

Protein Bioseparation: An Overview

1.1 Introduction

Protein bioseparation which refers to the recovery and purification of protein products from various biological feed streams is an important unit operation in the food, pharmaceutical and biotechnological industry. For the purpose of simplicity, these industries will be collectively referred to as *bioprocess industries* throughout this book. Protein bioseparation is at the present moment more important in the bioprocess industry than at any time before. This is largely due to the phenomenal developments in recent years in the field of modern biotechnology. More and more protein products have to be purified in larger quantities. A further boost to protein bioseparation is likely to come from the developing science of *proteomics*.

The purpose of this chapter is to provide the reader with an overview on protein bioseparation. Different aspects of protein bioseparation are discussed in the book edited by Sadana [1]. In order to read about *bioseparations* in general, refer to the book by Belter *et al.* [2].

1.2 Proteins

A protein is a biopolymer composed of basic building blocks called amino acids. Naturally occurring proteins are made up of up to 20 different amino acids. Proteins are by far the most abundant biopolymers in living cells (constituting about 40 to 70 percent of dry cell weight) and have diverse biological functions:

- a. Structural components (e.g. collagen, keratin)
- b. Catalysts (e.g. enzymes, catalytic antibodies)

- c. Transport molecules (e.g. haemoglobin, serum albumin)
- d. Regulatory substances (e.g. hormones)
- e. Protective molecules (e.g. antibodies)

A protein molecule can be a single poly-(amino acid) chain or may comprise more than one poly-(amino acid) chain, held together by covalent bonds or by non-covalent interactions. A protein usually coils up and folds into a specific 3-dimensional configuration, depending on the intrinsic properties of the protein as well as on the environment in which the protein exists. The structure of a protein can be defined at different levels, these being:

- a. Primary
- b. Secondary
- c. Tertiary
- d. Quaternary

The primary structure of a protein is the sequence of amino acids present in the poly-(amino acid) chain/s. The secondary structure describes the local structure of linear segments of the protein molecule. The three most common types of secondary structure are the alpha helices, the beta sheets, and the turns. The tertiary structure is the three-dimensional arrangement of all the atoms present in a single poly-(amino acid) chain. The quaternary structure describes the arrangement of the poly-(amino acid) chains (or subunits) in a particular protein. For details on proteins, refer to the following books [3–5].

1.3 Protein products

As mentioned in the previous section, proteins have a diverse range of biological functions. Proteins also have a diverse array of applications. A large number of protein based products have been commercialised. These can be classified into the following broad categories:

- a. Food and nutritional products
- b. Pharmaceutical products
- c. Industrial catalysts

- d. Diagnostic products
- e. Proteins used for other miscellaneous applications

Some of the protein-based products are listed in Table 1.1. The first two named categories follow intuitively from the importance of proteins in living systems. A large number of protein products are used as foods, food additives and as nutraceuticals. These are obtained from various microbial, plant and animal sources. Depending on their specific applications, these need to be processed (e.g. purified) to varying degrees. By the rule of thumb, nutraceuticals have greater purity requirements than do food additives and these in turn have to be processed to a greater extent than foods.

Pharmaceutically useful proteins are frequently referred to as *therapeutic proteins*. Most of the recent developments in the area of protein bioseparation are centred on therapeutic proteins.

Enzymes, which are biological catalysts, can be used *in vitro* for industrial scale catalysis. These enzymes are referred to as industrial enzymes and are produced in large quantities. Another major use of enzymes is in diagnostics. Enzymes are also used as components of detergent formulations and cosmetic products.

1.4 The requirement for protein bioseparation

Most protein-based products need to be purified before they can be used. The need for purification is due to the following:

- a. Reduction in bulk
- b. Concentration enrichment
- c. Removal of specific impurities (e.g. toxins from therapeutic products)
- d. Prevention of catalysis other than the type desired (as with enzymes)
- e. Prevention of catalysis poisoning (as with enzymes)
- f. Recommended product specifications (e.g. pharmacopoeial requirement)
- g. Enhancement of protein stability
- h. Reduction of protein degradation (e.g. by proteolysis)

Table 1.1. Protein products.

Proteins

<i>Food / Food additives / Nutraceuticals</i>
Egg albumin
Casein
Soy proteins
Whey protein concentrate
Protein hydrolysates
Alpha lactalbumin
Beta lactoglobulin
Lysozyme
<i>Pharmaceuticals</i>
Monoclonal antibodies
Serum albumin
Serum immunoglobulins
Factor VIII
Tissue plasminogen activator
Urokinase
Streptokinase
Insulin
Erythropoietin
Alpha and beta interferon
Factor IX
<i>Industrial enzymes</i>
Hemicellulase
Glucose isomerase
Alpha amylase
Penicillin G acylase
Alkaline proteases
Cellulases
<i>Diagnostic enzymes</i>
Peroxidase
Glucose oxidase
<i>Miscellaneous</i>
Enzymes used in cosmetic products
Detergent enzymes
Digestive enzymes
Enzyme based silage additive

Some of the characteristic features of most protein products are:

- a. These products are present at very low concentrations in their respective biological feed streams
- b. These products, are present, along with large numbers of impurities, some of which are only slightly different from the products themselves
- c. These products are thermolabile
- d. These products are sensitive to operating conditions, such as pH and salt concentration
- e. These products are sensitive to chemical substances, such as surfactants and solvents
- f. The quality requirements for these products are frequently very demanding

These above-mentioned factors imply that an ideal protein bioseparation process must combine high productivity with high selectivity of separation, and must be feasible at *mild* operating conditions.

1.5 Economic aspects of protein bioseparation

The isolation and purification of proteins from the product streams of bioreactors and other biological feed streams is widely recognised to be technically and economically challenging. Protein bioseparation quite often becomes the limiting factor in the successful development of protein based products. The isolation and purification cost can be a substantial fraction of the total cost of production for most products of biological origin. Table 1.2 shows the bioseparation cost as approximate proportion of cost of production for certain protein based products. As clearly indicated by these figures, bioseparation cost is the major cost and this is an incentive for developing cost-effective isolation and purification processes.

1.6 Protein bioseparation methods

A myriad of protein bioseparation techniques is available. Some of these protein isolation and purification techniques are discussed in the following books [1, 6–10]. When developing a bioseparation process for a specific

Table 1.2. Cost of protein bioseparation.

Product	Approximate relative price	Bioseparation cost as % of total cost of production
Food/additives	1	10–30
Nutraceuticals	2–10	30–50
Industrial enzymes	5–10	30–50
Diagnostic enzymes	50–100	50–70
Therapeutic enzymes	50–500	60–80

protein, the following should be taken into consideration:

- a. The volume or flow rate of the feed stream
- b. The relative abundance of the protein in the feed stream
- c. A profile of the impurities present
- d. The intended application of the protein, along with particular product specifications
- e. The market price of the protein

Protein bioseparation techniques can be classified into three broad categories:

- a. High-productivity, low-resolution
- b. High-resolution, low-productivity
- c. High-resolution, high-productivity

Most conventional protein bioseparation processes rely on a scheme, which is best described as RIPP (Removal, Isolation, Purification and Polishing) [2]. Biological feed streams are generally dilute with respect to the target proteins, which need to be separated from a large number of impurities. Such a feed stream would easily overwhelm a high-resolution separation device. Therefore, low-resolution, high-productivity techniques are used first to reduce the volume and the overall concentration of the process stream. This is followed by high-resolution, low-productivity techniques to obtain the pure target protein. However, with the advent of high-resolution, high-productivity techniques, it is frequently possible to shorten, if not totally replace the RIPP scheme.

Table 1.3 lists some of the more commonly used protein bioseparation techniques. Note that ultrafiltration is listed in two categories since the

Table 1.3. Protein bioseparation techniques.

High-productivity, low-resolution

Cell disruption
 Precipitation
 Centrifugation
 Liquid-liquid extraction
 Microfiltration
 Ultrafiltration
 Supercritical fluid extraction

High-resolution, low-productivity

Ultracentrifugation
 Packed bed chromatography
 Affinity separation
 Electrophoresis
 Supercritical fluid chromatography

High-resolution, high-productivity

Fluidised bed chromatography
 Membrane chromatography
 Ultrafiltration
 Monolith column chromatography

resolution in an ultrafiltration process depends very much on how it is operated. Some of the other protein bioseparation techniques are briefly discussed below.

1.6.1 Cell disruption

Different types of cells (e.g. microbial, animal and plant) produce proteins either intracellularly or extracellularly. For recovering intracellular proteins, the cells have to be disrupted. Different cell disruption techniques are listed in Table 1.4.

1.6.2 Precipitation

Proteins can be partially purified using precipitation techniques. The main advantage of these techniques is that very large process volumes can be handled. Proteins can be precipitated using (a) salting out salts

Table 1.4. Cell disruption methods.

Physical methods

Disruption in ball mill or pebble mill
Disruption using colloid mill
Disruption using French press
Disruption using ultrasonic vibrations

Chemical methods

Disruption using detergents
Disruption using enzymes
Combination of detergent and enzymes
Disruption using solvents

like ammonium sulfate and sodium chloride, (b) solvents like ethanol, methanol and acetone, and (c) concentrated acids or alkali. Precipitation processes are generally favoured at low temperatures. After precipitation, the precipitates are separated from the bulk liquid (also called the supernatant) using centrifugation or filtration.

1.6.3 **Centrifugation**

A centrifuge is a device that is used for separating precipitated proteins from a solution by spinning the samples at rotation speeds typically ranging from 1000–10000 revolutions per minute. Centrifugation may be carried out at two different scales:

- a. Analytical centrifugation
- b. Preparative centrifugation

Analytical centrifuges are used in research laboratories and in the industry for small-scale separation and sample preparations (i.e., 1–1000 ml). Preparative centrifuges handle larger sample volumes (i.e., 1 to several thousand litres).

1.6.4 **Ultracentrifugation**

An ultracentrifuge is a special type of centrifuge, which is operated at a much higher speed, e.g. 30000 revolutions per minute. Ultracentrifuges

of both analytical and preparative scales are available. These are used to separate proteins in solution.

1.6.5 Column chromatography

Chromatography relies on the distribution of components to be separated between two phases: a stationary (or binding) phase and a mobile phase, which carries these components through the stationary phase. In its simplest form the stationary phase is present in the form of a packed bed within a column, hence the term column chromatography. The mixture of components enters a chromatographic column along with the mobile phase, and each individual component is flushed through the system at a different rate. The rate of migration of a component depends on its interactions with the stationary phase, as well as on the mobile phase flow rate. Different types of columns are used for chromatographic separations. Packed beds are most commonly used. Other types include packed capillary columns, open tubular columns and monolith columns.

Different types of separation chemistries are used for chromatographic separation of proteins:

- a. Ion exchange
- b. Reverse phase partitioning
- c. Hydrophobic interaction
- d. Size exclusion
- e. Supercritical fluid extraction
- f. Affinity interaction

1.6.6 Electrophoresis

Electrophoresis refers to the separation of components by employing their electrophoretic mobility (i.e., movement in an electric field). The mixture is added to a conductive medium, followed by the application of an electric field across it. Positively charged components will migrate towards the negative electrode, negatively charged components will migrate towards the positive electrode, while neutral components will remain immobile. Electrophoresis can be classified into two types, depending on the medium

in which the separation is carried out:

- a. Gel electrophoresis
- b. Liquid phase electrophoresis

1.6.7 Membrane chromatography

Column chromatography has several major limitations. Some of these limitations could be overcome by using synthetic microporous membranes as chromatographic media [11,12]. In membrane chromatography, the transport of proteins to their binding sites takes place by convection and hence these processes are fast. Thus, the high resolution of a chromatographic process can be combined with the high productivity of a membrane separation process.

1.6.8 Microfiltration

Microfiltration relies on the use of microporous membranes for the separation of micron-sized particles from fluids. The various applications of microfiltration include:

- a. Cell harvesting from bioreactors
- b. Virus removal for solutions
- c. Clarification of fruit juice and beverages
- d. Water purification
- e. Air filtration (for sterilisation)
- f. Media sterilisation in bioreactors

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