EXPERIMENT 3: SEPARATION BY GEL FILTRATION AND ELECTROPHORESIS

A. GEL FILTRATION CHROMATOGRAPHY

BACKGROUND:

Gel Filtration Chromatography (GPC), also called size exclusion chromatography/Gel Permeation Chromatography/Molecular Sieve Chromatography, is a technique used for separating chemical substances by size, based upon their migration through a stationary bed of a porous, semisolid substance (gel). The method is very useful for separating enzymes, proteins, peptides and amino acids from each other and from substances of low molecular weight (e.g. salts and enzyme cofactors). Small molecules diffuse into the interior of the porous stationary phase particles so that their flow is restricted, while large molecules are unable to enter the pores and tend to flow unhindered. Thus, the components of highest molecular weight leave the bed first, followed by successively smaller molecules. The bed materials most extensively used are agarose, polyacrylamide (Bio-Gel) and a polymer prepared from dextran and epichlorohydrin (Sephadex). The dry polymers are usually suspended in suitable agents to form a homogeneous, semisolid mixture.

When a gel filtration material is placed in an aqueous solution it swells. The degree of swelling is dependent upon the degree of cross-linking of the material; the lower the cross-linking the more swelling and the more fragile the gel will be. After a gel has been equilibrated in a starting buffer it is poured into a column and allowed to settle (column packing can occur by gravity flow or with the application of pressure). When a mixture of compounds is allowed to pass through the column filled with the gel, the compounds will penetrate into the gel matrix to an extent governed by their molecular weights. As buffer is passed through the column the compounds will be separated on the basis of their size. The smaller a compound is, the longer it will be retained on the column.

When a compound is too large to penetrate the gel matrix it passes through the column by traveling around the swollen gel particles. Such a compound is eluted in the void volume, $V_0$, which represents the volume of buffer contained in the column. Blue Dextran 2000 is a dextran with a molecular weight of ~ 2 million Da. It is often used to determine the $V_0$ of a GPC column for two reasons. It is too large to penetrate the gel matrix, and its blue color makes easy to see. Skewing or streaking of the blue band as it moves down the column indicates a poorly packed column. The blue band should move uniformly down the column but will become broader with movement down the column because of lateral diffusion (especially at slower flow rates).
Nomenclature in GPC Chromatography

In a GPC column the stationary phase has two “volumes” associated with it. The volume inside the pores ($V_i$) and the mobile phase volume outside of the pores ($V_o$).

$V_o$ - Column void volume. This is a measure of the mobile phase in the column.

$V_i$ - Volume inside the sorbent pores (stationary phase volume)

$V_t$ - Total volume of the column ($V_t = V_{\text{bead}} + V_o$), also called $V_{\text{bed}}$

$V_e$ - Elution volume for a compound

$V_n$ - Adjust elution of a compound ($V_e - V_o$)

Unfortunately, it is difficult to determine $V_i$, the volume of the little pores. So, let’s cheat a little and say that the volume of the “stationary phase” ($V_i$) is the volume of the pores plus the volume of the beads that contain them. So, the general equation for partition chromatography

$$V_e = V_o + KV_i$$

Becomes

$$V_e = V_o + K_{av} V_s$$

Where $V_s$ is the volume of the beads plus pores. So, $K$ isn’t a true partition coefficient, and we have to change its name to $K_{av}$.

This pseudo partition coefficient is calculated as:

$$K_{av} = \frac{(V_e - V_o)}{(V_i - V_s)}$$

A plot of $K_{av}$ versus log molecular weight for a number of globular proteins gives a straight line relationship. However, the $V_e$ actually depends on the shape of the molecule, so the dependence
on molecular weight is only approximate.

**Other Important Equations:**

The column you used in this experiment is a 1.1 i.d. x 50 cm column

\[
\text{Volume of a cylinder} = \pi r^2 h
\]

\[
\text{Cross-sectional area of a cylinder} = \pi r^2
\]

**REAGENTS:**

**Blue Dextran 2000:**

Blue dextran is composed of a dextran with a molecular mass of 2,000,000 Da with blue dye covalently attached. It is often used to determine the void volume of a gel permeation column. Its Uv-Vis $\lambda_{\text{max}}$ is 621 nm.

**Myoglobin:**

Myoglobin is a protein found in the muscle cells of animals. It functions as an oxygen-storage unit, providing oxygen to the working muscles. There is a close chemical similarity between myoglobin and hemoglobin, the oxygen-binding protein of red blood cells. Both proteins contain a molecular constituent called heme, which enables them to combine reversibly with oxygen. The heme group, which contains iron, imparts a red-brown color to the proteins. The molecular mass of myoglobin is 16,952 Da. Its Uv-Vis $\lambda_{\text{max}}$ is 409 nm.

**Vitamin B12** (Cyanocobalamin)

A complex water-soluble organic compound that is essential to a number of micro-organisms and animals, including humans. Vitamin B$_{12}$, known as the anti-pernicious-anemia factor, is also known as the extrinsic factor—a substance from outside the body—that aids in the development of red blood cells in higher animals. The vitamin, which is unique in that it contains a metallic ion, cobalt, has a complex chemical structure. The molecular mass of B$_{12}$ is 1,355 Da and its $\lambda_{\text{max}}$ is 361 nm.

**Horse Radish Peroxidase:**

Enzyme involved in the oxidation of ascorbate and phenols using hydrogen peroxide as one its substrates. For today’s’ lab we will a guaiacol/hydrogen peroxide substrate to test column fractions for peroxidase activity. In the reaction catalyzed by peroxidase, guaiacol is oxidized to tetra-hydroguaiacol in the presence of hydrogen peroxide. Guaiacol is a clear uncolored solution but turns brown when oxidized. Hence, the activity of the enzyme can be followed photometrically at 420 nm. The column buffer for the lab is 0.1 M Sodium Acetate pH 6.0/0.4 M Sodium Chloride. This buffer system was primarily chosen because the pH optima for peroxidases is pH 6.0. The other components of the sample mix are not pH dependent, at least in the range of pH 4 to pH 8. The addition of 0.4 M NaCl helps prevent ion exchange between the
sample components and the Sephadex. Sephadex has several free carboxylic acids which attracts positively charged sample components. The high concentration of Na\(^+\) ions in the column buffer shields the overall negative charge of the Sephadex, ensuring that the only separation process taking place in the G 75 column is molecular sieving.

**Blue Dye #1**

Blue dye #1 is a blue dye with a molecular weight of 363. It can be used to measure the total volume gel permeation columns. It’s the same dye that is covalently attached to the blue dextran molecule and can be measured similarly at 621 nm.

200 ul of a mixed sample will be added to your column, which is equilibrated in a 0.1 \(M\) acetate, 0.4 \(M\) NaCl, pH 6.00. The final concentration of each sample component is:

- 5 mg/mL of Blue Dextran 2000
- 50 ug/mL peroxidase
- 2 mg/mL cyanocobalamin
- 2 mg/mL myoglobin
- 0.25 mg/mL Blue Dye #1.

**PROCEDURE:**

**Preparation of column and application of sample.** In order to expedite the experiment, the G75 Sephadex column will be prepared for you in advance. This is a lengthy procedure and must be carefully done to provide a column with excellent resolution. The column used in this experiment is a 1.1 i.d. x 50 cm glass column. The column is packed by first adding degassed (bubbles interfere with column packing) column buffer (0.1M sodium acetate pH 6.0/0.4M sodium chloride) to about 50\% total volume of the column. The G75 Sephadex was prepared earlier by mixing dry Sephadex G 75 powder with column buffer in a vacuum flask and gently swirling to completely swell the gel to its final volume (3-24 hrs). The swelled Sephadex is completely degassed without mechanical mixing (i.e. stirrer bar would crush the delicate sephadex beads). Instead, the Sephadex is periodically swirled by hand under vacuum to degas it (approximately 30-60 min). A packing funnel is attached to the top of the column, which has its outlet closed (i.e. column stop cock is closed), and the swelled Sephadex is carefully transferred from the vacuum flask into the packing funnel by slowly pouring it down a glass rod (to prevent rapid mixing which creates air bubbles). The column stop cock remains closed as the Sephadex gently sinks by gravity into the column, displacing the pre-added degassed column buffer upward to the packing funnel. The Sephadex gel continues to pack by gravity until all of it leaves the packing funnel and resides in the column. At this point, the stopcock of the column is opened and the column packs under gravity flow (buffer is
captured by a beaker below the column) for several column volumes. The Sephadex is prevented from leaving the column by a porous fritted disk at the bottom of the column, which only allows the column buffer to pass through. The column stop cock is then closed and the packing funnel is removed from the top of the column. At this point, a flow adapter is attached to the analytical column, and gently pushed down until the bottom of the adapter sits about 0.5 cm above the gravity packed Sephadex bed. The flow adapter rubber o-ring is expanded by closing the flow adapter trigger, which prevents column buffer and sample from exiting the top of the column. The flow adapter tubing is then attached to one end of the peristaltic pump with a tubing connector and care is given not to trap any air in the tubing. The other end of the peristaltic tubing is placed in a buffer reservoir (500 mL vacuum flask) containing degassed column buffer. This is now a closed system which prevents air from entering the column. It also prevents column buffer from leaving the column even when the column stop cock is open. Air is the bane of chromatographers because it dries out the Sephadex (destroying resolution). At this point, the column stop cock is opened and the peristaltic pump turned on at a flow rate which is 20% faster than the desired sample flow rate (i.e. 1.2 mL/min if the desired sample flow rate will be 1.0 mL/min). At least two column volumes of column buffer is pushed through the column (via peristaltic pump). This pressure flow causes the Sephadex bed volume to shrink slightly, which requires the flow adaptor to be subsequently adjusted by releasing the o-ring and carefully pushing the adapter down until it resides again just above the Sephadex bed. The o-ring on the flow adaptor is then re-engaged, locking the flow adapter to the column. The flow adapter bottom area contains a fritted disk, which gently disperses the sample and column buffer evenly over the entire surface area of the gel bed (the flow adapter prevents column and sample flow from disrupting the top of the gel bed as this outcome changes bed height and shape, reducing chromatography resolution and precision). The porous disk also traps sample particulates from getting on the Sephadex, an outcome which rapidly deteriorates the Sephadex gel. Typically, all samples are centrifuged at 13,000 X G, to remove particulates. Periodically, the disk must be replaced as sample build up on the disk causes back pressure on the column, which will reduce flow rate and deteriorate column performance. Once the column has been prepared as above it is connected to a fraction collector. The TA’s will demonstrate how to use the peristaltic pump and fraction collector.
Do not let the column go dry under any circumstances.

1) Measure the bed volume of your stationary phase. This will be equivalent to the total volume of the stationary phase in the column ($V_t$). Measure the diameter of the column (cm) and height of the stationary phase (cm). The volume of a cylinder $v = \pi r^2 h$. This will give units of cm$^3$ or mLs. Calculate the exact flow rate of your column by turning on the peristaltic pump and collecting the eluate into a 25 mL graduated cylinder. Your column flow rate should be about 0.8-1.0 mL/min. Time how much volume comes through in 10 minutes and calculate your flow rate by dividing time collected into volume collected. You will use this information to program the fraction collector to collect 1.0 mL fractions.

3) Once you have determined your exact flow rate and have programmed the fraction collector, randomly check several tubes in the fraction collector to make sure they are collecting column eluate. The TA will demonstrate how to manually cycle through your tubes. Once you have assured yourself the tubes are properly collecting eluate, turn off the peristaltic pump and return the fraction collector arm to its initial position by pressing the END button. Many a chromatographers’ day has been ruined by returning to the fraction collector only to find the eluate has missed the collection tubes and is instead dripping onto the lab bench 😞.

4) 200 ul of mixed sample will be provided to you in a 1.5 mL snap cap bullet shaped vial. Be careful not to shake the contents or your sample or it will be dispersed on the sides of the vial, requiring centrifugation. The peristaltic pump should be off. Remove the tubing from the column reservoir (500 mL vacuum flask), and carefully place it in your sample so that the tubing touches the bottom of the sample vial. Care should be taken not apply excessive pressure to the tubing which could prevent sample from getting in the tubing (the tubing has been cut at a 45° diagonal to help prevent this outcome). Turn the pump on, and carefully watch as the sample moves up the tubing. Stop the pump before the entire sample is removed. This action keeps air from gaining access to the column. Remove the tubing from the sample container, wipe off the end of the tubing with
a Kimwipe and submerge it back into the column reservoir. Start the pump, but not the fraction collector. Owing to the combined color of Vit B₁₂, Blue Dextran, and Blue dye, your sample will be easy to follow as it travels up the column tubing, enters the flow adapter, and finally is dispersed evenly onto the column Sephadex bed. The moment you see the colored sample entering the Sephadex gel, push the Start button of the fraction collector to begin collecting fractions.

6) Continue collecting eluate in 1 mL fractions until the blue dye#1 has all come off the column (usually ~ 45 tubes).

ANALYSIS

1) The Blue Dextran 2000 should move uniformly down the column in a narrow band and be collected in Fractions 10-12.

2) The next substrate off the column will be the peroxidase. The maximum peroxidase activity should be in approximately Fraction 14 but ask your TA for guidance.

To quantitatively visualize and locate the peroxidase peak do the following:

A) Start with the first test tube the TA indicates should have activity.

B) Adjust the spectrophotometer to read absorbance at 420 nm.

C) Zero against 3.0mL of the quaiacol/hydrogen peroxide substrate solution.

D) Add a 100 ul aliquot from each fraction your TA has directed you to take to a separate tube containing containing 3.0 mL of peroxidase substrate.

E) Exactly 3 min later determine the absorbance at 420nm in your Spec 20.

3) In the mean time have your lab partner(s) begin to read the remaining volume of liquid (~900 µl) in each fraction using the Shimadzu Uv-Vis spectrophotometer. Transfer the fraction into a microcuvette and read absorbance at λ 621 nm, 409, and 361 nm (blue dextran/blue dye#1, myoglobin, B12). Save this data to your USB drive for future plotting in Excel.

DATA HANDLING:
1. Record all absorbance readings in **Table #1**.

<table>
<thead>
<tr>
<th>Test tube number</th>
<th>Volume (mL)</th>
<th>Absorbance at 621 nm Blue Dextran</th>
<th>Absorbance at 420 nm Peroxidase</th>
<th>Absorbance at 499 nm Myoglobin</th>
<th>Absorbance at 361 nm B12</th>
<th>Absorbance at 621 nm Blue Dye#1</th>
</tr>
</thead>
</table>

2. Normalize the area of each peak curve by dividing the data by the greatest absorbance (the apex of each peak) for each wavelength. This will normalize all peak heights to 1. Calculate absorbance values relative to this value. Report these data in a separate table (**Table #2**). Note: the blue dye #1 peak will not achieve an absorbance maximum due to the presence of blue dextrin. Do not worry about this outcome, because the blue dye#1 peak will not be used for theoretical plate calculations described in Task 4. below.

3. Prepare a graph of the elution volume (x axis) versus the Uv-Vis normalized data for each compound applied to the GPC column (**Figure #1**).

4. Prepare a second graph of $K_{av}$ and $V_e/V_0$ (plot these two together on the same graph) versus the logarithm of the molecular weight peroxidase, myoglobin, and B12 but not for Blue Dextran 2000 or Blue Dye#1 (**Figure #2**). Calculate the equation for x and y using linear regression analysis. Report the $R^2$ value for these lines.

   Report calculated data in **Table #3** as follows:

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW</th>
<th>logMW</th>
<th>$V_0$</th>
<th>$V_t$</th>
<th>$V_e$</th>
<th>$V_n(V_e-V_0)$</th>
<th>$V_i(V_t-V_0)$</th>
<th>$K_{av}$</th>
</tr>
</thead>
</table>

5. Evaluate the theoretical plate numbers for peroxidase, myoglobin, and B12 using the formula $N = 8 \left( \frac{V}{\beta} \right)^2$ where:

   $N$ = number of theoretical plates
   
   $V$ = elution volume of a compound
   
   $\beta$ = is the width of the elution peak at the height ($\lambda_{max} / 2.72$)

   **To calculate $\beta$**

   $\beta$ is calculated by measure the peak width at ($\lambda_{max} / 2.72$). Using **Figure #1**, first determine where the apex of the peak is. Since this data corresponds to a normalized value of 1, the $\lambda_{max} / 2.72$ will equal 1/2.72 = .37. Measure the width of this peak, at half height, in terms of volume.

   Report Data in a **Table #4** as follows:
<table>
<thead>
<tr>
<th>Compound</th>
<th>Elution volume (mL)</th>
<th>Peak width (mL)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myoglobin</td>
<td></td>
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<tr>
<td>B12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue Dye#1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Chromatography Questions:**

1. Explain why the data points for Blue Dextran and Blue Dye#1 should not fall on the straight line drawn in the second graph.

2. Based on your data, indicate how well your column performed as a measure of how well the Blue Dextran 2000 and Blue Dye#1 were resolved.

3. If you had an unknown protein with a $K_{av}=0.21$, what would be its molecular weight and $V_e$ based on your calibration data obtained in lab 9 show your calculations)?

4. What advantages (if any) are there to plotting $K_{av}$ vs. $V_e/V_o$ against molecular weight?

5. Does your calculated $V_t$ compare well with your determined $V_t$? If not explain why.
B. Electrophoresis