Lab (6): 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 (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]

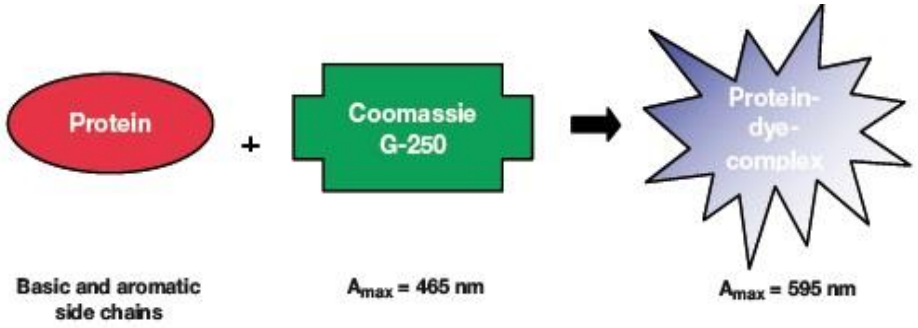

Figure 3. Protein estimation principle using the Bradford method.[9]

Materials:

Chemical

Prepared crude extract, 40% pellet, dialyzed sample, BSA standard solution (5 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 (5000 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**	**-**	**-**	
Unknown	**-**	**-**	**100**	

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		
Unknown	_____	

References:

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