Texas A&M University-Corpus Christi
CHEM4402 Biochemistry II Laboratory
Laboratory 12: Gel Permeation Chromatography

During the last laboratory period, we prepared cell-free extracts of GFP from fully induced bacteria. Today, we will further purify GFP using gel permeation chromatography. Chromatography, along with solvent extraction, precipitation, filtration and centrifugation, is one of the primary methods used for the purification of biomolecules. Experiments to determine properties such as molecular weight, three-dimensional structure, protein function or catalytic activity require it to be in a fairly pure state. Other cellular constituents such as lipids, other proteins, carbohydrates or small metabolites can interfere with the collection of data or impair the activity of the protein We therefore need methods to remove as much cellular "debris" as possible.

Some methods, such as pulverization of tissue followed by centrifugation or solvent extraction are crude but effective. For example, extraction of ground tissue with a non-polar solvent, such as chloroform, does a remarkable job of removing most hydrophobic material (lipids and many proteins). The remaining sample can then be subjected to additional fractionation steps to remove remaining contaminants. These take advantage of differences in physical or chemical properties (e.g. molecular weight, polarity, solubility or binding affinity) to fractionate the compound of interest away from the remaining cellular material.

One of the most powerful fractionation methods is chromatography. Chromatography makes use of stationary and mobile *phases* that have different affinities for the compound of interest. As its name implies, the *stationary* phase is immobile, and generally serves to retain the biomolecule of interest. The *mobile* phase serves to transport the biomolecule-containing mixture through the stationary phase, which preferentially binds the biomolecule of interest, allowing poorly retained molecules to pass on. In column chromatography the stationary phase is generally a solid media while the mobile phase is a liquid buffer or solvent.

There are three basic types of column chromatography: ion exchange, size exclusion and affinity. Ion exchange chromatography separates compounds on the basis of electrical charge. The ion exchange stationary phase, or resin, can be classified as either a **cation** or **anion** exchanger. Cation exchange resins are negatively charged, and bind positively charged molecules (cations), allowing negatively-charged molecules to pass on. Anion exchange resins do the same for negatively-charged molecules. Size exclusion chromatography, often referred to as *gel permeation* chromatography, separates compounds on the basis of size (figure 1). The stationary phase for gel permeation chromatography is composed of very small, inert, porous beads. The size of the pores defines the size of molecule retained; the smaller the pore, the smaller the molecule that can be retained, allowing larger molecules to pass on and become separated. Affinity chromatography, on the other hand, relies upon a physical interaction between the biomolecule to be separated and a ligand attached to the chromatography media. A prime example is the use of maltose as a ligand. Maltose binds to, and separates, proteins which have an affinity for carbohydrates.

In today's experiment we will be using gel permeation chromatography to separate GFP from the rest of the material in the crude cellular extract. Recall that our only purification step thus far has been a centrifugation step to remove much of the large, insoluble cell debris. We now use

chromatography to separate GFP from most of the remaining cellular protein, lipid, nucleic acids, carbohydrate and other small metabolites.

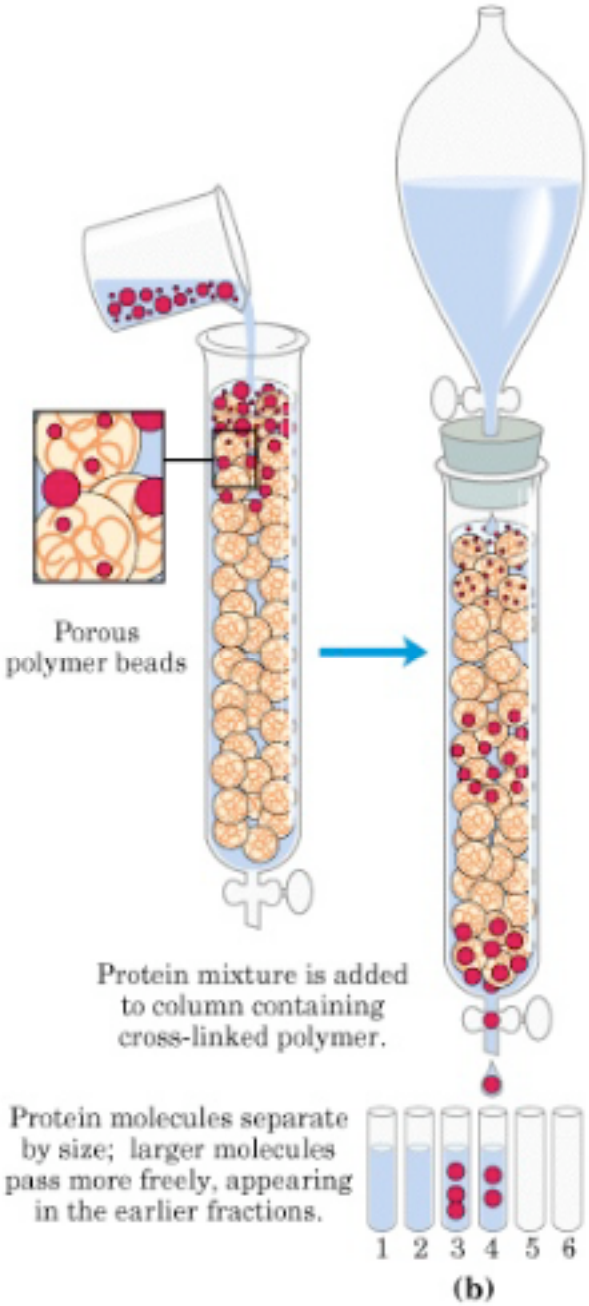

Figure 1. Gel permeation (size exclusion) chromatography (from Lehninger Principles of Biochemistry, Nelson & Cox, 3rd Ed., 2000).

Materials

25 ml Sephadex G-50 resin	200 ml 50 mM ammonium bicarbonate
100 ul SDS loading buffer	30 x 1 cm column
3 microcentrifuge tubes	column stand
11 glass test tubes (13 x 100 mm)	column stopcock
spectrophotometer	waste beaker

Column specifications

Dimensions	30 cm x 1 cm
Bed height	20 cm
Flow rate (gravity)	approximately 2 ml/min (~ 1 drop/2 sec)
Sample loading	GFP solution
Temperature	room temperature
Mobile phase	50 mM ammonium carbonate
Stationary phase	Sephadex G-50

Procedure

Follow the instructions below to pour a gel permeation chromatography column and load the GFP solution prepared during the last laboratory period. Collect 10 fractions from the column and measure the absorbance at 476 nm, the wavelength of maximum absorption for GFP. Save an aliquot from the fraction with the highest absorbance reading (*max*) and from those fractions just before (*max-1*) and after (*max+ 1*) the highest reading fraction. These will be analyzed on a SDS-PAGE gel next week, along with samples from the timed induction series also collected last week.

A. Column Packing

1. Mount a chromatography column on a column stand at your station. Adjust the clamps until the column is vertical.
2. Secure the stopcock in place on the bottom of the the column and turn the handle to the **closed** (12 o'clock) position. Obtain 200 ml of 50 mM ammonium bicarbonate buffer.
3. Check for leaks by filling the column 1/2 full with with buffer. Open the stopcock (9 o'clock position) and drain the buffer into a waste beaker. Turn the stopcock back to the **closed** position.
4. Obtain a bottle of Sephadex G-50 media ("gel"). You should notice two layers in the bottle. The bottom layer is Sephadex G-50 while the top layer is excess 50 mM ammonium bicarbonate. Swirl the bottle to resuspend the Sephadex media. **As demonstrated by your instructor**, steadily pour the slurry and fill the column in one smooth motion with no stops.
5. Allow the sephadex to **settle for three minutes** in your column.
6. Open the stopcock and allow the column to pack by **slowly draining buffer** into a waste beaker until the Sephadex G-50 gel stops settling (i.e. the media volume no longer changes). At this point the column is packed. Continue draining buffer until it is approximately 1 cm above the packed. **Do not allow the buffer to drain below the top of the gel!**

B. Sample Loading and Fraction Collection

1. Number ten test tubes along with a blank tube (B) and place them in a rack.
2. Collect your GFP solution (15 ml tube) from freezer and thaw in a 37ºC water bath.
3. Fill your blank tube with 2.0 ml of 50 mM ammonium bicarbonate. Using this tube as a guide, mark the rest of your test tubes with a small line to indicate the 2.0 ml volume level**.**
4. Carefully drain the last 1 cm of buffer from the top of your packed gel. The buffer level should now be just even with the top of the gel. Take care not to let any portion of it to run dry.
5. Using a disposable glass pipet, transfer your cell-free extract to the top of the gel by *gently swirling the tip of the pipet around the inside edge of the glass column, just above the top of the gel*. Take care not to disturb.
6. Prepare to take fractions by removing the waste beaker and placing your test tube rack (containing your sample tubes) under the stopcock. Be sure that your tubes are in the correct order for collecting fractions (1-10). From this point on, you will collect the solution (eluant) coming off of the chromatography column into the test tubes. Fill each test tube to the 2.0 ml mark you made in step 2 before moving on to the next tube. Each filled tube is called a **fraction**.
7. **Begin taking fractions now**. Slowly open the stopcock and allow the sample to flow completely into the gel, collecting the eluant in test tube #1. Adjust the flow rate to 1 drop every 2 seconds. When the sample on top of the column has moved completely into the gel, close the stopcock to stop any further flow.
8. Using a new glass pipet, add 50 mM ammonium bicarbonate buffer to a height of approximately 5 cm above the top of the packed column. Open the stopcock and resume taking fractions. Adjust your flow rate to 1 drop every 2 seconds. Do not close the stopcock from this point on.
9. Carefully continue to add 50 mM ammonium bicarbonate buffer to the top of the gel while you take fractions. The best way to do this is to work as a team, with one lab partner collecting the fractions while the other maintains a consistent 5 cm "column" of buffer on top of the gel. Maintaining a consistent volume of buffer provides a constant flow rate through the column.
10. Continue taking 2 ml fractions until the 10th tube is filled. The easiest way to do this is to adjust your column until the stopcock is just above the tubes in the rack. When a tube is filled to the 2 ml mark, simply slide the rack to the next tube position.

C. Spectrophotometry

1. Turn on the spectrophotometer and set the wavelength to 476 nm. Allow spectrophotometer to warm up for 10 minutes.
2. Fill your column with ammonium bicarbonate buffer and open the stopcock completely. Wash your chromatography media of any residual proteins by adding additional buffer until you have run 100 ml through the column.
3. **Add an additional 2 ml of ammonium bicarbonate buffer to each of your samples and the sample blank**, bringing the total volume up to 4 ml.
4. Zero the spectrophotometer with your sample blank, which contains only buffer. Record the absorbance of each of your samples at 476 nm. Be sure the exterior of each tube is dry prior to placing it in the spectrophotometer.
5. Based on your A_{476} readings, **transfer 25 ul from the fraction with the highest reading (*max)*** to a microcentrifuge tube. **Add 25 ul of SDS loading dye**. Repeat for the fractions just before (*max-1)* and after (*max+1*) your highest reading sample. You will analyze these samples on a polyacrylamide gel next lab period. Be sure to mark each microcentrifuge tube with the appropriate fraction number and your initials. Place in your lab section's freezer box along with your GFP induction samples from last week.

D. Cleanup

1. Ask your instructor to remove the column media back to the bottle. They will do this using forced air. **Do not pour the sephadex down the sink or into the trash.**
2. Flush your column well with distilled water from the sink, as demonstrated by your instructor.
3. Remount your column on your stand in an *inverted* position to dry. Place a paper towel beneath the column to absorb any water that drips off.
4. Rinse the stopcock thoroughly and place on your paper towel to dry. Be sure to also save the column top and the nipple that covered the column outlet.
5. Pour your test tubes into your waste beaker. Used test tubes may be disposed of in the **glass waste containers**. Pour the contents of your waste beaker down the sink and wash well with soap and water. Rinse once with distilled water and place on dish rack to dry.

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Reading Assingment: Lehninger Ch. 3.3 *Working with Proteins*

1. Prepare a graph of your A_{476} absorbance values (Y-axis) vs. fraction number (X-axis) using Microsoft Excel (or equivalent). Prepare a legend that contains a figure no. as well as a one-sentence description of your graph. As part of the legend text, also identify which fractions had the highest absorbance reading (*max*) as well as the max_{-1} and max_{+1} readings (6 pt).

2. How does *High Performance Liquid Chromatography* differ from regular column chromatography? (2 pt)

3. Suppose you have a mixture of 4 proteins with the isoelectric (*pI*) points listed below. Which protein(s) will be retained on a *cation* exchange column when the mobile phase consists of a buffer with pH = 7.0? Why? (Recall: What is the charge on a molecule when the pH is greater than it's pI?, less than pI?) (2 pt)

Protein	pI
A	2
B	5
C	7
D	8

4. Laboratory performance (2 pt).