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REVIEW ARTICLE

Isolation Techniques of Proteins: A Review

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ABSTRACT

Proteins are the structural component of muscles in human beings. They have gained interest of scientists for their role in providing energy and formation of enzymes and hormones. Due to this reason various analytical techniques are used nowadays to increase their production or extraction from natural sources of animals and plants. The proteins obtained from animal sources are more complete as compared to plant sources as they contain all the necessary amino acids. The proteins are analyzed before they are administered into the body or used in the form of drugs. The present review briefly highlights the techniques that are used for their isolation and analysis.

INTRODUCTION

Proteins are nitrogen containing substances made up of amino acids and are important structural component of muscles and tissues. They can be used as an energy source and also plays a vital role in the production of enzymes, hormones and blood via hemoglobin [1, 2].

SOURCES OF PROTEIN

The main sources of proteins are animals and plants and they are also made synthetically. The synthetic sources are available in market as protein supplements. Complete proteins are considered to be those obtained from animal sources (e.g. eggs, milk, meat, fish and poultry) as they contain all essential amino acids while the ones obtained from plants lack one or two essential amino acids. Therefore, if a person is vegetarian he/she needs to consume a whole lot of fruits, vegetables and legumes in order to achieve complete protein nutrition [3-5].

Animal Sources

The proteins from animal sources include whey, bovine colostrums, casein, etc. [6-8]. (Figure **1**).

Figure 1. Animal sources of protein.

Plant Sources

On the other hand, the protein sources from plants were described briefly by Massey [9] and may include soy, walnuts, wheat, barley, peanuts, etc. (Figure **2**).

Figure 2. Plant sources of protein.

TECHNIQUES USED FOR THE ISOLATION OF PROTEINS

The plant tissues are rich in proteases which make it difficult to isolate proteins from a plant cell. Cell disruption is essential prior to protein extraction because they are present inside the protein bodies of a cell. The general procedure depends on the type of plant, its fragments (e.g. leaf, fruit, seed, etc.) or even the stage of development of a plant [10]. Various chemical and physical methods are used to break cell wall. Some of the techniques involved in the disruption of cells include:

i) Mechanical homogenization
ii) Pressure homogenization
iii) Ultrasound homogenization
iv) Osmotic or chemical lysis
v) Temperature treatments

SOLUBILIZATION/ PRECIPITATION OF PROTEINS

The solubilization of proteins is essential in separating proteins selectively from a mixture of substances present in a sample (Figure **3**). This step is considered to be most crucial as other substances might interfere in protein assay [11]. Various methods are used for this process and are discussed as follows:

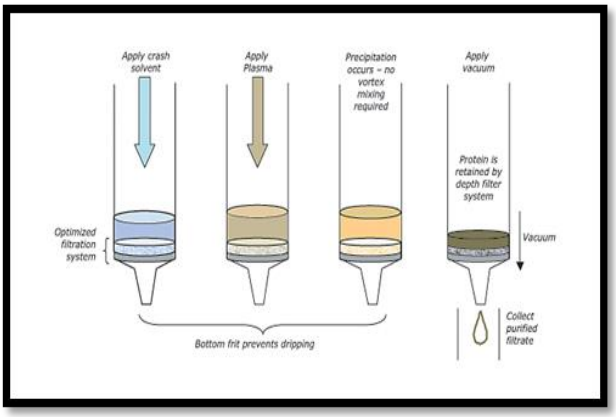

Figure 3. General representation of protein precipitation.

Organic Solvents

Organic solvents are mainly used to extract proteins from tissues of cereals, legumes, fruits, etc. Trichloroacetic acid (TCA) and acetone are most commonly used for the purpose of precipitation in the extraction of proteins from plants [12]. A high pH value and negative charge of TCA with the addition of acetone causes a rapid denaturation of the protein, along with precipitation, thereby instantly arresting the activity of proteases and other modifying enzymes. The only

disadvantage of proteins obtained via TCA is that they are difficult to re-dissolve [13].

Aqueous Solutions

Use of water for the extraction of proteins is advantageous over other organic solvents due to the fact that it is not flammable, explosive or toxic for human use. The properties of proteins and their extraction depend upon the type of extraction process and different factors including pH, ionic strength of medium, salt concentration and net charge [14]. For the extraction of animal proteins various studies have been performed on the rheological and functional properties obtained from the by-products of meat and fish [15-18]. However, for plants it is difficult to develop an ideal method for protein extraction because of the presence of cell wall along with the metabolic and structural characteristics of plant tissues. Some recent studies have reported the use of calcium and sodium salts for protein extraction from vegetables [19-23]. The use of additives such as TCA or carboxymethylcellulose (CMC) has been found helpful for the optimization of the recovery of protein precipitates [24].

Aqueous Enzymatic Extraction

De Moura and co-workers [25] used a two-stage counter current aqueous enzymatic extraction process for soybean. They used small amount of water with high oil and protein contents in contrast to those in which single stage enzymatic extraction was employed. Aqueous enzymatic protein extraction is proven as environmental friendly, safe, and a cheap alternative to extract oil and protein simultaneously [26]. This process avoids protein damage which may occur during the process of refining, thus improving their nutritional and functional properties [27, 28]. Although enzymatic extraction produces no toxic chemicals, it has some disadvantages such as the time consumption and high cost of enzymes making it uneconomical. Use of immobilized enzyme decreases the overall cost by allowing the reuse of enzymes.

Subcritical Water

Subcritical water is simply water which maintains its liquid state in the temperature range of 100–374 °C under pressurized conditions. Its unique properties include low dielectric constant and higher ion product than the normal water, thus making it a promising extraction solvent for various compounds including proteins [29]. Some studies have also demonstrated the ability of subcritical water for extraction of proteins from rice bran and soybean with high protein yields and good functional properties [30. 31].

Protein Enrichment Methods

Once the protein has been isolated, there are few steps needed prior to the analysis of the sample by mass spectrometry (MS). There exists no single analytical method covering the protein range present in a specific sample. The purpose of enrichment methods is to obtain extensive fractions of the proteins of interest. Some of those methods are discussed in the following sections:

Centrifugation

Centrifugation is used for various reasons. It may be an initial step to separate cellular structures where proteins of interest are present, e.g., mitochondria, membrane, or nucleus. It may also involve multiple steps such as the cellular homogenate is extracted in different layers on the basis of their molecular weight, size, and shape (Figure **4**). Centrifugation is also used to fractionate a protein mixture into different fractions. This separation is based on the coefficient of sedimentation, expressed in Svedberg (S) units. The smaller the S value, the slower a molecule moves in a centrifugal field. Separation relies on mass, shape, and protein density [32, 33].

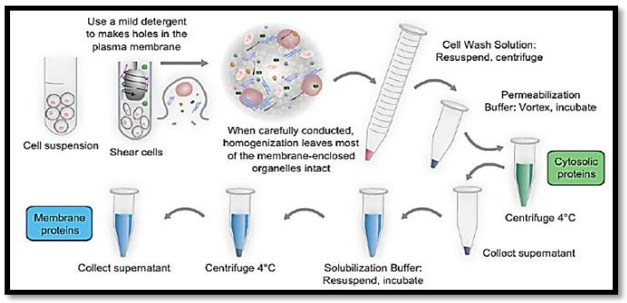

Figure 4. Isolation/ enrichment of membrane proteins via centrifugation.

Precipitation

Among different compounds that cause precipitation, the most widely used is ammonium sulfate [34]. The addition of abundant amounts of this salt or others such as sodium chloride into a protein solution aggravates protein interactions by its aggregation that results in precipitation, referred as 'salting-out' process. Salt concentration required for precipitation varies from one to another as it promotes selective protein separation. This approach has been used to separate soybean proteins, glycinin and β-conglycinin [35].

Another method of protein enrichment is immune-precipitation. The separation depends upon the binding of an antigen to its antibody to form the antigen-antibody complex. It offers high recovery of proteins and is widely used for food allergens [36].

Electrophoresis

This technique separates out a mixture of proteins based on charge, charge / mass ratio, size, or shape. This method is mostly used as an analytical and preparative tool, often employed as a pre-fractionating technique [37, 38].

Chromatography

Liquid chromatography (LC) is widely used in protein pre-fractionation prior to in-depth analysis. Among LC fractionation methods, ion-exchange chromatography (IEC) is most commonly used. Acidic proteins are obtained by anion-exchange whereas basic proteins are withdrawn by cation-exchange chromatography. The milk proteins have been fractionated from sheep milk by IEC as reported by Gomez-Ruiz [39].

Reverse phase LC (RP-LC) isolates proteins according to their hydrophobicity by adsorbing them on a stationary phase carrying hydrophobic groups, and eluted with increasing concentration of an organic solvent, generally acetonitrile. RP-LC is used in combination with IEC and MS for structural analysis. Pre-fractionation with chromatographic methods is also used to identify modifications such as glycosylation or phosphorylation, mainly examined using affinity chromatography (AC). It utilizes highly specific biological interactions (i.e., antigen-antibody, receptor-ligand, enzyme-substrate / inhibitor, etc.). The examples of AC are heparin chromatography, chiefly used for studying microbial proteins, or lectin chromatography that is specially used for glycoproteins [40, 41].

Immobilized metal affinity chromatography (IMAC) is used for fractionating phosphoproteins. It is based on the formation of coordinate bonds between basic groups on protein surface and metal ions. Major drawbacks include little or no binding to iron or gallium charged resins at neutral pH, and the use of low-pH buffers may also provoke protein denaturation or precipitation in the column [42].

Digestion of Proteins

The digestion of proteins involves the gathering of specific peptides that help promote their interpretation by MS or in other methods of protein analysis. For this purpose, various proteolytic agents are used which are specific for bond cleavage including enzymes such as trypsin, many endoproteases as well as chemical agents like hydroxylamine or cyanogens [43]. There are two types of digestion which are mainly used for proteins, i.e. 'in-gel' and 'in-solution' digestion. Most

proteomic studies perform in-gel digestion which is carried out mainly by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The reason to this is that it offers a high dynamic range of analysis of protein mixtures, i.e. ratio of lowest to highest amounts of protein is detectable or removal of impurities of low molecular weight prior to analysis. In-gel protein digestion, for the very first time was established by Rosenfeld [44]. The steps of the method include distaining, reduction, alkylation, cleavage of proteins into peptides by enzymes, and extraction of peptides from gel. Protein digestion by trypsin has most widely been used among the other enzymes for cleaving the amino bonds because of its high purity and low cost of production. Despite of this, tryptic digestion consumes a lot of time for digestion, ranging from several hours to half a day [45].

Many efforts have been made for the development of fast and efficient digestion methods. Keeping this in mind many alternative methods have recently been employed that involve the use of electromagnetic waves or radiation such as microwaves, ultraviolet radiation, and infrared radiation (IR) for speeding up protein digestion [46, 47]. The most promising among these radiations is IR because of safety purposes as compared to other radiations.

Analysis of Proteins

Extraction, identification and analysis of proteins can be carried out by various analytical techniques. Extra and intra cellular proteins are usually isolated and analyzed quantitatively by spectrophotometry [48, 49]. Analysis of proteins via staining of different dyes [50], like Coomassie brilliant blue dye and Lawsone (hennotannic acid), and their color intensity measurement has been achieved by a software. This type of image processing is chiefly applied to biological samples for the diagnosis of disease [51] and for drug analysis [52]. Previously, chemical or enzymatic methods were used to examine the structure of purified proteins, and products of their reactions were detected by ultraviolet (UV) absorbance or fluorescence spectroscopy. At present, other groups of methodologies are gaining popularity for the analysis of proteins, which is MS-based approach. They are of two main types, top-down and bottom-up analysis. Top-down methods analyze whole proteins whereas bottom-up approach examines the peptides from proteins which are already digested. During the analysis via MS, the mass-to-charge ratios (*m/z*) of molecular species are determined. On collecting the data, the compounds in a sample can be analyzed by comparing against the standard with known masses. From whole protein analysis (top-down), to peptide analysis (bottom-up), each particle to be measured has a *m*/*z* detectable by the MS [49].

The development of two techniques, namely, electrospray ionization (ESI) [53] and matrix assisted laser desorption/ionization (MALDI) [50] has made polypeptides accessible to MS. This leads to the development of new mass analyzers and complex multistage instruments. Measurement of molecular mass of a polypeptide and determining additional structural features including the amino acid sequence or the site of attachment can also be done by MS.

CONCLUSION

Solubilization, enrichment methods and digestion of proteins are carried out by a variety of techniques. The analysis of proteins is a crucial step and this mainly depends upon its type. Quantitative determination via mass spectrophotometry and qualitative analysis by the use of different dyes are employed in protein-drug analysis and diagnosis of disease. The development of new techniques has led to

the discovery of multistage instruments which provide additional features for the proteins to be analyzed.

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