# Protein Separation Techniques

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## Introduction

Protein separation techniques are based on the widely different chemical, physical and biological properties of various protein molecules. The number and complexity of these techniques are rapidly increasing as our understanding of protein molecules expands. Currently, many specialized column materials for use in chromatographic separation of proteins are commercially available. Additionally, sophisticated fraction collection and protein detection equipment is being used to facilitate protein separation. The purpose of this review is to briefly outline some of the common methods currently being used for separating proteins. It should be emphasized that the large and rapidly growing technology associated with protein separation cannot be adequately covered in this brief review. Consequently, it is intended to provide a base of information from which interested readers can expand their studies of specific protein separation techniques.

## Experimental Uses for Protein Separation Techniques

Protein separation techniques have traditionally been used to isolate and to purify specific proteins in order to facilitate studies of their enzymatic, physical, chemical and structural properties. These kinds of studies are necessary in order to elucidate the biological role of individual proteins in the cell and to understand the mechanism by which the activity of specific enzymes are controlled.

Because protein separation techniques are based on the chemical, physical and enzymatic properties of proteins, the behavior of a specific protein during a separation protocol can reveal a great deal about that protein. For example, ion exchange chromatography can give an indication of the relative net charge on the protein at a given pH; gel permeation chromatography can be used to determine molecular size (Stokes radius); affinity chromatography can be used to analyze the interaction of a protein with specific substrates, inhibitors, activators or antibodies; and hydrophobic interaction chromatography can be used to examine the hydrophobicity of specific proteins.

Protein purification techniques can also be used to monitor the interactions of specific purified proteins. Studies of in vitro interactions between two or more proteins from the same tissue or cell often yield information about how the proteins interact in vivo. In food systems, the interaction between specific proteins can affect their functionality in the system. Consequently, it is often of great interest to ascertain whether two purified proteins interact and how this interaction affects their properties.

## Protein Purification Techniques

### A. Gel Permeation Chromatography (1-4)

Gel permeation chromatography separates protein molecules according to their size and shape, with large, nonspherical molecules eluting more rapidly than small, spherical molecules. Gel permeation resins may be thought of as beads containing numerous uniform conical pits on their surface. Molecules that are larger than the diameter of the pit do not enter it and consequently pass through the column quickly. Small molecules can enter the pits. The smaller the molecule, the deeper it can go into these conical pits and the longer it will remain there. Consequently, the presence of the pits impedes the progress of small molecules through the column more than it does the progress of large molecules.

In order to standardize a gel permeation column, it is necessary to calculate the fraction of the stationary gel volume that is available for diffusion of a given solute species ($K_{av}$). This is done by using the following parameters:

- elution volume ($V_e$) - the volume at which a given protein elutes
- total volume ($V_t$) - volume of the packed column bed
- void volume ($V_o$) - elution volume of the molecules only distributed in the mobile phase of the gel because they are larger than the diameter of the largest pits in the gel.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

$K_{av}$ for a given protein is proportional to the log of the molecular radius of that protein. Under optimal conditions, protein molecules do not bind to gel permeation resins.

Both column length and flow rate affect the degree of resolution obtainable using gel permeation chromatography. Since resolution is directly proportional to the square root of the column length, a relatively longer column is desirable. Resolution is also improved by reducing the flow rate of the column. Because increasing column length and decreasing flow rate both increase the time required to elute the protein

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of interest from a gel permeation column, this increased time factor must be balanced against the increased resolution it provides.

**B. Ion-exchange Chromatography (5-11)**

An ion exchanger consists of an insoluble support matrix containing chemically bound positive (anion exchanger) or negative (cation exchanger) groups and mobile counter ions. The counter ions may be reversibly exchanged with other ions of the same charge without altering the insoluble matrix or the chemically bound groups.

Protein molecules bind electrostatically and reversibly to ion exchangers if their net charge is opposite to that of the exchanger. All proteins are amphoteric polyelectrolytes, which means that their net charge is dependent upon the pH of the solution in which they are suspended. At low pH, the net charge on most proteins is positive (binds to cation exchangers) and at high pH the net charge is usually negative (binds to anion exchangers). At the point of zero net charge, the isoelectric point, protein molecules are not bound to any type of ion exchanger. Since protein molecules can bind to either anion or to cation exchangers (depending upon pH), the stability of the protein at various pH's is usually the most important factor in choosing an ion exchanger for specific protein separations.

Once proteins have been bound to an ion exchange resin, selective elution can be accomplished in several ways. For example, altering the column buffer pH towards the isoelectric point of a specific protein will cause that protein to lose its net charge, desorb and elute from the column. Consequently, if a pH gradient is applied to an ion exchanger containing bound protein, each protein will elute as its isoelectric point is reached and protein separation will be accomplished. Changes in ionic strength of the column buffer can also be used to selectively release proteins from ion exchange resins. At low ionic strength, competition for charged groups on the ion exchanger is at a minimum and even slightly charged proteins are bound strongly. Increasing the ionic strength of the column buffer (by using a linear gradient) increases the competition and interferes with the interaction between the ion exchanger and the protein. Weakly charged molecules (those closest to their isoelectric pH) are eluted at lower ionic strength than more highly charged molecules.

Chromatofocusing is a specialized form of ion-exchange chromatography that requires a resin containing charged groups with high buffering capacity. The column is equilibrated at a given starting pH and protein is loaded. The column is then eluted with buffer of a different pH than the starting buffer. In this way, a pH gradient is formed within the column as the eluting buffer titrates the ion exchanger. For example, in order to produce a descending pH gradient, (pH 9-6) the ion exchanger must be at a higher pH than the eluent. Consequently, a basic buffering group is required on the ion exchanger so an anion exchanger must be used. The column is first equilibrated at pH 9 and the pH of the eluent is set at pH 6. The eluent contains a large number of differently charged molecules and as they migrate down the column, the most acidic of these bind to the basic groups on the anion exchanger. In this way, the pH at successive points in the column is gradually lowered as elution proceeds and more eluent is added to the column. A given protein is released from a particular portion of the column when the pH in that region of the column reaches the isoelectric pH of the protein. The protein then moves through the column as additional exchanger is titrated to the isoelectric pH of the protein. Elution of different proteins is in descending order of their isoelectric points.

**C. Hydrophobic Interaction Chromatography (12).**

In this type of chromatography, proteins are separated based on the strengths of their hydrophobic interactions with an uncharged resin containing hydrophobic groups. These interactions are facilitated by increasing ionic strength. Consequently, proteins are often adsorbed to a hydrophobic resin in the presence of high concentrations of neutral salt (e.g., NaCl).

Selective elution of adsorbed proteins is achieved by altering the eluent in a way that causes desorption based on the strength of the hydrophobic interaction of individual proteins with the hydrophobic matrix. This can be accomplished by lowering the ionic strength of the eluent, lowering the polarity of the eluent by including substances such as ethylene glycol, including detergent in the eluent, or raising the pH of the eluant.

**D. Affinity Chromatography (13-14)**

Affinity chromatography is a type of adsorption chromatography in which the column bed material has biological affinity for the particular protein to be isolated. Specific adsorptive properties of the bed material are obtained by covalently coupling an appropriate binding ligand to an insoluble matrix. The covalently attached ligand then binds a specific protein from a complex mixture of proteins and the bound protein is retained on the matrix. Unbound proteins are then washed away and the bound protein is released by altering the composition of the eluent. Highly selective protein isolations are achieved due to natural biological specificities. For example, enzymes can be purified by using affinity columns containing a substrate analog, specific inhibitor or cofactor for that enzyme. Antigens can be purified by using affinity columns containing the appropriate antibody. Similarly, antibodies can be purified on affinity columns containing covalently bound antigen.

Affinity chromatography provides opportunities for the isolation of proteins based on their biological function and thus differs greatly from conventional chromatography techniques in which separation depends on gross physical and chemical differences between proteins. This is a powerful technique that is currently being utilized extensively in isolating and purifying proteins.

**E. Other Types of Chromatographic Resins.**

In addition to those already mentioned, many other resins have been successfully utilized in specific protein isolations. For example, both hydroxylapatite (15) and Cibacron Blue F3-GA (coupled to an insoluble matrix) (16) have been effective in specific protein purification protocols.

**F. Preparative Isoelectric Focusing (17).**

Basically, this technique amounts to electrophoresis of proteins in a pH gradient. Small molecules with high buffering capacity are incorporated into a granulated gel (Sephadex G-75 or similar gel) and when a current is applied to the gel, these molecules migrate to their isoelectric pH (where they have zero net charge) and remain there. Because of the high buffering capacity of these small molecules, a pH gradient is formed in the gel. In this system, proteins move to their isoelectric pH and are thus separated according to their differing isoelectric points. The gel acts as a support matrix which holds the separated proteins in their respective positions once separation has occurred.

Once the individual proteins have been separated according to their isoelectric point, individual sections of the gel (about 1 cm wide) are removed. Buffer is added to these individual gel sections to make a slurry which is poured into a small column. Because most proteins are large enough to be completely excluded from the gel matrix, it is possible to remove the protein from the gel by washing this column with 2 to 3 volumes of the appropriate buffer.

This procedure provides a high-resolution method for purifying small quantities of a particular protein. One of the major limitations of this method is that many proteins precipitate at their isoelectric pH and this may interfere with separation.

**G. High Performance Liquid Chromatography (18-20).**

Although this technique shows promise for the future, it is not yet very practical for large scale preparation of large protein molecules. It may be useful for specific applications, especially for small peptides, or as a substitute for preparative isoelectric focusing. It is very rapid and might be a possible alternative for purifying small quantities of an extremely labile protein.

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## Discussion

*Question:* I'd like to have you tell us how you decide which of these techniques you use for which proteins. Is that by category and by their nature?

*Answer:* It depends a lot on the characteristics of the protein. In general, when you start out a protein purification, you have extracted the protein and it is in a dilute solution which is difficult to work with. The first thing you have to do is to concentrate the protein. Usually this concentration can be accomplished by using a technique such as salting out with ammonium sulfate which concentrates the protein without harming it. The big drawback to ammonium sulfate precipitation is that some molecules (especially small ones) may not precipitate. Another method which may be used to concentrate proteins in isoelectric precipitation, which involves precipitating the protein at its isoelectric pH. There are some problems inherent with this technique in that it is possible that individual proteins will be denatured by the change in pH that is required to cause precipitation. In many cases, it is also possible to concentrate a dilute protein solution by binding the proteins to an ion exchange resin, precipitating the resin and then resuspending it in a small volume of a buffer that causes the protein to be released.

After you have concentrated the extracted protein, you can begin to think of chromatography. As a general rule of thumb, if you know nothing about the proteins that you are working with, it is probably best to start a chromatographic purification by using a gel permeation column. The reason for this is that gel permeation doesn't require any specific interaction between the protein molecules and the gel matrix making up the column. Consequently the purification does not depend upon a hydrophobic interaction or an ionic interaction that might be interfered with by substances present in a crude protein mixture.

In developing a protein purification protocol, you try to take advantage of as many properties of the protein as possible in order to separate that protein from contaminating proteins. Consequently, if you choose a gel permeation column (which separates on the basis of size) as the initial column, subsequent columns should be ionic exchange or hydrophobic in nature. In this way, proteins of similar size obtained from the gel permeation column can be separated according to their charge or their hydrophobicity. As you develop the procedure, you should attempt to choose subsequent columns to exploit a different property of the protein.

As you work through a protein purification procedure, you need to do two things: 1) you need to have an assay for the presence of the protein so that you can follow it through the purification; 2) you need to monitor the purity of the protein preparation by using SDS polyacrylamide gel electrophoresis. Along these lines, it is good to make a table showing protein yield (mg), and specific activity (activity/mg) for each step of the purification procedure. In a purification procedure, these two things are often traded off against one another. For example, you may be willing to give up a significant amount of the total enzyme protein in order to increase the specific activity (homogeneity) of the protein that you retain. Consequently, at the end of a purification procedure, you may only have 20% of the total enzyme protein present initially, but that 20% will hopefully be pure or very close to it.

Purification procedures for different proteins tend to be unique for that protein. Procedures are developed in a stepwise manner. That is, following each chromatographic step, the purity and activity of the active fraction is assessed. A decision is then made as to what type of column might be most effective in further purifying this fraction. For instance, a fraction separated according to molecular size and shape on a gel permeation column might be assayed by using isoelectric focusing. If this fraction contained peptides varying greatly in their isoelectric points, an ion exchange column should be a very effective step in further purifying a specific protein present in the fraction.

*Question:* Are all of these techniques that you have covered with us nondenaturing so that you can look at the functional properties of the protein?

*Answer:* Right, these techniques are all nondenaturing in general. The only thing you would have to be careful of is that the protein you are interested in is not denatured by specific conditions used in one of the procedures. For instance, with hydrophobic columns, if you alter the pH of the eluent or if

you use ethylene glycol to change the polarity of the eluent, there is always the possibility that the protein you are interested in might be denatured. Consequently, you need to follow the specific activity of the enzyme very closely during purification and any purification step that decreases the specific activity should not be used.

*Question:* When you say "denature," are you talking about an irreversible change?

*Answer:* For the purposes of this conversation, yes, although many denatured proteins can be partially or completely renatured under the proper conditions.

*Question:* One thing that we've tried to do with an Amicon cell is to concentrate proteins, particularly myofibrillar proteins. We find that when you get to a particular concentration, they aggregate and stop up the cell.

*Answer:* Myofibrillar proteins do tend to aggregate at higher concentrations under some conditions.

*Comment:* We've used Amicon cells a lot and they work up to a limit. We've been able to concentrate protein to between 2 and 10 mg ml and we have been able to live with that protein concentration.

*Question:* I presume you've had some experience using the separation techniques you've described in purifying the calcium-activated protease (CAF). How did you apply these techniques in that situation?

*Answer:* The calcium-activated protease comprises less than 0.5% of the total protein extracted from muscle tissue in a low ionic strength buffer. We currently purify the protease to greater than 90% homogeneity by using ion-exchange, gel permeation and hydrophobic chromatography. We monitor the acitivity of the enzyme by using a protease assay and its purity by using SDS polyacrylamide gel electrophoresis. Because we know where the protease bands are located on an SDS gel, we are able to make extensive use of SDS polyacrylamide gel electrophoresis in monitoring the purity of the enzyme at each stage of the purification procedure. This is very important because, during the later stages of the purification procedure, increases in specific activity may not accurately reflect the effectiveness of a purification step. For example, a purification step resulting in only a 10% increase in specific activity may remove a major contaminant from an enzyme preparation that is already 80% homogeneous. Consequently, small increases in specific activity may be extremely significant when dealing with a relatively homogeneous enzyme preparation. We were able to identify the bands corresponding to the calcium-activated protease by cutting out protein containing bands from a nondenaturing polyacrylamide gel, electrophoretically removing the protein from these gels and assaying the protein for calcium activated protease activity. The electrophoretically-purified calcium-activated protease was then run on an SDS polyacrylamide gel to show the banding pattern of the protease. In the last two steps in the purification of the calcium-activated protease, we run SDS gels on individual column fractions and pool fractions based on the purity of the protease in each fraction. In this way, we can be very selective about the homogeneity of the protease in the pooled fractions. This procedure greatly increases the homogeneity of the calcium-activated protease preparation.

*Question:* What about measuring the molecular weight of fibrous proteins in relationship to globular proteins?

*Answer:* The only way to do that using chromatographic techniques is to denature the proteins by using urea or guanidine HCl and then measure their elution volume from a calibrated gel permeation column.

*Question:* What about molecular size and shape (Stokes radius)?

*Answer:* The best way to do that would be to measure the elution volume of a protein from a gel permeation column in a nondenaturing buffer. The calculations are described in the body of the presentation.

*Question:* What do you mean by a calibrated gel permeation column?

*Answer:* Spherical proteins of known molecular weight can be purchased in column calibration kits. To calibrate a gel permeation column, the elution volume of each of these proteins from the column is determined. Then you graph the Stokes radius vs elution volume. This standard graph can then be used to calculate the Stokes radius of a protein whose elution volume is known.

*Question:* Is there anything unique that you could tell us about myofibrillar proteins versus stroma proteins with regard to protein separation techniques.

*Answer:* The biggest difference between these two classes of proteins is their solubility. With the connective tissue proteins, you often must use a denaturing buffer system in order to keep them soluble during the separation procedure. This limits the types of chromatography that you can use.

One thing I want to mention is that the manufacturers of chromatography resins are a very good source of information about chromatographic techniques since it is to their advantage to see to it that you have good experiences with chromatography. Pharmacia, Bio-Rad, and LKB, to mention a few, provide information on use of specific resins that they manufacture. Additionally, if you have a specific problem, it is often very helpful to call the manufacturer and talk to their technical representatives. These people are usually knowledgable and cooperative and provide a very useful source of information.

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