Lab (6-7): Detection and quantitative estimation of proteins by different methods

Aim:

- To detect the presence of a protein or peptides using biuret test.
- To determine the concentration of extracted protein by different methods.

Introduction:

The quantitation of protein content is important and has many applications in clinical laboratory practices and in research especially in the field of biochemistry. The accurate quantitation of protein content is a critical step in protein analysis. (1) Protein quantitation is often necessary before processing protein samples for isolation, separation and analysis by chromatographic, electrophoretic and immunochemical techniques. Depending on the accuracy required and the amount and purity of the protein available, different methods are appropriate for determining protein concentration. (2)

The simplest and most direct assay method for proteins in solution is to measure the absorbance at 280 nm (UV range). Instead, several colorimetric and fluorescent, reagent-based protein assay techniques have been developed that are used by nearly every laboratory involved in protein research. Protein is added to the reagent, producing a color change or increased fluorescence in proportion to the amount added. The most commonly used techniques involve biuret test, Bradford test, bicinchoninic acid assay (BCA assay) and Lowry test. (2)

No one reagent can be considered to be the ideal or best protein assay method. Each method has its advantages and disadvantages (Table 1). The choice among available protein assays is usually based on the compatibility of the protein assay method with the samples. Additionally, one must consider potential interfering substances included in samples that may affect certain assay methods, as well as the accuracy, reproducibility and incubation time desired. Therefore, successful use of protein assays involves selecting the method that is most compatible with the samples to be analysed, choosing an appropriate assay standard, and understanding and controlling the particular assumptions and limitations that remain. The objective is to select a method that requires the least manipulation or pre-treatment of the samples to accommodate substances that interfere with the assay. (2)

There are different important criteria for choosing an assay including compatibility with the sample type and components, assay range and required sample volume, protein-to-protein uniformity, speed and convenience for the number of samples to be tested, availability of spectrophotometer or plate reader necessary to measure the color produced (absorbance) by the assay. (2)

Table 1. Comparison of various methods used for total protein concentration determination.

Method	Sensitivity	Time	Reagent	Interferences	Disadvantages and comments
Biuret	Low 1-20 mg	Moderate 20-30min	Alkaline copper sulphate	Zwitterionic buffers, Some amino acids	Similar color with all proteins. Destructive to protein samples.
Lowry	High ~ 5 µg	Slow 40-60min	Cu^{+2} Folin–Ciocalteau	Ammonium sulphate, glycine, Zwitterionic, buffers, Mercaptans	Time-consuming. Color varies with proteins. Destructive to protein samples.
Bradford	High ~ 1 µg	Rapid 15 min	Coomassie Brilliant Blue G-250	Strongly basic Buffers, detergents Triton X-100, SDS	Stable color, which varies with proteins. Reagent commercially available. Destruction to protein samples. Discoloration of glassware.
BCA	High ~ 1 µg	Slow 60 min	Cu^{2+}, bicinchoninic acid	EDTA, DTT, Ammonium sulphate	Compatible with detergents. Reagents commercially available. Destructive to Protein samples.
Spectroph -otometric (A_{280})	Moderate 50-100 µg	Rapid	-	Purines, pyrimidines, Nucleic acids	Useful for monitoring column eluents. Nucleic acid absorption can be corrected. None-destructive to protein samples. Varies with proteins.

Protein concentration is determined by reference to a standard curve consisting of known concentrations of a purified reference protein. Because proteins differ in their amino acid compositions, each one responds somewhat differently in each type of protein assay. Therefore, the best choice for a reference standard is a purified, known concentration of the most abundant protein in the samples. This is usually not possible to achieve, and it is seldom convenient or necessary. In many cases, the goal is merely to estimate the total protein concentration, and slight protein-to-protein variability is acceptable. Generally, bovine serum albumin (BSA) works well for a protein standard because it is widely available in high purity and relatively inexpensive. [(2)]

For greatest accuracy in estimating total protein concentration in unknown samples, it is essential to include a standard curve each time the assay is performed. Typically, standard curves are constructed using at least two replicates for each point on the curve.[(2)]

Experiment (1). Qualitative detection of proteins by biuret test:

Principle:

In this reaction, peptide bonds in the proteins and peptides treated with an alkaline solution of dilute copper sulphate $CuSO_4$ (biuret reagent) forming a purple coloured complex (Figure.1). The colour density is proportional to the amount of proteins present. This test is specific for the peptide bond, positive result (turning from blue to purple colour) will be given if the substance has two or more peptide bonds (three or more amino acids). Despite its name, the reagent does not in fact contain biuret ($(H_2N\text{-}CO\text{-})_2NH$). The test is named so because it also gives a positive reaction to the peptide-like bonds in the biuret molecule. [3]

Materials:

Chemical

Prepared crude extract, biuret reagent, distal water.

Preparation of solutions

1) Biuret reagent

Dissolve 3 g of $CuSo_4.5H_2O$ and 12 g of sodium potassium tartarate in 1000 ml dis. H_2O. Then add 600 ml of 2.5 M NaOH and complete the volume to 2 L by adding dis. H_2O. Store the reagent in dark bottle to protect from light.

Equipment and Glassware

Test tubes, test tube rack, pipette, pipette pump

Protocol:

1. Label three test tubes as **A** and **B**
2. **In tube A:** add 1 ml of animal crude extract.
3. **In tube B:** add 1 ml of water.
4. Add 1 ml of biuret reagent to all tubes and mix well.

Results:

Tube	Observation
Animal crude extract	
Water	

Experiment (2). Quantitative estimation of proteins by Lowry assay:

Principle:

In the past this has been the most commonly used method for determining protein concentration, although it is tending to be replaced by the more sensitive methods described below. The Lowry method is reasonably sensitive, and the sensitivity is moderately constant from one protein to another. When the Folin reagent (a mixture of sodium tungstate, molybdate and phosphate), together with a copper sulphate solution, is mixed with a protein solution, a blue-purple colour is produced which can be quantified spectrophotometrically. The method is based on two chemical reactions: the first reaction is based on Biuret reaction, which is the reduction of copper ions under alkaline conditions to form a complex with peptide bonds. The second reaction is the reduction of Folin-Ciocalteu reagent by the copper-peptide bond complex, which subsequently causes a color change of the solution into blue with an absorption in the range of 650 to 750 nm detectable with a spectrophotometer. The resultant strong blue colour is partly dependent on the tyrosine and tryptophan content of the protein sample. (4)

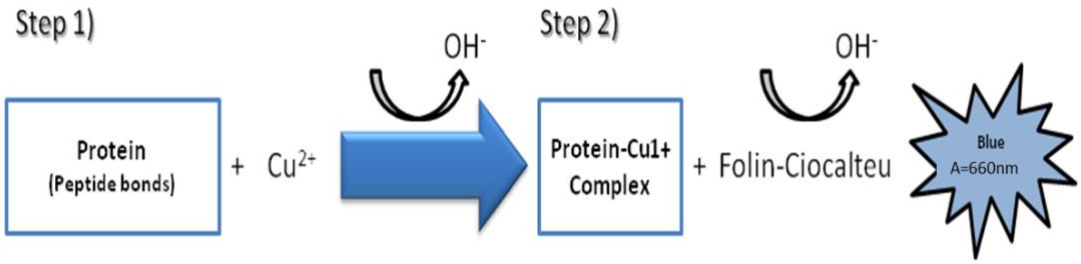

Figure 1. Series of reaction on Lowry method.(5)

Materials:

Chemical

Prepared crude extract, 40% pellet, dialyzed sample, BSA standard solution (100 mg/dl), Folin-Ciocalteu reagent, Reagent Am reagent B, reagent C, distal water.

Equipment and Glassware

Test tubes, test tube rack, pipette, pipette pump, plastic cuvettes, spectrophotometer.

Preparation of solutions

2) Reagent A:

Dissolve 2 g Na_2CO_3 (2%), 0.4 g NaOH (0.4%), 0.16 g sodium, potassium tartarate (0.16%), 1g SDS (1%) in water and make up to 100 ml. Store it at room temperature.

3) Reagent B:

4% $CuSO_4.5H_2O$, Dissolve 0.4g $CuSO_4.5H_2O$ in a little volume of water and make up to 10 ml. Store at room temperature.

4) Reagent C:

100 parts of reagent A + 1 part reagent B. Take 100 ml reagent A and add 1ml reagent B.

5) Folin-Ciocalteu reagent:

Dilute commercial reagent by 1: with water. Prepare fresh.

Protocol:

1. Set up 7 tubes as follows:

Tube	Water (ml)	Bovine serum albumin (BSA) Standard solution (100 mg/dl) (ml)	Sample [unknown concentration] (ml)
Blank	1	-	-
A	0.8	0.2	-
B	0.6	0.4	-
C	0.4	0.6	-
D	0.2	0.8	-
E	-	1.0	-
Animal crude extract (D1)	-	-	1.0
Animal crude extract (D2)	0.5	-	0.5
Plant crude extract (D1)	-	-	1
Plant crude extract (D2)	1.8	-	0.2
40% pellet	-	-	1
Dialysed sample	-	-	1

2. Add 3 ml of reagent C (include copper and alkaline reagent) for all tubes.
3. Mix and let the tubes stand at room temperature for **15 min**.
4. Add 0.3 ml of Folin-Ciocalteu reagent. Mix well after each addition.
5. Let the tubes stand at room temperature for **45 min**.
6. Read absorbance at **660 nm** against the blank.
7. Calculate the protein concentration for each standard solution using $C_1 \times V_1 = C_2 \times V_2$ formula.
8. Plot standard curve for absorbance at 660 nm against protein concentration (mg/dl) using results for solutions (A-E).
9. From the standard curve, estimate the concentration of the protein present in your crude extracts.

Results:

Test tube	Protein concentration (mg/dL) [X- axis]	Absorbance at 660 nm [Y- axis]
Blank		
A		
B		
C		
D		
E		
Animal crude extract (D1)	_____	
Animal crude extract (D2)	_____	
Plant crude extract (D1)	_____	
Plant crude extract (D2)	_____	
40% pellet	_____	
Dialysed sample	_____	

Experiment (3). Quantitative estimation of proteins by biuret assay:

Principle:

Biuret method is based on copper ions Cu^{2+} binding to peptide bonds of protein under alkaline condition to give a violet colour that have a maximum absorbance at 540 nm. The intensity of the color, and hence the absorption at 540 nm, is directly proportional to the protein concentration, according to the Beer–Lambert law (Figure 2). [6]

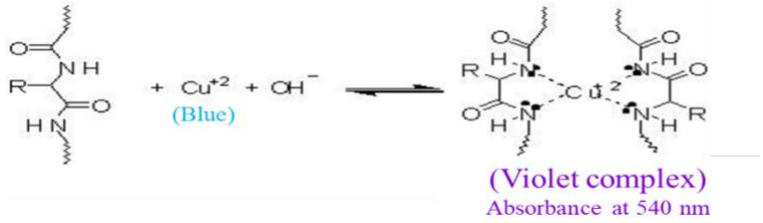

Figure 2. The formation of biuret complex in biuret reaction.[7]

Materials:

Chemical

Prepared crude extract, 40% pellet, dialyzed sample, BSA standard solution (5 g/l), biuret reagent, distal water.

Equipment and Glassware

Test tubes, test tube rack, pipette, pipette pump, plastic cuvettes, spectrophotometer.

Protocol:

1. Set up 12 tubes as follows:

Tube	Water (ml)	Bovine serum albumin (BSA) Standard solution (5 g/L) (ml)	Sample [unknown concentration] (ml)	Biuret reagent
Blank	2	-	-	3 ml
A	1.6	0.4	-	
B	1.2	0.8	-	
C	1	1	-	
D	0.8	1.2	-	
E	0.6	1.4	-	
F	0.4	1.6	-	
G	-	2	-	
Animal crude extract (D1)	-	-	2	
Animal crude extract (D2)	1	-	1	
Plant crude extract (D1)	-	-	2	
Plant crude extract (D2)	1.8	-	0.2	
40% pellet	-	-	2	
Dialysed sample	-	-	2	

2. Let the tubes stand at room temperature for **10 min**.
3. Read absorbance at **540 nm** against the blank.
4. Determine the protein contents from BSA standard curve.

Results:

Test tube	Protein concentration (g/L) [X- axis]	Absorbance at 540 nm [Y- axis]
Blank		
A		
B		
C		
D		
E		
F		
G		
Animal crude extract (D1)	_____	
Animal crude extract (D2)	_____	
Plant crude extract (D1)	_____	
Plant crude extract (D2)	_____	
40% pellet	_____	
Dialyzed sample	_____	

Experiment (4). Quantitative estimation of proteins by Bradford test:

Principle:

The Bradford reagent consists of the dye Brilliant Blue G in phosphoric acid and methanol or ethanol. This method relies on forming a complex by the binding of the dye Coomassie Brilliant Blue G-250 to the proteins resulting in a shift in the absorption maximum of the dye from 465 to 595 nm. The absorption at 595 nm is proportional to the amount of protein present in the sample. The dye reagent reacts primarily with arginine residues and less so with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. The practical advantages of the method are that the reagent is simple to prepare and that the colour develops rapidly and is stable (Figure 2). [4,8]

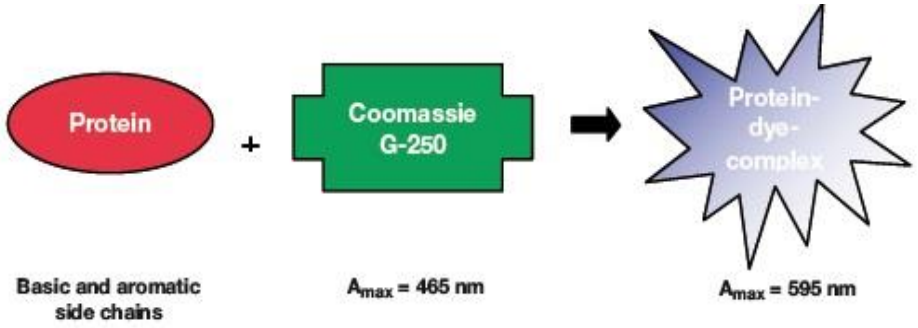

Figure 3. Protein estimation principle using the Bradford method.[9]

Materials:

Chemical

Prepared crude extract, 40% pellet, dialyzed sample, BSA standard solution (2 g/l), Bradford reagent, distal water.

Preparation of solutions

1) Bradford reagent

Dissolve 100 mg of Coomassie brilliant-G250 in 50 ml of 95% ethanol, add 100 ml of 85% w/v phosphoric acid and then complete the volume to 1 L by adding by adding dis. H_2O. Store the reagent in dark bottle to protect from light. After the dye has completely dissolved, filter through Whatman#1 filter paper just before use. Filtration may have to be repeated to get rid of all blue components. Keep in dark bottle.

Equipment and Glassware

Micropipette, tips, plastic cuvettes, spectrophotometer.

Protocol:

1. In plastic cuvettes ad the following:

	BSA standard solution (150 µg/ml) volume (µl)	Distal water (µl)	Sample (µl)	Bradford reagent (µl)
Blank	**-**	**100**	**-**	**1000**
A	**5**	**95**	**-**	
B	**10**	**90**	**-**	
C	**20**	**80**	**-**	
D	**40**	**60**	**-**	
E	**60**	**40**	**-**	
F	**80**	**20**	**-**	
G	**100**	**-**	**-**	
Animal crude extract (D1)	**-**	**90**	**10**	
Animal crude extract (D2)	**-**	**80**	**20**	
Plant crude extract (D1)	**-**	**90**	**10**	
Plant crude extract (D2)	**-**	**80**	**20**	
40% pellet	**-**	**80**	**20**	
Dialyzed sample	**-**	**80**	**20**	

2. Mix the content of each tube.
3. Incubate for **15 min** at room temperature.
4. Read the absorbance at **595 nm** against blank.
5. Determine the protein contents from BSA standard curve.

Results:

Test tube	Protein concentration (µg/ml) [X- axis]	Absorbance at 595 nm [Y- axis]
Blank		
A		
B		
C		
D		
E		
F		
G		
Animal crude extract (D1)	_____	
Animal crude extract (D2)	_____	
Plant crude extract (D1)	_____	
Plant crude extract (D2)	_____	
40% pellet	_____	
Dialyzed sample	_____	

References:

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