ejpmr, 2024, 11(7), 171-177

SJIF Impact Factor 7.065

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Review Article

ISSN 2394-3211

EJPMR

A COMPREHENSIVE REVIEW ON THE ANALYSIS OF PROTEINS

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Article Received on 30/04/2024 **Article Revised on 20/05/2024** **Article Accepted on 10/06/2024**

ABSTRACT

Peptides and proteins are large biomolecules composed of long chains of L-amino acids and play key functions in living organisms. It is important to have standardized analytical techniques in order to assess the amount of protein in diet. There are several ways to determine the protein content of food products, including the Bradford, Kjeldahl, Lowry and total amino acid content methods. These biomolecules are characterized using a range of methods, one of which is high-performance liquid chromatography (HPLC), which has been utilized widely. The purpose of this review article is to describe the analytical methods used to determine the protein concentration with its advantages and disadvantages.

KEYWORDS: Protein, amino acid, Kjeldahl method, Dumas method, HPLC, Ion-exchange and Size exclusion chromatography.

1. INTRODUCTION

The food components in the diet that provide energy like proteins, carbohydrates, and fats play a significant role in the growth and maintenance of the body. The primary nutrients found in food include vitamins, minerals, proteins, fats, and carbohydrates. Food also includes water and dietary fibers. Carbohydrates and fats mainly provide energy to our body. Minerals and proteins are essential for the development and function of our bodies. The body uses proteins for a vast array of additional purposes, including enzymatic activity and the passage of nutrients and other biochemical substances across cellular membranes.[1] Good quality proteins must be consumed by the body through diet in order to maintain these vital processes. Muscle proteins are broken down more quickly when there is insufficient consumption of dietary proteins that contain critical amino acids. This stunts muscle growth and causes debility.[2] Proteins are polypeptide structures made up of one or more extended chains of residues from amino acids. They perform a vast range of biological tasks, such as transporting materials, initiating metabolic reactions, replicating DNA, and giving cells structural support. A chain of amino acids makes up the macromolecules known as proteins. There are approximately 20 different types of amino acids that vary in size, polarity, shape, hydrophobicity and chemical reactivity. As a result, each protein has a unique molecular structure with a unique amino acid sequence. Determining the concentration of proteins in a solution is a crucial part of the analysis and has numerous uses in different clinical and research labs.[3]

The nutritional quality of protein in a food product can be characterized by several factors, such as (i) providing enough protein to support optimal growth, (ii) maintaining an amino acid balance, (iii) the degree of protein digestion and absorption, or (iv) indispensable amino acids relative to amino acid requirements.[4] Although protein is known to be the nutritional component that can halt and even reverse the loss of muscle mass and strength, the majority of human dietary intervention studies on the health of the muscles that have been conducted to date have focussed on protein derived from animals.[5]

Standardized analytical techniques are essential for estimating the amount of protein in food. There are a number of techniques used in the food industry to determine the amount of protein in food, including the Bradford, Kjeldahl, Lowry, and total amino acid content techniques.[6]

Importance of Analysis

Protein analysis is important for

1. Determination of biological activity: Proteins are crucial to nutrition and food science. Proteolytic enzymes, which tenderize meats, pectinases, which ripen fruits, and trypsin inhibitors, which are present in legume seeds, are a few examples of these.

2. Examining functional properties: Different proteins have certain purposes in food. For example, gliadin and glutenin in wheat flour are used to build bread, and

casein in milk coagulates to form cheese products and froth in egg albumen.[7]

Classification of Proteins

Classification of proteins is done on the basis of the following

- Shape
- Constitution
- Nature of molecules.

Based on shape

• **Scleroproteins, or fibrous proteins:** Are found in mammals and are not soluble in water. Collagen, actin, myosin, and keratin in hair, claws, feathers, and other tissues are examples of fibrous proteins. These proteins are coiled, resistant to proteolytic enzymes, and exist in thread-like structures to create fibers.

• **Globular proteins:** These proteins dissolve in water, in contrast to fibrous proteins. They consist of polypeptides that coil around one another to produce round or oval molecules, such as insulin, albumin, and hormones like oxytocin.

Based on the Constitution

• **Simple proteins:** Only amino acids make up these proteins. such as prolamins, globulins, albumins, etc.

• **Conjugated proteins:** These are complicated proteins that have been endowed with the properties of an entity known as a non-amino acid. Also known as the prosthetic team. These fall into the following categories

a) Nucleoproteins: A mix of nucleic acid and protein
b) Mucoproteins: >4% combination of carbs and proteins
c) Glycoproteins: A less than 4% mixture of proteins and carbohydrates
d) Chromoproteins: A blend of colored pigments and proteins.
e) Lipoproteins: A lipid and protein combination.
f) Metalloproteins: Protein and metal ion combination.
g) Phosphoproteins: Phosphate group combined with proteins.

• **Derived proteins:** The breakdown products that result from the hydrolysis of proteins by acids, alkalies, or enzymes are referred to as derived proteins.

Based on the characteristics of molecules

• **Acidic proteins:** These include blood types and exist as anions with acidic amino acids.
• **Basic proteins:** They are rich in basic amino acids like arginine and lysine and exist as cations.[7]

Functions of Proteins

In every system in the human body, proteins serve vital purposes. These long chains of amino acids are essential for the following processes

- DNA synthesis and repair
- Catalyzing chemical reactions
- Material transportation throughout the cell
- Chemical signal reception and transmission
- Stimulus-response
- Structural support
- Repair and maintenance
- Hormones: One hormone that controls blood sugar levels is insulin, a little protein.[24,26]

2. Methods for determination of Protein concentration

A broad range of techniques have been designed for quantifying protein content. The basic concepts of these techniques include figuring out nitrogen, peptide bonds, aromatic acids, proteins UV absorptivity, free amino groups, light scattering characteristics, and dye binding ability.

a) Kjeldahl Method

The Kjeldahl method was developed in 1883 by Johann Kjeldahl to evaluate organic nitrogen. Sulfuric acid is used in the Kjeldahl technique to break down proteins and other organic food ingredients in a sample in the presence of a catalyst. Ammonium sulfate is produced by converting all of the organic nitrogen. After the digest is neutralized with an alkali, it is distilled into a solution of boric acid. Standardized acids are used to titrate the generated borate anions, which in the sample are transformed into nitrigen. Since nitrogen also originates from non-protein components, the analysis result indicates the food's crude protein composition.

Principles

A) Digestion

Complete oxidation and conversion of nitrogen to ammonium sulfate is achieved through digestion with sulfuric acid and the addition of powdered potassium permanganate.[7]

$$\text{Protein} \xrightarrow[\text{Heat, catalyst}]{\text{Sulfuric acid}} (NH_4)_2SO_4$$

B) Neutralization

The diluted digest is neutralized, and then it undergoes distillation into a known volume of standard acid that has iodate and potassium iodide.[7]

C) Titration

Liberated iodine is titrated with standard sodium thiosulfate.[7]

Advantages

- Applicable to all varieties of foods
- comparatively easy to use and affordable
- Micro Kjeldahl method has been adapted to measure microgram amounts of proteins; it is still accurate and the approved method for crude protein content.

Disadvantages

- Calculates total organic nitrogen rather than simply nitrogen found in proteins.

- Needs at least two hours to finish
- Less accurate than the biuret technique
- Reactive corrosive agent.[7]

b) Methods using UV-Visible spectroscopy

Many techniques based on UV-Visible spectroscopy have been developed to quantify protein concentration.

Near UV Absorbance

UV radiation can be absorbed and used to detect a wide variety of bioanalytes. Protein, including those found in tissues, absorbs UV light strongly because tryptophan, tyrosine, phenylalanine, and other amino acid side chains contain aromatic rings. Tyrosine and tryptophan absorb UV light more readily than phenyl alanine does, and tryptophan is the only amino acid that absorbs UV light at a maximal wavelength of 275–280 nm. However, when phenyl alanine and disulfide connections are formed between two cysteine residues, an absorbance of 260 nm is seen. By using this method, one can simply calculate the protein content by determining the measured sample solutions absorbance at 280 nm and its molar absorptive capacity.[8]

$$A = abc$$

Where: A = absorbance
a = absorptivity
b = cell or cuvette path length
c = concentration.

Advantages

- UV absorption spectroscopy is comparatively sensitive and can detect protein concentrations as low as 10 μgcm^3.
- Protein samples can be recovered and used again, making it a non-destructive procedure.

Disadvantages

- The primary drawback is the existence of chromophores, such as nucleic acids, which also absorb UV light at 280 nm and interfere with protein concentration measurements.[8]

Far UV Absorbance

The concentration of proteins that do not absorb near UV radiation, their quantity can be determined using "far UV absorption," which accounts for the absence of aromatic amino acids and disulfide linkage. Peptide linkages with wavelengths between 190 and 220 nm are the primary chromophores in this spectral range, while aromatic residues and disulfide bonds are the primary chromophores in the near UV spectral region. However, oxygen absorbs UV light considerably at 190 nm. Moreover, deuterium arc sources produce less light at this wavelength than at others. Thus, the protein concentration can be measured to be less than $1\mu gcm^{-3}$, and it is convenient to detect the absorption at a wavelength of 205 nm in the far UV region. Working at these lower wavelengths is only challenging because of the absorbance of UV radiation of buffers and additional components.[9]

Most commonly used methods are listed below as follows

i. Biuret Method

The Biuret Protein test is among the easiest and most often used assays. The main principle behind this assay is that in an alkaline environment, cupric ions combine with nearby peptide bonds in proteins to generate a complex that is intensely purple in color.[10] When cupric ions combine with peptide bonds (substances that have two or more peptide bonds, such as big peptides, biuret, and all proteins) in an alkaline environment, a violet-purplish color is generated. At 540 nm, the absorbance is measured. The protein concentration is directly correlated with the color intensity (absorbance).[7]

Procedure

i. 1 ml of protein solution (1–10 mg protein/ml) is combined with 5 ml of biuret reagent. The reagent contains copper sulfate. NaOH and potassium sodium tartarate are utilized in the alkaline solution to stabilize the cupric ion.
ii. The absorbance is measured at 540 nm against a blank for the reagent after it has been left at room temperature for 15 or 30 minutes.
iii. If the reaction mixture is not clear, filtering or centrifuging must be done before reading absorbance.
iv. Bovine serum albumin (BSA) is used to create a standard concentration versus absorbance curve.[7]

Applications

The protein content of meat, grain, soybeans, and animals has all been measured using the biuret method. Another popular technique for determining the protein concentration of isolated proteins is the biuret method.

Advantages

- Cheaper than the Kjeldahl method; quick (takes less than 30 minutes to complete); most straightforward approach for protein analysis.
- Less commonly occurring color derivations compared to Lowry, UV absorption, or turbidimetric procedures.
- The biuret reaction is mostly unaffected by very few things besides the proteins found in the diet.
- It is unable to identify nitrogen from non-protein or non-peptide sources.

Disadvantages

- Compared to the Lowry method, this is less sensitive; it requires at least 2 to 4 mg of protein for the assay.
- If bile pigments are present, they may contribute to absorbance.
- High concentrations of ammonium salts can disrupt the reaction.

- varied proteins have varied colors; gelatin has a pinkish-purple color.
- If there are high amounts of fat or carbohydrates in the final solution, opalescence may occur.
- This is not an absolute method; color must be compared to standardized proteins (like BSA) or to the Kjeldahl nitrogen method.[7]

ii. Lowry Method

The Lowry assay, which is one of the most accurate methods for detecting protein content, is based on both the Folin-Ciocalteau reaction and the biuret reaction. It can determine the range of protein concentrations in a sample, from 0.01 to 1.0 mg/mL in sample.[11] Tyrosine and tryptophan residues in the proteins reduce the Folin-Ciocalteau phenol reagent (phosphomolybdic-phosphotungstic acid), which is used with the biuret reaction in the Lowry technique. At 750 nm (high sensitivity for low protein concentration) or 500 nm (low sensitivity for high protein concentration), the bluish color that has evolved is read.[7]

Procedure

i. The proteins that need to be analyzed are diluted to a suitable dosage (20 to 100 mg).
ii. After adding the K Na tartrate-Na, CO solution, it is allowed to sit at room temperature for 10 minutes.
iii. After cooling, add the $CuSO_4$-K Na Tartrate-NaOH solution and incubate for 10 minutes at room temperature.
iv. After adding the freshly made Folin reagent, mix and incubate for 10 minutes at 50°C.
v. At 650 nm, absorbance is measured.
vi. Carefully designed BSA standard curve is used to estimate the protein concentration of the unknown.[7]

Applications

Protein biochemistry has made extensive use of the Lowry method due to its sensitivity and ease of use. It hasn't, however, been extensively utilized to identify proteins in food systems without first extracting the proteins from the mixture of foods.

Advantages

- Extremely sensitive
- 10–20 times more sensitive than the 250 mm UV absorption method
- 50–100 times more sensitive than the biuret method
- Many times, more sensitive than the ninhydrin method
- More specific than most other methods
- Less affected by sample turbidity
- Relatively simple can be completed in 1 to 15 hours.

Disadvantages

For the following reasons, the Lowry procedure requires careful standardization for particular applications

- Compared to the biuret approach, color varies more with various proteins.
- There is no strict correlation between color and protein concentration.
- Monosaccharides, hexoamines, lipids, sucrose, and phosphate buffers all cause different degrees of interference with the process.
- The process is interfered by high concentrations of reducing sugars, ammonium sulfate, and sulfhydryl compounds.[7]

iii. Bradford (Bio-Rad) Method

The Bradford assay measures the amount of protein in cell fractions using a dye-based methodology. Protein concentrations between 10 and 100 µg/ml are measured using it. This assay depends on the strong binding of negatively charged Coomassie Brilliant Blue (CBB)G-250 dye to hydrophilic arginine and hydrophobic amino acid residues in the protein sample under acidic conditions, which forms a blue color complex. The assay relies on a shift in spectral shift from 465 nm to 595 nm. The intensity of the color complex, which can be determined at 595 nm, depends on the concentration of protein. This colorimetric assay is quicker, more reliable with the majority of buffers, salts, and solvents, and less susceptible to interference from different substances found in protein samples.[12]

Procedure

i. Coomassie Brilliant Blue G-250 is acidified with 85% phosphoric acid after being dissolved in 95% ethanol.
ii. The Bradford reagent is combined with samples containing proteins (1–100 µg per mL) and standard BSA solutions.
iii. Absorbance at 595 nm is read against a reagent blank.
iv. The BSA standard curve is used to measure the protein concentration in the sample.[7]

Applications

Potato tubers, beer products, and Worts have all had their protein contents successfully determined using the Bradford method. The method for measuring protein amounts in micrograms has been enhanced. The Bradford method has been widely utilized in protein purification because of its speed, sensitivity, and lack of interferences compared to the Lowry method.

Advantages

- Rapid; reaction time is 2 min.
- Reproducible.
- Extremely sensitive; many times, more sensitive than the Lowry technique
- No cations like K, Na, or Mg^{+2} interfering
- No interference from ammonium sulfate
- Does not interfere with polyphenols or carbohydrates like sucrose
- Measures proteins or peptides having molecular masses of roughly equal to or higher than 4,000 Dalton's.

Disadvantages

- Interfered by detergents that are both ionic and non-ionic, including sodium dodecyl sulfate and Triton X-100. However, with the right controls, mistakes resulting from trace levels (0.1%) of these detergents can be corrected.
- Quartz cuvettes can bind to the protein-dye complex. Glass or plastic cuvettes are required for the analyst to use.
- Different proteins have different colors. It's important to choose the standard protein carefully.[7]

iv. Bicinchoninic Acid (BCA) Assay

The BCA assay is a copper-based colorimetric assay that relies on the biuret reaction, in which, in an alkaline environment, the protein backbone chelates cupric ions and reduces them to cuprous ions. An intense purple result is produced when these ions react with the chemical dye bicinchoninic acid. This product can be quantified spectrophotometrically at 562 nm, in comparison to a reference curve of absorbance from different amounts of bovine serum albumin (BSA).[13]

Procedure

- Combine the protein solution with the BCA reagent (one step) which contains copper sulfate, sodium carbonate, BCA sodium salt, and NaOH and pH 11.25.
- Incubate for 30 minutes at 37°C, or 2 hours at room temperature, or 30 minutes at 60°C. The chosen sensitivity determines the temperature to be used. A greater color response is obtained at higher temperatures.
- Compare the 562 nm solution to a blank for the reagent.
- Use BSA to create a standard curve.[7]

Applications

The BCA method has been used in protein isolation and purification. The suitability of this procedure for measuring protein in complex food systems has not been reported.

Advantages

- Sensitivity is comparable to the Lowry method; however, the sensitivity of the micro-BCA method (0.5 mg to 10 mg) is slightly better than that of the Lowry method.
- The Lowry method is more difficult than one-step mixing.
- Compared to the Lowry reagent, the reagent is more stable.
- The reaction is unaffected by buffer salts and non-ionic detergents.
- Denaturing reagents at medium concentrations (3M urea or 4M guanidine-HCI) do not cause interference.

Disadvantages

- Color changes with time. The time allotted for reading absorbance must be closely monitored by the analyzer.
- Compared to the Lowry method, reducing sugars causes more interference. Ammonium sulfate at high amounts also causes interference.
- Protein color differences are comparable to the Lowry technique.
- The absorbance does not respond linearly to concentration.[7]

c) Ion-Exchange Chromatography

Ion-exchange chromatography separates biomolecules based on reversible charge-charge interactions between the charged groups on proteins and an ion-exchange support that carries the opposite charge. A low ionic buffer is used to wash the column after the solutes have been bonded. Proteins that are neutral or have the same charge as the ion exchange support are easily removed during the washing step because they are not drawn to the medium. The target-bound protein molecules are eluted by either raising the buffer's ionic strength or adjusting its pH to vary the ionic interactions between the target biomolecules and ion-exchange media.[14]

Applications

- Good desired protein recoveries, rapidity, and high resolution are the main benefits of Ion-exchange Chromatography.
- The technique of ion-exchange chromatography is highly effective at resolving differences between two proteins that differ only in one charged amino acid.
- The method is widely applied in the characterization and purification of proteins.
- Protease, alkaline phosphatase, and biotinylated proteins are frequently separated using ion-exchange chromatography.
- As a means of analysis Ion-exchange chromatography is widely used to characterize charged variations, especially those of monoclonal antibodies, in order to do protein profiling.[15,16]

Advantages

- The popularity of the methods is based on the high resolution.
- Simple salt buffers are sufficient.

Disadvantages

- Incompatibility with mass spectrometry-especially in case of ionization mode.[27]

d) Size Exclusion Chromatography

Large molecules like proteins or polymers are the main types of molecules that are analysed using size exclusion chromatography (SEC).[17] In SEC, separation is accomplished by means of the porous matrix's molecular sieve characteristics. SEC matrices are made up of

various beads with slightly varied pore diameters. Smaller molecules run longer and more winding paths in media rather than running straight paths outside the media as larger molecules do resulting in more retardation of small molecules as compared to large molecules.[18]

Applications

- The SEC has applications because it is easy to use and has special features that are not present in other chromatographic techniques.
- SEC's main benefit is that it gently interacts with the sample without affecting it, preserving the proteins biological function.
- Since aggregates are frequently regarded as impurities, SEC is also used in analytical laboratories to quantify the aggregates in biopharmaceuticals, such as when erythropoietin's higher molecular aggregates are determined.[23]

Advantages

- Preferred technique for the detection and investigation of protein aggregation.[19,20,21]
- Quantitative reproducibility.

Disadvantages

- Limitations in pH selection.[28]

CONCLUSION

Protein analysis by standardized analytical techniques is essential for estimating the amount of protein in food. So quantification by chromatography methods is better when compared to spectroscopic methods.

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