Part 3
Separation of Complex Protein Mixtures into Individual Components

**Purpose:** The purpose of Part 3 is to use a chromatographic technique called Gel Filtration chromatography and Polyacrylamide Gel Electrophoresis to isolate and purify individual components from complex biological mixtures.

**Key Concepts/Terms:**
- Adsorption Chromatography
- Affinity Chromatography
- Denaturation
- Gel Filtration Chromatography
- Native and SDS PAGE
- Ion Exchange Chromatography
- Partitioning
- Stationary vs. Mobile phase

**Background**

A problem continually faced by biochemists in the laboratory is the separation, purification and identification of one or more biological compounds from a complex mixture. Chromatography is one of the most convenient methods for achieving such separations and can be useful in the separation of large (gram) or small (picogram) amounts of materials. The selection of a particular form of chromatography to achieve a separation is dependent on the material to be isolated. Often several different types of chromatography are used sequentially to achieve the complete purification of a compound from a complex mixture.

The basis of all forms of chromatography is the partitioning or distribution of compounds between two immiscible phases – e.g., liquid/liquid, solid/liquid or gas/liquid. One phase is “stationary” and may be a liquid, gel, or solid. A “mobile” phase flows over or through the stationary phase and usually a liquid or gas. The selection of stationary and mobile phases in a chromatographic system is based on the chemical characteristics of the molecules of interest. Various types of chromatography are available to separate different kinds of biological molecules. Some major types include:

i) **adsorption chromatography** – used to isolate low molecular weight compounds (mw’s generally below 500) using polar/non-polar properties between a solid stationary phase and a mobile liquid or gas phase;

ii) **ion exchange chromatography** used to separate charged molecules (low or high molecular weight compounds) between a stationary ion exchange resin (solid or gel) and a mobile phase (liquid);

iii) **gel filtration chromatography** – used to separate molecules based on size between a solid or gel phase (stationary) and a liquid phase (liquid);

iv) **affinity chromatography** – used to separate molecules (low or high molecular weight) based on their affinity/specificity for a macromolecule/functional group bonded to a solid support and a liquid or mobile phase (will be used in enzyme lab later this term).
In practice, chromatographic separations are usually accomplished by one of three means: column, thin layer, or paper. Each of these chromatography methods have a specific advantage, application, and method of operation. You will use all of these methods during the term.

_Gel Filtration Chromatography._ The separation of molecules based on their molecular size and shape is referred to as “gel filtration” chromatography and utilizes the molecular sieve properties of a variety of porous materials. The most common of such materials are polymeric organic compounds that possess a three dimensional network of pores. Large molecules that are completely excluded from the pores (i.e., too big) will pass through the interstitial spaces around the gel particles, while smaller molecules can freely enter the pores (depending on their size). Because large molecules do not enter the gel pores (i.e., excluded), they are not retained by the particles and travel with the mobile phase very quickly. Molecules that are small enough to enter the pores take a longer, more torturous route through the gel particles (both inside and outside) and therefore move much more slowly (i.e., they are retained on the column for longer periods).

Crosslinked dextrans (trade name Sephadex), agarose (Sepharose, BioGel A, Sagavac), polyacrylamide (BioGel P), polyacryloylmorphine (Enzocryl Gel) and polystyrenes (BioBeads S) are the common gels used for gel filtration chromatography. Sephadex gels are obtained by crosslinking hydrophilic dextrans with epichlorhydrin into an insoluble form (i.e., the gel) that retains the hydrophilicity character of dextran. By varying the degree of crosslinking, several types of Sephadex are commercially available and differ in the maximum size of pores that make them useful for separating molecules over a wide molecular size range.

_Electrophoresis_ is the separation of proteins or nucleic acids based on size and charge by running them through gels using an electric current. The two types of gels used are Agarose and Polyacrylamide gels.

Agarose gels, which are produced from agar, are linear polysaccharides consisting of alternating residues of D-galactose and 3,6-anhydro-L-galactose units with side chains of 6-methyl-D-galactose. Their gel properties are attributed to hydrogen bonding - both intermolecular (within) and intramolecular (between) bonding forming helices. Due to their hydrophilic nature and absence of charged groups, agarose gels, like dextran gels, cause very little destruction (i.e., denaturation) or adsorption of sensitive biochemical substances. By virtue of their greater porosity they complement the dextran gels. Whereas Sephadex is used for the fractionation of spherical molecules - e.g., globular proteins (mw up to 800 kD) - or randomLy coiled polymers (mw up to 200 kD), agarose gels can separate molecules and particles with mw up to several million Daltons.

Polyacrylimide gels are prepared by the polymerization of two monomers and a range of gels with differing porosities can be obtained. Acrylimide gels have characteristics very similar to the dextran and agarose gels, but have exclusion limits (i.e., pore sizes) ranging from 1.8 kD to 400 kD.

Two types of Polyacrylamide Gel Electrophoresis (PAGE) are used: Native gels through which proteins and nucleic acids are not denatured prior to or during the electrophoresis; and SDS PAGE in which the proteins are denatured with heat and Sodium Dodecyl Sulfate (a detergent). The beauty of this method is that the gel is essentially a plastic that can be made in and used in water. Review these methods in Chapter 3 of the textbook.

Lab Exercise-Part 3 First Lab Exercise Gel Filtration

**Materials**

1. 1.0M potassium acetate buffer, pH 6.0 2 liters
2. 0.1M potassium acetate buffer, pH 6.0 5 liters
3. Bovine Serum Albumin (BSA) fraction V 100mg to make 10mL
4. Blue dextran 100mg to make 10mL
5. Acetone 10mL
6. Cytochrome C or substitute 100mg to make 10mL
7. Flavin Mononucleotide (FMN or Riboflavin-phosphate) 100mg to make 10mL
8. Bradford reagent (Biorad) (200mL to make 1 liter of diluted reagent)
9. Sephadex G-75 30 grams
10. 95% ethanol 1 liter
11. One scinted glass filter for washing Sepadex
12. Whatman filter paper disc and support to filter diluted Bradford Reagent
13. Syringe filters (0.45 μm or 1 μm) 3

This week’s lab deals with the separation of the same colored compounds used during the previous lab on Spectroscopy, i.e., blue dextran (approx. mw 200,000,000 Da), cytochrome C (mw 12.3 kDa), FMN (mw 456.3 Da), and BSA (approx. mw 66 kDa). You will pour a Sephadex G-75 gel filtration column. Each group will attempt to separate a mixture of the three colored compounds from last week, plus BSA. The Bradford protein assay will be used to detect the BSA.

At each station will be a description of the gel material for pouring the column, a flask with the gel material, a column, buffer, a buffer reservoir, some tubing to attach the reservoir to the column, the sample to be loaded which is a mixture of the four compounds discussed above, and tubes to collect the eluant from the column.

The TA will describe how to pour the column, but you will need to figure out how much material to use and how to estimate the bed volume of the column (how much volume the column material takes up). The bed volume can not be calculated from how much of the gel slurry is poured in, because the column settles and packs differently depending on the shape of the column, the height of the buffer reservoir, etc. The diameter of the column is 1 cm. Try to get the height of the packed material to be between 20 and 25 cm high which is 20-25 times the diameter of the column.

Experiment

Pouring the column:
1. Pour 5 mL of buffer into the bottom of the column. Make sure the column is closed.

2. Put the funnel in the top of the column and pour in the slurry of G-75 Sephadex until the column is full and there is Sephadex in the funnel.

3. Let the column settle for at least 15 minutes. Then open the column. The height of the Sephadex will decrease. You may need to close the column and add more Sepadex to get a column that is about 20 cm high.

Loading the sample:
1. Open the column and let the buffer run out until the buffer meniscus just touches the top of the column material. It is important to NOT let the column run dry, but also not to have too much liquid on the column interface.

2. Pipet your sample onto the column with a Pasteur pipet by letting it slide down the glass just immediately above the column material. Circling the inside of the column with the pipet tip is the gentlest way to load your sample. DO NOT disturb the surface of the column by dropping the sample directly onto it.
3. Open the column and let the sample run into the column until the meniscus of the sample touches the column top. Close the column. Wash the walls of the column with buffer and run into the column as above. Add several mL of buffer **VERY GENTLY** to the column by circling the inside of the column with your Pasteur pipet tip. DO NOT disturb the surface of the column.

4. Hook up the column to the buffer reservoir and open the column. Make sure that the buffer from the reservoir is moving through the tube towards the column. If no buffer is moving through the tube from the reservoir, close the column, add more buffer carefully to the top of the column and reconnect the buffer reservoir securely.

**Running the column:**
  The column flow rate will depend on how well it is packed and the hydrostatic head. You will need a flow rate sufficient to elute all four samples from the column in about one half hour to allow you to complete the lab. If flow rate is extremely slow, it will be best (and faster) to re-pour the column.

**Collecting fractions:**
  As described in the background material above, you will want to collect the eluant from the column from the time that you load the sample.

1. Collect one mL fractions in the test tubes provided. The easiest way to do this is to pipet one mL into one tube and mark the height of the meniscus on the tube. Then mark all the rest of the tubes at the same height. This should allow you to collect one mL fractions manually with reasonable accuracy.
2. The volume collected up until the first compound elutes from the column is called the void volume. The volume where one half of the compound has eluted (usually the fraction with the greatest amount of that compound) is called the elution volume of that compound. To determine which fraction has the greatest concentration of each compound, you will need to read the sample in the Spec 20 at its maximum absorption wavelength that was determined last week.
3. You must add 2 mL of buffer to each 1 mL fraction so that it can be read in the Spec 20. Then, plot the absorption versus fraction number for each compound (they can all be plotted on the same graph).
4. To detect the BSA, you will have to do the Bradford assay, as described in last week's protocol. Once you have collected your fractions, the TA will discuss with you the best way to measure the absorption of the colored compounds and which fractions to test with the Bradford assay.

The graph should have the following information on it:
The column bed volume
The void volume of the column
The elution profile of each of the four compounds (absorbance vs. fraction number)
The names of the people in the group

**Lab Exercise-Part 3 Second Lab Exercise PAGE**

This week we will use PAGE to separate proteins from liver. Fresh supermarket liver will be ultra-sonicated to disrupt its membranes, then centrifuged to pellet the unbroken cells and debris. The supernate will be used in native PAGE to separate proteins based on their native size.

**Materials**
1. Precast 8-16% polyacrylamide, Tris-Glycine buffer, pH 8.7.
2. Sodium dodecyl-sulfate (SDS) – 10% solution
3. Tris-Glycine for SDS PAGE Buffer:
   - Tris-base 40 g
   - Glycine 45.5 g
   - Distilled water to 5 L

4. Sample Buffer: 1X Tris-Glycine SDS buffer 10 mL
   - Glycerol 20 mL
   - B-mercaptoethanol 0.2 mL
   - Bromophenol blue ~5 mg
   - Distilled water 9.8 mL

5. Coomassie Blue Stain: 1 g in 500 mL of 50/10.
6. Destaining Solution (10/10) 10% methanol (or ethanol) + 10% Acetic Acid.

7. Standard Protein Electrophoresis Ladder: NativeMark, Unstained Protein Standard, 20-1,200 kDa, Fisher LC0725
8. 1.5 mL centrifuge tubes.
9. Fresh chicken liver (Publix) and a Waring Blender.
10. PAGE electrophoresis chambers and power supplies.

**Experiment**

Preparation of Liver Extract. For the whole class, place about 15 g of chicken liver in to 50 mL of ice cold Tris-Glycine buffer. Homogenize (blend) for 2-3 minutes. Each pair pipettes 1 mL into a 1.5 mL centrifuge tube and centrifuge the crude cell extract at 12 to 14 k rpm for 5 minutes. Pipette off the clear supernate as your sample of liver cell cytoplasm. Prepare 3 serial 1:5 dilutions using Sample Buffer in micro-centrifuge tubes: this makes a concentration gradient from high concentration (liver cytoplasm sample) to increasingly lower concentration.

SDS PAGE gels. These gels have 10 lanes/gel plate; the plates should be in the electrophoresis chamber with bottom and top buffers. In the first lane add 5 µL of the standard protein ladder. In the next 4 lanes put 5 µL each of your sample and dilutions. One gel plate has enough lanes for 2 groups.

Attach the electrophoresis power supply to the electrophoresis chamber electrodes and begin with the voltage and amperage recommended by the supplier of the pre-cast PAGE gel. When the tracking dye (bromthymol blue) reaches the bottom of the gel plate. Turn off the power supply, carefully detach the electrical connection to the chamber, and remove your gel plate. Place it into the fixing solution (50/10) in a tray. After ~80 to 90 minutes, remove the gel plate and place it into the Coomassie Blue stain tray for ____ minutes. Then, transfer it to the destaining solution (10/10) to remove the blue stain from non-protein areas of the gel. After adequate destaining, photograph your plate showing the protein bands. Which dilution shows the cleanest result?

**Research Question, Part 3 Third Week**

Derive an interesting research question using native PAGE gels to resolve (answer) your question(s). You will set up your experimental design and run the native PAGE during the next week’s lab.

**Materials for Research Question.**
1. All the SDS PAGE materials above.
2. Alkaline phosphatase staining solution (Fisher)
3. Peroxidase staining solution (Fisher).