Swayam Course - Analytical Techniques

Week: 5, Module 13 - Protein estimation Techniques

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Objectives

To enumerate & state principles of the methods used for QUALITATIVE & QUANTITATIVE estimation of proteins.

- Qualitative tests- Heat test, Ninhydrin test
- Quantitaive tests-
- For total proteins
  - Near and Far UV absorption
  - Biuret reaction
  - Bradford assay
  - Lowry assay
  - BCA assay
- For specific or individual proteins
  - ELISA
  - Immuno PCR
  - Mass spectrometry
  - Protein quantification in single living cells
  - Nanoparticles and nanopore-based methods

1. Introduction

Proteins are the most abundant biomolecules, constituting more than 50% of the dry weight of the cell. They are known to perform many different biological functions. Qualitative and quantitative estimation of proteins in biological fluids, is based on the physico-chemical properties of proteins. Accurate protein quantitation is essential to protein studies in a multitude of research topics. A wide arary of different methods have been developed to quantitate both complex mixtures of proteins as well as a single type of protein.

2. Methods of protein estimation

2.1 Qualitative methods: include Heat test and Ninhydrin test.

2.2 Quantiative methods: comprise tests based on total protein estimation and individual or specific protein estimation.

Total protein quantitation methods comprise traditional methods such as the measurement of UV absorbance at 280 nm, Biuret, Bradford assays, Lowry assay and BCA assay.

Individual or specific protein quantitation methods include enzyme-linked immunosorbent assay (ELISA), Immuno PCR, mass spectrometry, Protein quantification in single living cells and Nanoparticles and nanopore-based methods.

2.1 Qualitative tests

2.1.1 Heat test

Based on principle of heat coagulation and precipitation of proteins

When the protein sample is heated, the proteins are denatured by heat due to increase in the kinetic energy of the molecules and by breakage of weak bonds like electrostatic interactions, hydrogen bonds and hydrophobic interactions. The denatured proteins becomes insoluble and precipitates out. Dilute acetic acid is added which provides an acidic medium and brings the pH close to isoelectric pH. At isoecectric pH the protein has minimum solubility and thus precipitates.

In urine analysis upon heating urine in an alkaline medium the phosphates also produce similar turbidity like proteins but the turbidity dissapears upon addition of acetic acid if the turbidity is due to phosphates but it persists if it is due to proteins.

2.1.2.Ninhydrin reaction: It is one of the most important reactions used for the qualitative detection of hydrolytic products of protein i.e. amino acids.

Principle: The amino acids are deaminated to CO_2, NH_3 and an aldehyde under heat. Ninhydrin reacts with NH_3 and forms a purple coloured complex, which can be measured at 570 nm. All amino acids, except proline, react with ninhydrin at ambient temperature, to form a blue colored complex, which intensifies to purple on heating. Proline forms a yellow coloured complex.

$$\text{Ninhydrin} + \text{Amino acid} \xrightarrow{CO_2 \quad NH_3} \text{Hydrindantin} + \text{Aldehyde}$$

$$Ninhydrin + hydrindantin + NH_3 \rightarrow purple\ colored\ complex$$

(Ruhemann's purple)

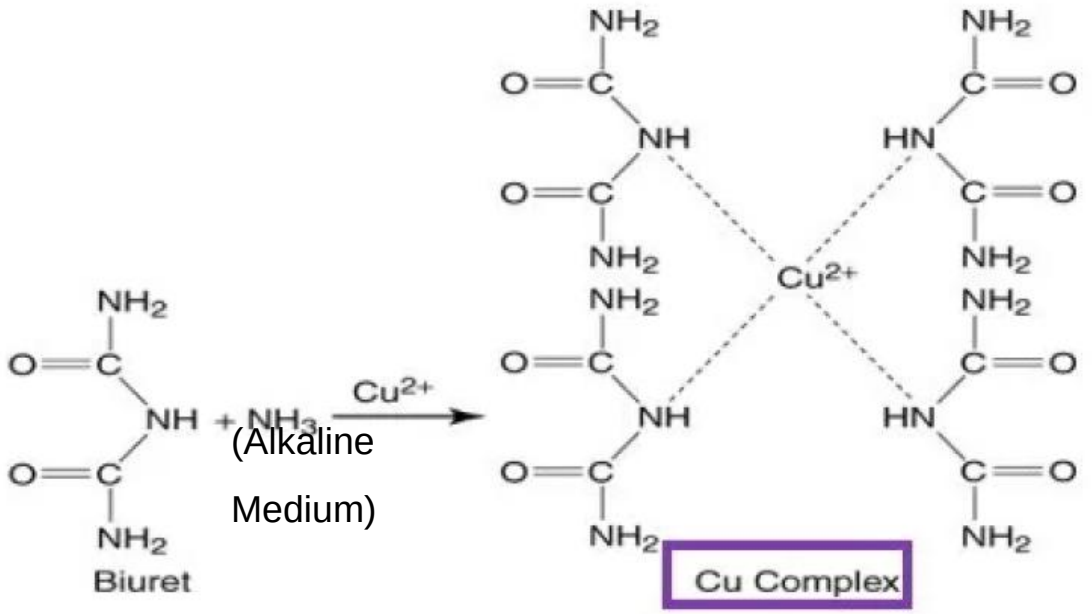
2.2 Quantitative tests:

2.2.1 Total protein quantitation methods

2.2.1.1 Ultraviolet (UV) absorbance at 280 nm (range: 0.1-100 ug/ml): Aromatic amino acids Tyrosine, Tryptophan and Phenylalanine give proteins their characteristic ultraviolet (UV) absorption at 280 nm, which is routinely used to estimate protein concentration. Peptide bonds also contribute to absorption, but at 190 nm i.e. far UV range. This method is simple, and can be performed with extremely small sample volume, as little as 0.5 µl. However, the protein sample must not contain any non-protein components with substantial absorption at 280 nm, such as nucleic acids contaminants. For pure protein samples, the exact amino-acid sequence of the analyzed protein must be known and an absorption coefficient specific to the particular protein must be calculated prior to determining the concentration of solution. This method is quickest, but error-prone and is incompatible with a broad array of protein extraction methods which frequently employ detergents and denaturing agents.

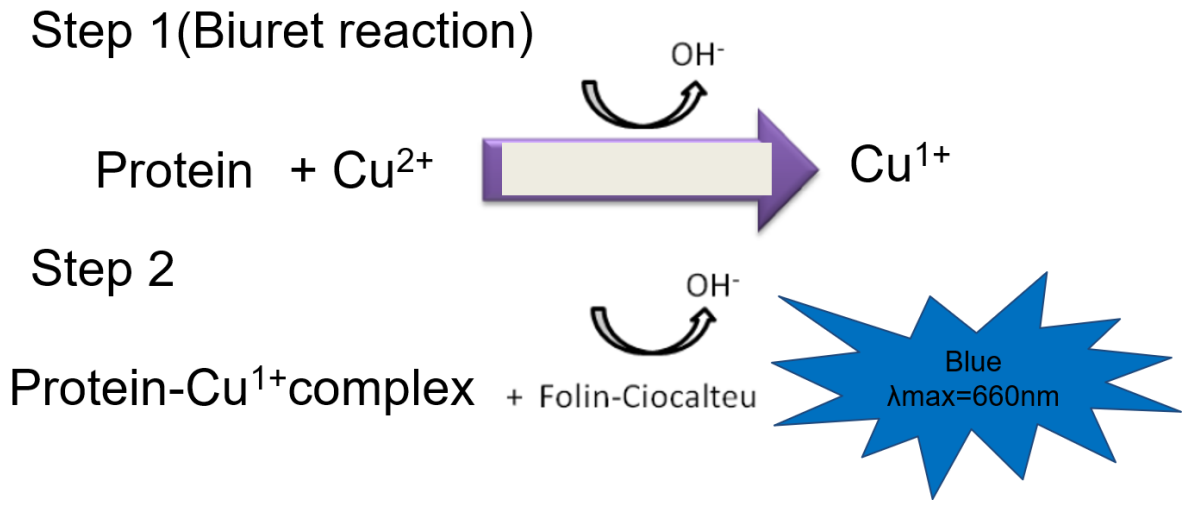
2.2.1.2 Biuret reaction: Peptide bonds react with Cu^{++} ions in alkaline solution, to form a purple colored chelate. Presence of at least two peptide bonds is essential for the reaction. The name of this reaction is derived from a similar reaction given by urea. When urea is heated to 180^{o}C, it forms biuret which in the presence of strong alkali, reacts with dilute solutions of copper sulphate, to form a violet coloured complex.

Absorption maximum of the colored complex is 540 nm. This method is absolute as it is based on reaction with peptide bonds. The main disadvantage is its lack of sensitivity. It cannot be used to estimate protein less than **1 mg/ml**. Amino acids and dipeptides do not give this reaction.

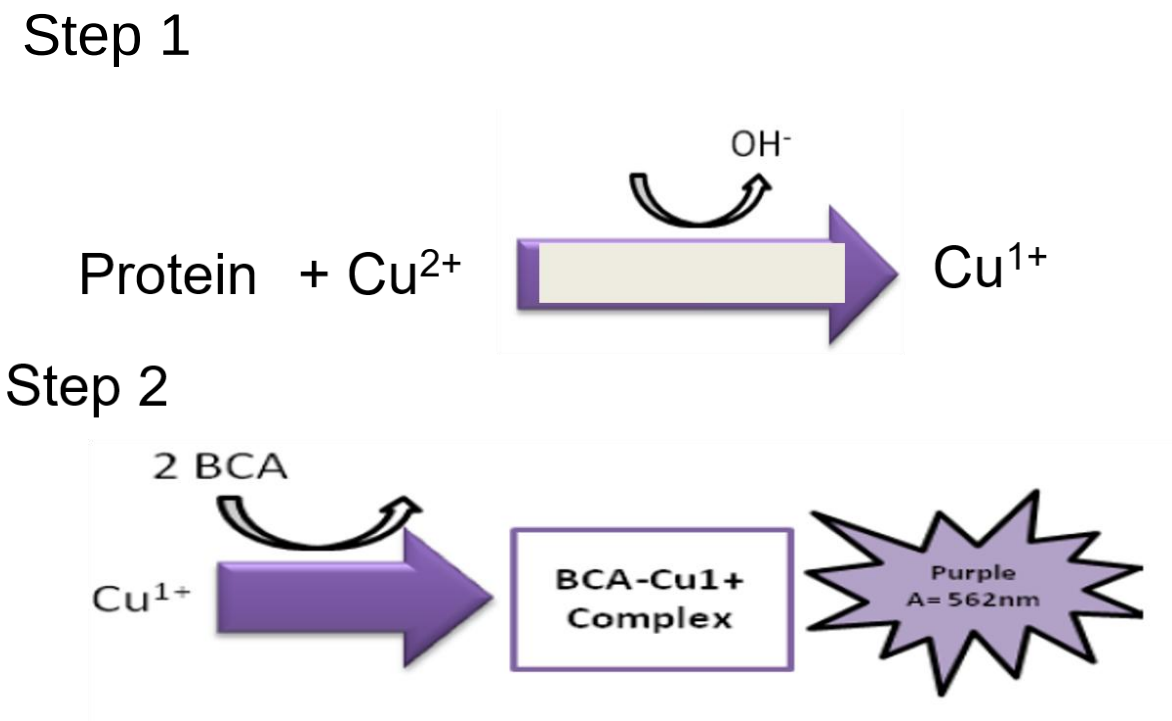


2.2.1.3 Bradford Method: Change in absorbance maximum of Coomassie Brilliant blue (from 465 nm to 595 nm) on binding to protein. Free dye binds with basic amino acids arginine and lysine in the protein. This method is simple and very sensitive. Colour development is rapid but not very stable. Sensitivity is **20 µg/ ml.**

2.2.1.4 Lowry's Method: This method involves the formation of a copper-protein complex in alkaline solution as in the Biuret reaction and the reduction of phosphotungstic and phosphomolybdic acids (Folin-Ciocalteu reagent) into tungsten blue and molybdenum blue respectively, by the copper catalysed oxidation of aromatic amino acids. λ_{max} is 660 nm. Sensitivity is **10 µg/ ml**.



2.2.1.5 BCA Method: Proteins reduce alkaline Cu (II) to Cu (I) in a concentration dependent manner. Bicinchoninic Acid **(BCA)** is a highly specific chromogenic reagent for Cu (I) forming a complex with an absorbance maximum at 562 nm. Because of this property, the resultant absorbance at 562 nm, is directly proportional to the protein concentration. Sensitivity is **0.5 µg/ ml**



2.2.2 Individual or specific protein quantitation methods:

A crucial step of many biomedical laboratory experiments is the quantitation of a specific protein in solution. Several techniques have been employed to accomplish that few of which are mentioned below.

2.2.2.1 Immunosorbent assays Enzyme-linked immunosorbent assays (ELISA): They are used to qualitatively and quantitatively analyze the presence or concentration of a particular soluble antigen such as a specific antibody, or peptide in liquid samples, such as biological fluids and cell culture supernatant. These assays make use of the ability of polystyrene plates to bind proteins, including antibodies, as well as the particular specificities of antibodies for target antigens. These assays incorporate a colorimetric endpoint that can be detected via absorbance wavelength and quantitated from a known standard curve of antigen or

antibody dilutions. There are 4 commonly used ELISA formats: direct, indirect, sandwich and competitive.

2.2.2.1.1 Direct ELISA: Direct ELISA use known quantities of monoclonal antibodies to determine the concentration of a particular antigen in a solution. For example, direct ELISA can determine the concentration of a specific cytokine secreted by a cell population after stimulation or inhibition by measuring the amount of that cytokine in the culture supernatant. First an antigen is immobilized in the well of an ELISA plate. The antigen is then detected by an antibody directly conjugated to an enzyme such as HRP.

2.2.2.1.2 Indirect ELISA: In indirect ELISA, antigen is adsorbed to a well in an ELISA plate. Detection is a two-step process. First, an unlabeled primary antibody binds to the specific antigen. Second, an enzyme conjugated secondary antibody that is directed against the host species of the primary antibody is applied.

2.2.2.1.3 Sandwich ELISA: In 'sandwich' ELISA method, the target antigen is sandwiched between two monoclonal antibodies that bind different epitopes on the same antigen. The 'capture' antibody is used to coat the wells of the assay plate. After washing and blocking, the solution to be measured is added to the wells to allow the specific antigen, if present, to be 'captured'. Once the excess is washed away, the 'detection' antibody, which is enzyme-linked, is added to the wells. Finally, a colorimetric substrate for the enzyme is added to the wells. Relative quantitation can be measured by simple color changes in the assay wells: the higher the color change, the higher the antigen concentration in the sample. Absolute quantitation can be performed with a standard curve that is generated from wells with known concentrations of the target antigen.

2.2.2.1.4 Competitive ELISA: Competitive ELISA is used to measure the concentration of an antigen or antibody in a sample by detecting interference in an expected signal output. Sample antigen or antibody competes with a reference for binding to a limited amount of labeled antibody or antigen, respectively. The higher the sample antigen concentration, the weaker the output signal, indicating that the signal output inversely correlates with the amount of antigen in the sample. For example, to detect an antigen, first a known antigen is used to coat a multiwell plate. Following standard blocking and washing steps,

samples containing unknown antigen are added. Labeled detection antibody is then applied for detection using relevant substrates (e.g. 3,3',5,5'-Tetramethylbenzidine or TMB). If there is a high concentration of antigen in the sample, a significant reduction in signal output will be observed. In contrast, if there is very little antigen in the sample, there will be very little reduction in the expected signal output.

2.2.2.2 Immuno PCR (IPCR): Immuno PCR is a technique that combines the sensitivity of the nucleic acid amplification by PCR with the specificity of the antibody based assays resulting in an increase of the detection sensitivity. Typically, it is possible to obtain a 100–10,000-fold increase over the detection limit of the ELISA in several applications. I-PCR was first described by Sano et al. in 1992. The combination of quantitative PCR with I-PCR (qI-PCR) allowed the quantification of low-abundant biomarkers in complex biological samples that are difficult to detect by classical immunoassays. A capture antibody is immobilized on the surface of a microtiter plate and used to bind the target analyte. A second antibody, which has been coupled to an oligonucleotide, is then bound. The DNA is amplified and detected via real time PCR (Figure 1).

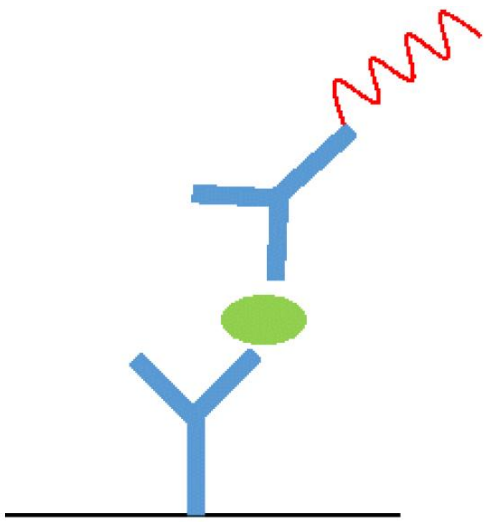


Figure 1. Schematic representation of Immuno PCR

2.2.2.3 Protein mass spectrometry Protein mass spectrometry is a relatively new and developing method for protein quantitation. Besides protein characterization, an important step in proteomic analysis is the possibility to quantify a specific protein.

Many techniques have been introduced and implemented for protein quantitation by mass spectrometry. When protein labeling is possible, one protein or peptide sample is labeled with a stable heavier isotopes (e.g., 13C or 15N) while a second sample (internal standard) is labeled with a lighter isotope (e.g., 12C or 14N). The samples are mixed and the mass differences due to the labels make it is possible to analyze the ratio of the two sample peak intensities by a mass analyzer, which corresponds to their relative abundance ratio. Alternative methods allow protein quantitation by mass spectrometry without labeling the samples.

2.2.2.4 Protein quantification in single living cell: Protein expression can be monitored in living cells by genetically linking a protein of interest to a reporter protein or epitope, such as green fluorescent protein (GFP). Linking the protein of interest to the reporter with a particular cisacting hydrolase element called a "Protein Quantitation Reporter" (PQR) allows the protein and reporter to be transcribed together but translated as separate functional proteins. This can ensure stoichiometric expression of both the protein and the reporter; thus, the protein quantity can be inferred from the fluorescent intensity (Figure 2).

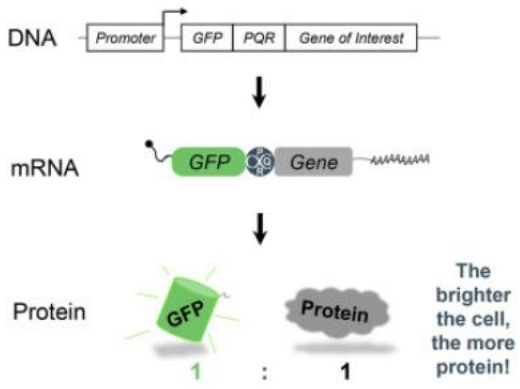


Figure 2. Schematic representation of Protein quantitation in single living cell

2.2.2.5 Nanoparticles and nanopore-based methods This new strategy for determination of protein concentrations in simple and complex solutions takes advantage of the optical properties of nanoparticles. The wavelength at which certain nanoparticles absorb light shifts due to binding other molecules or to aggregation. Because the light absorption range is in the visible spectrum, this shift is perceived

as a change in color. Nanoparticles have been so far used in conjunction to protein-binding particles, for example, antibodies, peptides or aptamers. Rogowski et al, proposed a new type of nanoparticle sensor for the detection and quantification of proteins, which they called a “chemical nose”. Their sensor consists of a mixture of gold nanoparticles with different shapes displaying differential aggregation when interacting with different proteins. Based on their light absorption spectra, the particles allow the detection and quantitation of purified proteins in aqueous solutions or unpurified proteins in complex solutions (Figure 3).

Kong et al developed a method using long DNA carrier molecules and solid-state glass nanopores to measure the concentration of proteins in solution in the nanomolar range. In this method, DNA carrier molecules, able to bind proteins at specific locations, translocate through the nanopores with the help of an electric current. When translocating, the DNA causes a current drop signal. An additional drop is registered when a protein is attached to the DNA. The higher the protein concentration, the more “loaded” the carrier. The frequency of secondary current drop peaks also increases with increasing protein concentration (Figure 4).

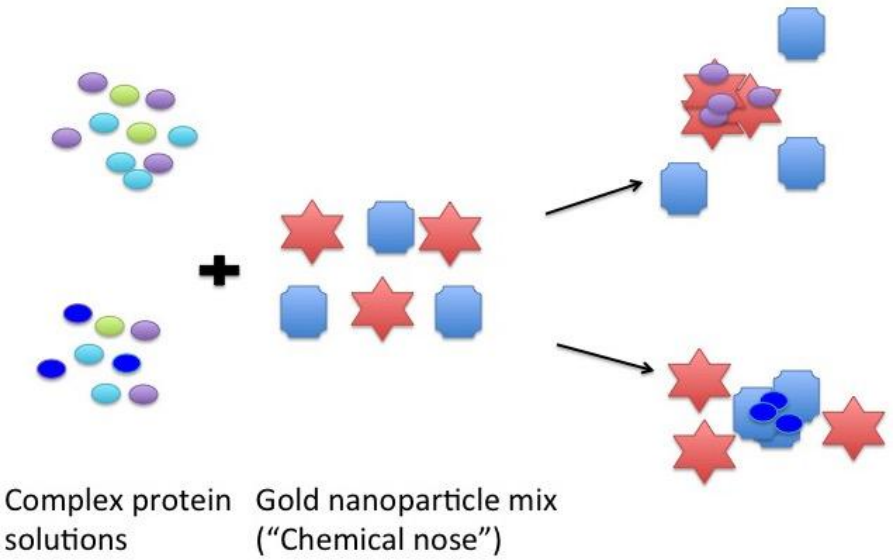


Figure 3. Schematic representation of the “chemical nose” technology

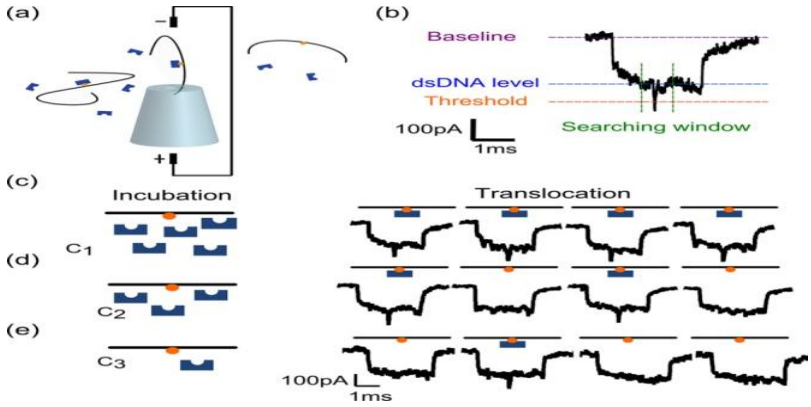


Figure 4. Schematic diagram of the DNA carrier molecules translocating through a nanopore: (a); Current drop peak caused by DNA translocation and secondary drop indicating “loaded” DNA (b); DNA-translocation events at decreasing protein concentration (c, d and e).

3. Summary

- Proteins are most abundant biomolecules
- Methods of protein estimation- qualitative and quantitative
- Qualitative- Heat test, ninhydrin test
- Quantitative
  - For total proteins- Near and Far UV absorption, Biuret reaction, Bradford assay, BCA assay, Lowry assay.
  - For specific or individual proteins- ELISA, Immuno PCR, Mass Spectrometry, Protein quantitation in single living cell, Nanoparticles and nanopore-based methods.