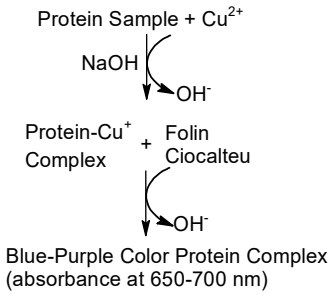

EXPERIMENT 3

ESTIMATION OF PROTEINS BY LOWRY'S METHOD

Structure

3.1 INTRODUCTION

Lowry's assay for total protein estimation is one of the most commonly used colorimetric assays. It is sensitive, highly reproducible, inexpensive and easy to perform. It relies on the reaction of copper with protein, but the sample is also incubated with the Folin- Ciocaltaeu reagent. Reduction of the Folin-Ciocaltaeu reagent under alkaline conditions results in an intense blue colour (heteropolymolybdenum blue) that absorbs at 750 nm. This method is the best used for protein concentrations of 0.01-1 mg/ml.

Most proteins estimation techniques use Bovine Serum Albumin (BSA) universally as a standard protein, due to its low cost, high purity and ready availability.

Expected Learning Outcomes

After performing this experiment, you shall be able to:

- describe to the principle working behind Fohin-ciocaltaen assay;
- distinguish between Biuret and Lowry's assay methods;
- enlist the composition and preparation of Analytical reagent; and
- significance of standard plot in estimating protein concentration in an unknown sample.

3.2 PRINCIPLE

The principle of this method is based on two reactions leading to formation of a cloured complex. Firstly, the Biuret reaction in which Cu (II) of the reaction mixture reacts with the peptide bond of proteins under alkaline conditions resulting in their reduction to cuprous ions [Cu(I)]. Secondly, Lowry's reaction in which the Folin- Ciocaltaeu reagent contains phosphomolybdic complex which is a mixture of sodium tungstate, sodium molybdate and phosphate, along with copper sulphate solution and the protein results in blue purple colour which can be assessed by measuring the absorbance at 650 - 700 nm. The phenolic group of the amino acid (tyrosine and tryptophan) residues will produce a blue purple colour due to the reduction of phosphomolybdotungstate to heteropolymolybdenum blue by the copper catalysed oxidation of the amino acids and its intensity depends on the amount of these aromatic amino acids present. The blue purple colour formed thus differs from protein to protein. The blue purple colour is formed due to the presence of tryptophan and tyrosine (Fig. 3.1).

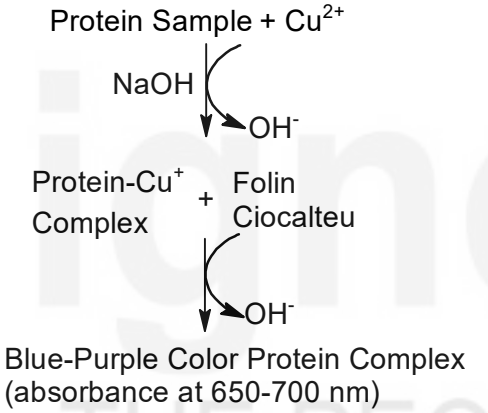

Fig. 3.1. Showing the flow of Lowry's reaction

3.3 MATERIALS REQUIRED

1. **Glassware and Equipment's**: Cuvette, Test tubes, Test tube stand, Pipette, Measuring cylinder and Spectrophotometer.

2. **Chemicals and Reagents:** Bovine Serum Albumin (BSA), Analytical Reagent, Folin's reagent (commercially available), Distilled water.

3. **Reagent Preparation:**

   1) Analytical reagent:

      a. 1% $CuSO_4$ – 50 ml

      b. 2% sodium potassium tartrate- 50 ml (i.e., 1gm in 50 ml H_2O)

      c. Alkaline Na_2CO_3 – 250 ml (i.e., NaOH=1gm, Na_2CO_3 = 5gm, total= 250 ml)

   2) **NOTE**: Prepare freshly before use: 196 ml of (c) + 2 ml of (a) + 2 ml of (b) = 200 ml of analytical reagent.

3) Folin's reagent = 50 ml (i.e., 25 ml of reagent + 25 ml of H_2O)

4) BSA stock- 100 mg/ml

3.4 PROCEDURE

i. A 100 µg/ml stock solution of BSA protein was prepared.

ii. 200 ml of analytical reagent and 50 ml of Folin's reagent was prepared.

iii. In test tube, with the help of pipette, dilution with volume of protein was made (10µg, 20µg, 30µg, 40µg, 50µg) by mixing distilled water to it. Another test tube with 1ml of unknown was taken.

iv. 2.5 ml of analytical reagent (AR) was added to all the test tubes and the test tubes were left to incubate at room temperature for 10 minutes.

v. 0.25 ml of Folin's reagent (FR) was added to all the test tubes and the test tubes were left to incubate at room temperature for 30 minutes.

vi. After this, readings of absorbance for all the test tubes were taken and a standard graph was plotted to calculate the concentration of unknown solution.

3.5 RESULTS

Observation Table

BSA (µg/ml)	**Volume of Stock BSA (ml)**	**Distilled Water (ml)**	**Analytical Reagent (ml)**	**Incubate for 10 minutes at Room Temp.**	**Folin's Reagent (ml)**	**Incubate for 30 minutes at Room Temp.**	**Absorbance 660 nm**
0	0	1	2.5		0.5		
20	0.2	0.8	2.5		0.5		
40	0.4	0.6	2.5		0.5		
60	0.6	0.4	2.5		0.5		
80	0.8	0.2	2.5		0.5		
100	1.0	0	2.5		0.5		

Note: The following formula can be used for estimating the concertation of unknown solution.

$$\frac{\text{OD of Test}}{\text{OD of Stadard}}\ x\ \text{Concentration of standard}$$

Result- The amount of BSA in unknown is _____ µg.

Discussion- The blue colour is developed by the reduction of the phosphomolybdic- phosphotungastic components in the Folin- Ciocalteau reagent due to the presence of amino acids. Tyrosine and Tryptophan are present in the protein also the colour developed by this reaction of the protein with the alkaline cupric tartrate are measured in Lowry's method.

3.6 PRECAUTIONS

The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9 to 10.5 is essential.

Advantages:

The method is sensitive down to about 10 µg/ml and is probably the most widely used protein assay despite of being only a relative method, subject to interference from Tris buffer, EDTA, nonionic and cationic detergents, carbohydrate, lipids, sulphydryl reagents and some salts.

Disadvantages:

i. The major disadvantage of the Lowry method is the narrow pH range within which it is accurate. However, we will use very small volume of sample, which will have little or no effect on pH of the reaction mixture.
ii. A variety of compounds will interfere with the Lowry procedure.

Ammonium ions, zwitter ionic buffers, nonionic buffers and thiol compounds may also interfere with the Lowry reaction. These substances should be removed or diluted before running Lowry assay.

SAQ

1. Distinguish between Biuret and Lowry's method.
2. Explain the principle of protein estimation using Lowry's method.
3. Enlist the reagents used in Lowry's method.
4. What is the absorbance wavelength used in Lowry's method?