.

A variety of compounds will interfere with the Lowry procedure. These include some amino acid derivatives, drugs, lipids, sugars, salts, nucleic acids and sulphydryl reagents [Dunn, 1992]. Price [1996] notes that ammonium ions,

zwitterionic buffers, nonionic buffers and thiol compounds may also interfere with the Lowry reaction. These substances should be removed or diluted before running Lowry assays.

Reagents

A. 2% Na_2CO_3 in 0.1 N NaOH

B. 1% NaK Tartrate in H_2O

C. 1% $CuSO_4 \cdot 5 H_2O$ in H_2O

D. 48 mL of A, 1 mL of B, 1 mL C

E. Phenol Reagent - 1 part Folin-Phenol [2 N] : 1 part water

[Reagents A, B and C may be stored indefinitely]

BSA Standard - 1 mg/ mL

Bovine Serum Albumin: 5 mg in 5 mL of water [1 µg / µl].

Procedure

[Run triplicate determination for all samples.]

1. Set up eleven sets of three 16 x 150 mm test tubes in rack.
2. Add BSA [0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µl] to these tubes.
3. Add 2 mL of solution D to each test tube.
4. Incubate for 10 minutes at room temperature.
5. Add 0.2 mL of dilute Folin-phenol solution to each tube.
6. Vortex each tube immediately.
9. Incubate at room temperature for 30 minutes.
10. Determine absorbance of each sample at 600 nm.

11. Plot absorbance vs mg protein to obtain standard curve.
12. Set up triplicate assays for all "unknowns".

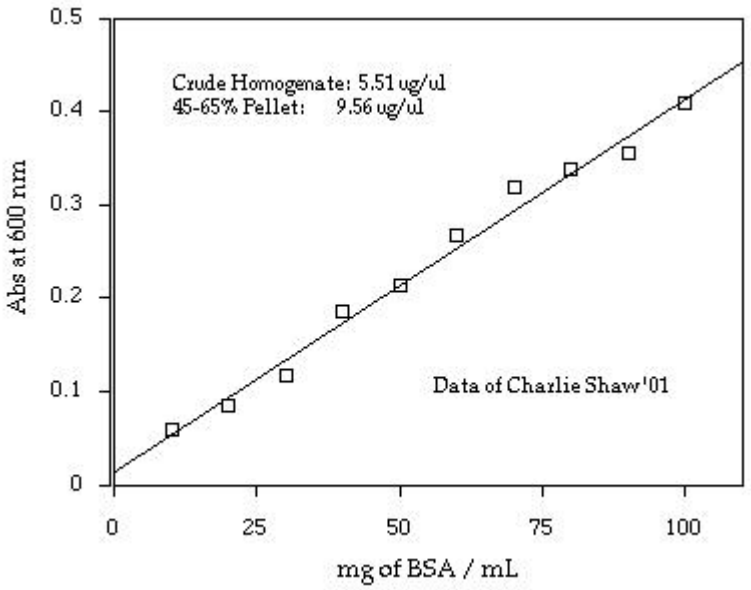


Figure. Protein concentrations of a curve

Protein-Dye Binding

Attempts have been made to determine protein concentration by using dyes. The method published by BRADFORD now predominates. It is based on the shift of the absorption maximum of Coomassie Brilliant Blue G 250 from 465 to 595 nm, which occurs when the dye binds to the protein.