Lab #5

SIZE EXCLUSION CHROMATOGRAPHY

Adapted from: C. Greene MJC

INTRODUCTION:

Our bodies contain thousands of different proteins which perform many different jobs. Digestive enzymes are proteins; some of the hormones signals that run through our bodies and the antibodies protecting us from disease are proteins. The information for assembling a protein is carried (in code) in the DNA of our genes. The sections of DNA which contain the code for making proteins are called genes. There are thousands of genes on each chromosome. Each gene codes for a unique protein. The gene which makes a digestive enzyme in your mouth is different from one which makes an antibody. Proteins vary greatly in size, and this principle can be used to separate certain proteins from others of differing size.

Proteins are often products sought to be used for medical purposes. Some of these proteins are purified in large quantities from a naturally-occurring source. Recently, many proteins for medical purposes have been made through genetic engineering and recombinant DNA technology. No matter what the source, a protein of interest is found in a mixture of a cell's other proteins. Some cells, such as bacteria, produce large quantities of up to two thousand different kinds of proteins.

Since 75% of the dry matter in living things is protein, biologists must often purify a protein of interest from other proteins in a cell. Determining the procedure for the purification of a particular protein is a challenging task for the biotechnology industry. To separate any of the macromolecules, scientists utilize their knowledge of the chemistry of these molecules, including; the molecular weight of the protein (size), its charge, and its shape.

Chromatography is commonly used in biotechnology for purifying biological molecules, like proteins, for medicine or other uses. Chromatography separates individual components from complex mixtures. Chromatography consists of a mobile phase (solvent and the molecules to be separated) and a stationary phase either, in paper or glass beads, called resin, (in column chromatography), through which the mobile phase (sample) travels.

Molecules travel through the stationary phase at different rates because of their chemistry.
In **size exclusion chromatography** (SEC), microscopic beads which contain tiny holes are packed into a column. When a mixture of molecules is dissolved in a liquid and then poured onto a size chromatography column that contains porous beads, large molecules pass quickly around the beads, whereas smaller molecules enter the tiny holes in the beads and pass though the column at a significantly reduced rate. Depending on the molecules, proteins many be separated based on their size and fractions containing the isolated proteins can be collected

The mass of beads within the column is often referred to as the column bed. The beads act as "traps" or "sieves" and function to filter small molecules which become temporarily trapped within the pores. Larger molecules pass around, or are "excluded" from, the beads. The column you will be using is prefilled with beads that effectively separate or "fractionate" molecules that

are below 60,000 daltons. As the liquid flows through the column, molecules below 60,000 daltons enter the beads and pass though the column more slowly. The smaller the molecules, the slower they move through the column. Molecules greater than 60,000 pass around the beads and are excluded from the column quickly - also referred to as the **exclusion limit** of a column. Different columns have different exclusion limits.

The liquid used to dissolve the biomolecules to make the mobile phase is usually called a buffer. The mixture of biomolecules dissolved in the buffer is called the sample. The sample is placed on the column bed and the biomolecules within the buffer enter the top of the column bed, filter through and around the beads, and ultimately pass through a small opening at the bottom of the column. For this process to be completed additional buffer is placed on the column bed after the sample has entered the bed. Then mobile phase liquid is collected, as drops, into collection tubes which are sequentially ordered. A set number of drops is usually collected into each tube. The larger molecules which pass quickly through the column will end up in the early tubes or "fractions". Then smaller molecules which penetrate the pores of the stationary phase end up in the later fractions.

Hemoglobin and vitamin B12 are the two molecules which are being separated in the lab activity. Hemoglobin, which is reddish/brown, has a molecular weight of 65,000 daltons and is thus excluded from the column resin beads. Hemoglobin will pass more quickly through the column and appear in the early collection tubes, or **fractions**. Vitamin B 12, which is pink, has a molecular weight of 1,350 daltons and is thus fractionated (slowed down) by the column. The vitamin B 12 molecules penetrate the pores of the beads, becoming temporarily trapped. As a result, they pass much more slowly through the column and should appear in the later fractions.

1. SIZE EXCLUSION CHROMATOGRAPHY SEPARATION:

Objectives:

- Demonstrate an understanding of the principles and operations of size exclusion chromatography
- Separate biological molecules from blood serum using size exclusion chromatography

Materials:

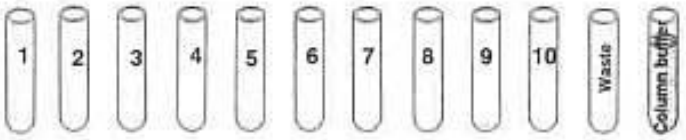
12 collection tubes
1 Size exclusion chromatography column
1 Column end cap
Disposable pipette / P-1000
Test tube rack

PROCEDURE: 

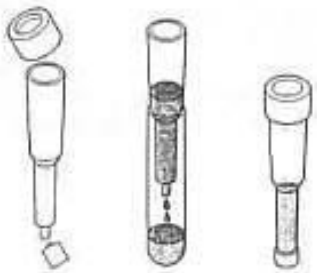
Follow the Laboratory Quick Guide for Size Exclusion Chromatography on the next two pages. Apply the solution provided by your instructor.

Laboratory Quick Guide
Size Exclusion Chromatography Kit

1. Obtain 12 collection tubes and label ten sequentially from 1 to 10. Label the tubes with your name and laboratory period. Label the final two tubes "Waste" and "Column Buffer". Using a clean pipette, transfer 4 ml of column buffer into the tube labeled "Column Buffer".

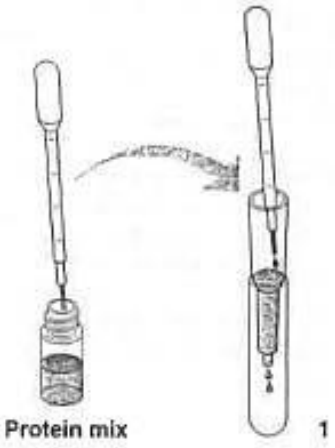


2. Remove the cap and snap off the end of the sizing column. Allow all of the buffer to drain into the waste tube. Observe the upper surface of the matrix and insure that all of the buffer has entered the column. Looking directly over and into the column, you should see the "grainy" appearance of the column matrix. Cap the bottom of the column.

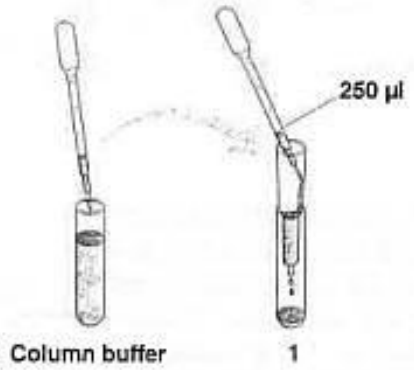


3. Carefully place the column onto tube 1. You are now ready to load (or the teacher may load) the protein sample onto the column.

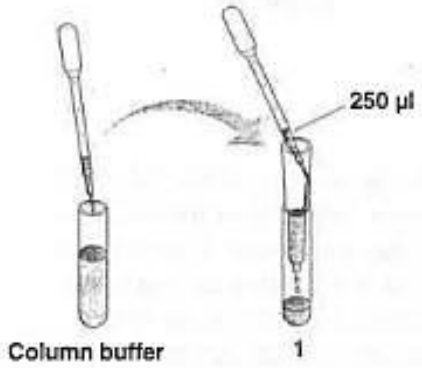
4. When you are ready to load the protein mix, uncap the column. It is important to uncap the column only when you are ready to load your protein—you do not want your column to run dry. Using a pipette, add one drop of protein mix onto the top of the column bed (your teacher may do the loading for you). The pipette should be inserted into the column and the drop should be loaded just above the top of the column so that it minimally disturbs the column bed.



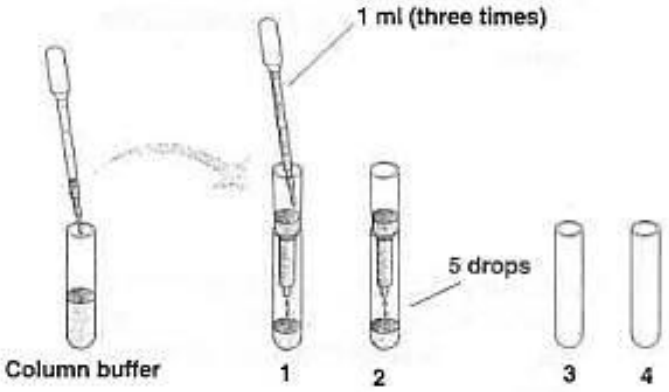
5. As soon as the drop of protein mix enters the column bed, carefully add 250 µl of column buffer to the top of the column. This is best done by inserting the pipette tip into the column so that it rests just above surface of the column matrix. Carefully let the buffer run down the side of the tube and onto the top of the bed. (Note: The size separation will work best when the column bed is left undisturbed). Begin to collect drops into tube 1.



6. Add another 250 µl of column buffer to the top of the column. Add the buffer as before, by placing the pipette just above the top of the column and letting the buffer run down the side of the tube. Continue to collect drops into tube 1.

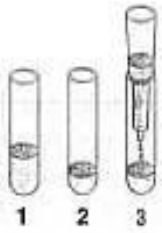


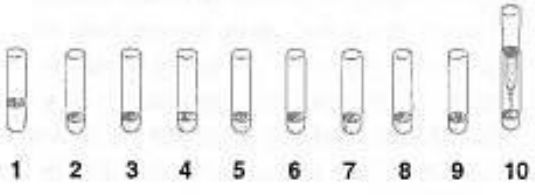
7. Add 3 ml of column buffer to the top of the column matrix. This can be done by adding 1 ml three times from the pipette. At this time the protein mix has entered the column far enough so that slight disturbances to the column bed will not affect the separation. Transfer the column to tube 2 and begin to count the drops that enter into each tube. Collect 5 drops of buffer into tube 2.



8. When 5 drops have been collected into tube 2, transfer the column onto tube 3. Collect 5 drops of buffer into each collection tube. When 5 drops have been collected into a tube, lift it off and transfer it to the next tube.

9. Continue collecting 5 drops into each tube. When you reach tube 10, collect a total of 10 drops. Cap the column and if your teacher instructs you to do so, parafilm or cover your fractions until the next laboratory period. Store the fractions in the refrigerator. Sketch your results.





SAMPLE COLLECTION TUBES:

Tube 1) Pre-collection
Tubes 2-10) 5 drops from column in each tube

2. DETERMINATION OF THE ABSORPTION SPECTRUM OF VITAMIN B12:

An absorbance spectrum (a plot of absorbance as a function of wavelength) is determined to select the optimal wavelength for analyzing a given compound. The optimal wavelength (Amax} for measuring absorbance is that wavelength that is most absorbed by the compound in question. This provides maximum sensitivity for your measurements.

An absorption spectrum is a visual representation of how well a particular photochemical absorbs different wavelengths of light. Absorption spectrums are useful pieces of information and can be employed in a number of ways in a research lab. Once the peak absorption (wavelength in which the photochemical absorbs or transmits the most amount of light) is determined, a spectrophotometer set at that wavelength can be used to quantify the amount of that substance in solution. The amount of light absorbed is directly proportional to the amount of substance in solution; the greater the amount of substance in solution, the greater amount of light absorbed and visa versa. By comparing the amount of light absorbed of an unknown amount of substance to that of a known quantity of the same substance, through the use of a standard curve, the quantity of the substance can be roughly determined. Since the technique is quick and does not require costly reagents, it is feasible to consider using spectrophotometry as a quantification technique. In this case, we are using absorbance to differentiate between tubes that contain vitamin B12 and those that do not.

Of course, the usefulness of the technique hinges on the fact that the substance being tested does in fact absorb light. Vitamin B 12 is considered to be a photochemical and should lend itself to being quantified using this technique.

DETERMINATION OF THE ABSORPTION SPECTRUM OF VITAMIN B12:

MATERIALS:
Vitamin B12 solution (5.625 µM or 562.5nM)
Cuvettes
P-1000 Micropipettor and blue tips
Spectrophotometer

Objectives:

1. Determine the Absorption Spectrum for Vitamin B12
2. Identify chromatography samples containing Vitamin B12 and those that do not

PROCEDURE: 

Follow the same general process that you utilized in the spectrophotometry lab.

SPECTROPHOTOMETER OPERATION:

1. Press in the power button (located on the back of the instrument} to the ON position.
2. Let the machine warm up for 10 min before you use it
3. Enter the desired wavelength [**380nm**]
4. Wipe off your cuvette containing the blank solution [**column buffer**] with a Kim Wipe and place it in the single cuvette holder.

   *Make sure that the cuvette is aligned with the light source. Be sure to have the **clear** faces of the cuvette facing towards the **front** of the machine!*
5. Close the sample compartment door.
6. Press the **AUTO ZERO** button on the keypad. The display shows "ZEROING ... " This should result in 0% Absorption.
7. Remove the blank.
8. Insert the Vitamin B12 Sample (provided) into the sample compartment and close the sample compartment door.
9. Read the absorbance displayed and record it
10. Remove the cuvette, but save the cuvette and its contents.
11. Adjust the Spectrophotometer to 390nm and repeat steps 8 – 10, ranging from wavelengths 380nm to 680nm.
12. Record your results on your worksheet.

3. SPECTROPHOTOMETRIC DETECTION OF VITAMIN B12:

An absorbance spectrum (a plot of absorbance as a function of wavelength) is determined to select the optimal wavelength for analyzing a given compound. The optimal wavelength (Amax} for measuring absorbance is that wavelength that is most absorb

MATERIALS:

Tubes collected from Size Exclusion Chromatography separation
Cuvettes
P-1000 Micropipettor and blue tips
Spectrophotometer

Objectives:

1. Use the Absorption Spectrum for Vitamin B12 to identify chromatography samples containing Vitamin B12
2. Demonstrate the separation of Vitamin B12 and Hemoglobin from blood serum

PROCEDURE: 

Follow the same general process that you utilized in the spectrophotometry lab.

SPECTROPHOTOMETRIC DETECTION OF VITAMIN B12:

1. Use the spectrophotometer to detect the presence of vitamin B12 in your chromatography samples.

   NOTE: You may need to increase the volumes of your samples to read them in the spectrophotometer – check with your instructor. What solution should you use to do this?

   NOTE: You will set the spectrophotometer for the Amax you determined for vitamin B12 in part 2 of this exercise.

   NOTE: You should use column buffer to blank the spectrophotometer to 0% Absorption.

NO CONTENT