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

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Protein quantification

- 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.

- **Protein quantitation** is often necessary before processing protein samples for *isolation*, *separation* and *analysis* by different techniques.

- Depending on the accuracy required and the amount and purity of the protein available, different methods are appropriate for determining protein concentration.

Different methods of protein quantification

Methods:

1. **Direct assay:** measure the absorbance at 280 nm (The simplest and most direct assay method), ***Why?***

2. **Colorimetric/fluorescent and reagent-based protein assay:** Protein is added to the reagent, producing a color change or increased fluorescence in proportion to the amount added.

The most commonly used reagent-based techniques involve:

- **Biuret test.**
- **Bradford test.**
- **Bicinchoninic acid assay (BCA assay).**
- **Lowry test.**

Choosing the compatible method

- No one reagent can be considered to be the ideal or best protein assay method.
- Each method has its advantages and disadvantages. *(Table 1)*

How to choose the appropriate method:

- Compatibility of the protein assay method with the sample → *Interfering substances*
- Availability
- Accuracy
- Sensitivity
- Incubation time desired

→ 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.

Choosing the compatible method

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 TritonX-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.

Determination of protein concentration

- 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.

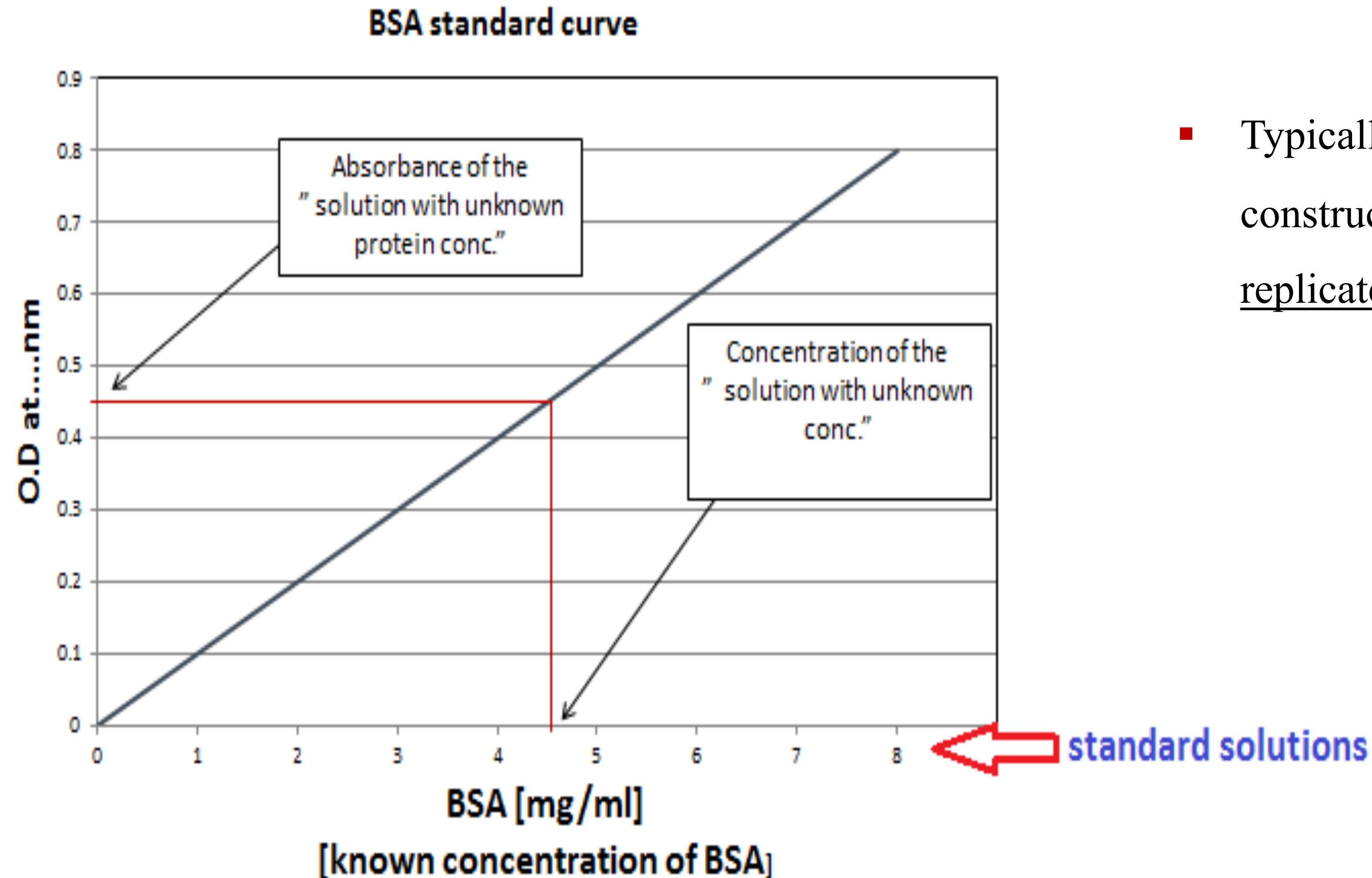
How to choose a reference standard? → purified, known concentration of the most abundant protein in the samples

→ 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.

→ Bovine serum albumin (BSA) works well for a protein standard → 1- widely available in high purity 2- relatively inexpensive.

Determination of unknown concentration by standard curve



- Typically, standard curves are constructed using at least two replicates for each point on the curve.

Practical part

Experiment 1. Qualitative detection of proteins by biuret test

Objective:

- To detect the presence of a protein or peptides using **biuret test.**

Principle:

- In this reaction, peptide bonds in the proteins and peptides treated with an alkaline solution of dilute copper sulphate CuSO4 (biuret reagent) forming a purple coloured complex.
- The colour density is proportional to the amount of proteins present.
- Two or more peptide bonds.
- 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.

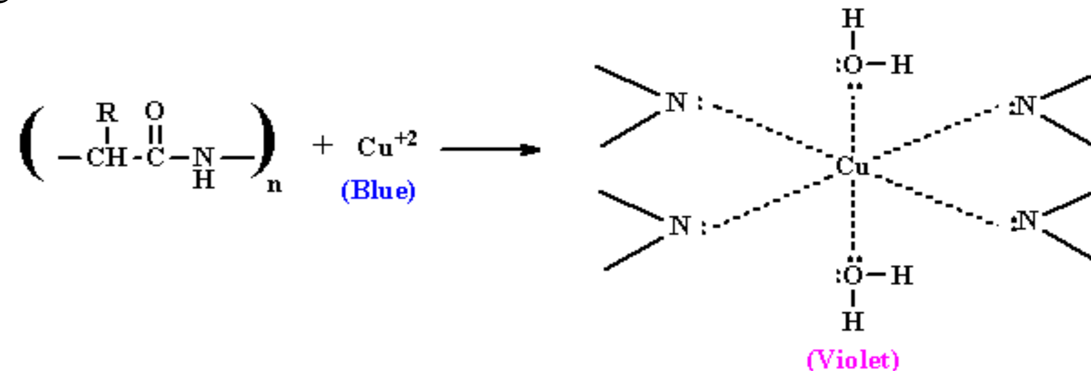


Figure 1. The formation of biuret complex in biuret reaction.

Experiment 1. Qualitative detection of proteins by Biuret test

Method:

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	

Blue color is the Biuret reagent color

Experiment 2. Quantitative estimation of proteins by Lowry assay

Objective:

- To determine the concentration of extracted protein by **Lowry assay.**

Principle:

- Replaced by the more sensitive methods.
- The sensitivity is moderately constant from one protein to another.
- The method is based on two chemical reactions: 1st reaction is based on Biuret reaction. 2nd reaction is the reduction of Folin-Ciocalteu reagent (sodium tungstate, molybdate and phosphate) by the copper-peptide bond complex, → a color change of the solution into blue *(650 to 750nm)*.
- The resultant strong blue colour is partly dependent on the tyrosine and tryptophan content of the protein sample.

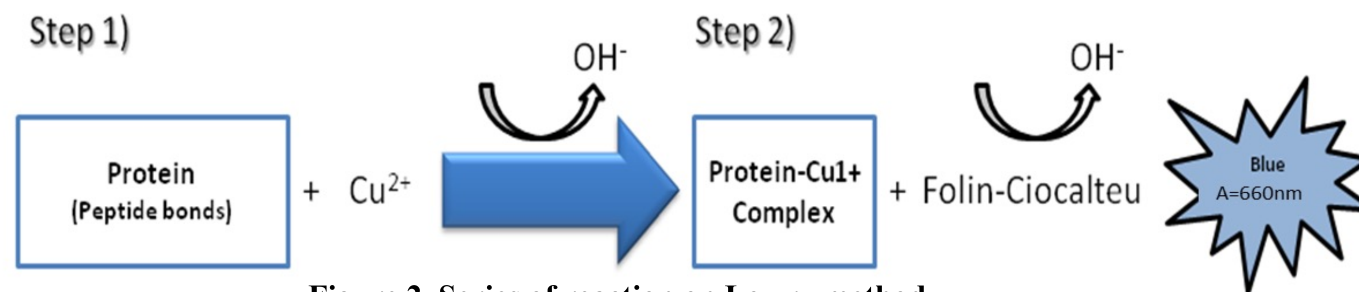


Figure 2. Series of reaction on Lowry method.

Experiment 3. Quantitative estimation of proteins by Biuret assay

Objective:

- To determine the concentration of extracted protein by **biuret assay.**

Principle:

- Biuret method is based on copper ions Cu2+ 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.

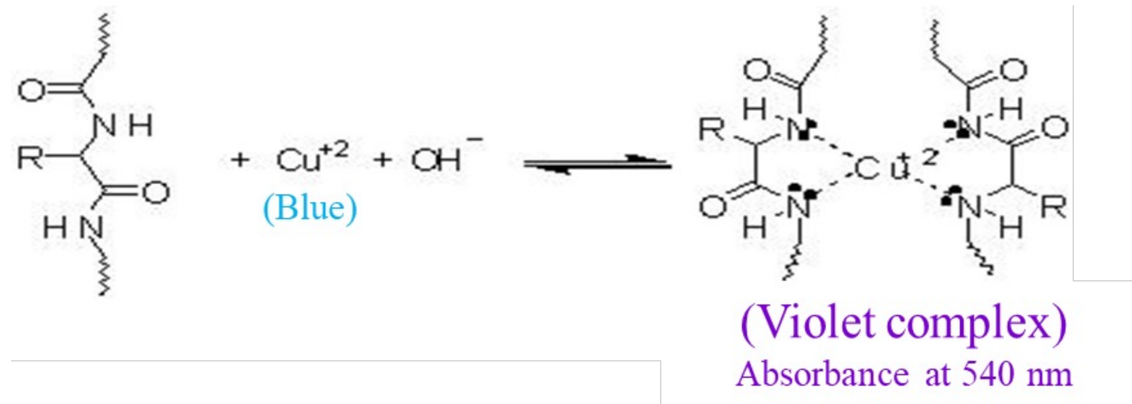


Figure 3. The formation of biuret complex in biuret reaction

From lower to higher concentration

There is a linear relationship between purple color developed and concentration.

Experiment 3. Quantitative estimation of proteins by Biuret assay

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)	____________________	

Table 1. Concentration of standard BSA solution and their absorbance at 540 nm.

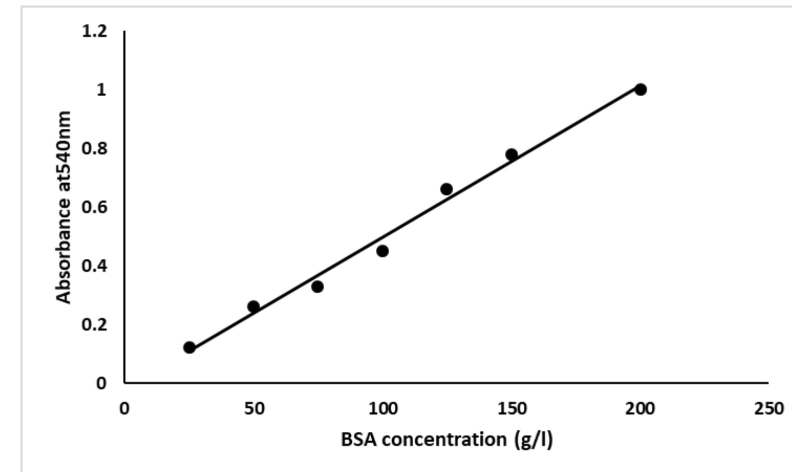


Figure 4. Standard curve of BSA using biuret method.

Homework

- A crucial step of many biomedical laboratory experiments is the quantitation of a specific protein. Several techniques have been employed to accomplish that. *Name 3 techniques.*