

Lab Week 3 – Separation of Complex Mixtures into Individual Components

Purpose: The purpose of this week’s lab is to use a chromatographic technique called gel filtration chromatography to isolate and purify individual components from complex biological mixtures.

Key Concepts/Terms:

Adsorption Chromatography	Gel Filtration	Ion Exchange
Affinity Chromatography	Chromatography	Chromatography
Chromatography	Immiscible phases	Partitioning
Denaturation		Stationary vs. Mobile phases

Laboratory Skills:

- (i) How to prepare a gel filtration column.
- (ii) How to run a gel filtration column to separate components from a mixture.
- (iii) How to collect fractions as they elute from the column.
- (iv) How to use the Bradford Assay to detect proteins in solution.

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 (Sephacrose, 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.

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 double 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.

The main application of gel filtration chromatography is in the purification of biological macromolecules. Viruses, proteins, enzymes, hormones, antibodies, nucleic acids and polysaccharides have all been separated and purified by use of appropriate gels. Mixtures of lower molecular weight compounds may also be separated. Thus, amino acids can be separated from peptides, and peptides obtained from the partial hydrolyses of a protein can be fractionated, as can oligonucleotides from a nucleic acid hydrolysate.

Materials

1. 1.0M potassium acetate buffer, pH 6.0 2 liters
2. 0.1M potassium acetate buffer, pH 6.0 5 liters
3. 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. DNP-glutamic acid 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 scintered glass filter for washing sepadex
12. Whatman filter paper disc and support to filter diluted Bradford Reagent
13. Syringe filters (0.45 u or 1 u) 3

Lab Exercise

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 daltons), cytochrome C (mw 12.3 kdaltons), a DNP amino acid (see black board for the mw of the specific DNP amino acid used for your lab), and BSA (approx. mw 66 kdaltons). You will pour an 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.

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 Sephadex to get a column that is between 20 and 25 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

Revised 05/03 krd and jcm