

Determining the Molecular Weight of Amylase by Gel Filtration/Size Exclusion Chromatography

Introduction

In previous exercises you have isolated organisms that degrade starch, you have partially characterized the enzyme that degrades starch, amylase, and you have even cloned the amylase gene. As a biotech scientist, your next step would be to isolate the enzyme away from other proteins present in the culture supernatant. Biologists often try to purify a protein of interest from other proteins in a cell. Part of protein purification and characterization is determining the molecular weight of a protein. This week you will be purifying and determining the molecular weight of amylase from *Bacillus licheniformis*.

Learning objectives

Conceptual

- Proteins can be separated from one another by their physical and chemical properties including:
 - Size
 - Charge
 - Solubility
 - Affinity for other molecules
- The separation of proteins can be used analytically to determine various properties of a protein or be used to purify proteins for analysis or for commercial production.

Practical

- The use of standard curves to characterize an unknown
- Calculation of the molecular weight of a protein by gel filtration chromatography.
- The use of enzyme assays to detect the presence of proteins.

Underlying Science

Chromatography is used to separate organic compounds on the basis of their charge, size, shape, affinity, or solubility. A chromatography set up consists of a mobile phase (the solvent and the molecules to be separated) and a stationary phase either of paper (in paper chromatography), a porous solid matrix or a resin, (in column chromatography) through which the mobile phase travels. Because of their chemical properties, molecules travel through the stationary phase at different rates and are separated from one another. Three basic types of column chromatography are illustrated in Figure 1 below.

In gel filtration chromatography (Figure 1A), microscopic, polyacrylamide beads containing small pores are packed into a column. A protein sample is applied to the top of the column and passes through the column with the solvent. As the sample moves through the column, *smaller molecules pass through the pores in the beads* taking a longer path through the column than *larger molecules that cannot enter the beads and simply travel around them*. THEREFORE PROTEINS ARE SEPARATED BASED UPON THEIR SIZE

WITH THE LARGER THE MOLECULES PASSING THROUGH THE COLUMN FASTER THAN SMALLER ONES. (NOTE: This is exactly opposite of what happens in an agarose gel). The solvent containing the proteins is collected into a series of tubes as it emerges from the bottom of the column. The samples collected in these tubes are referred to as "fractions" and contain the separated proteins. In gel filtration chromatography, larger proteins are present in the first fractions while smaller proteins are present in the later ones.

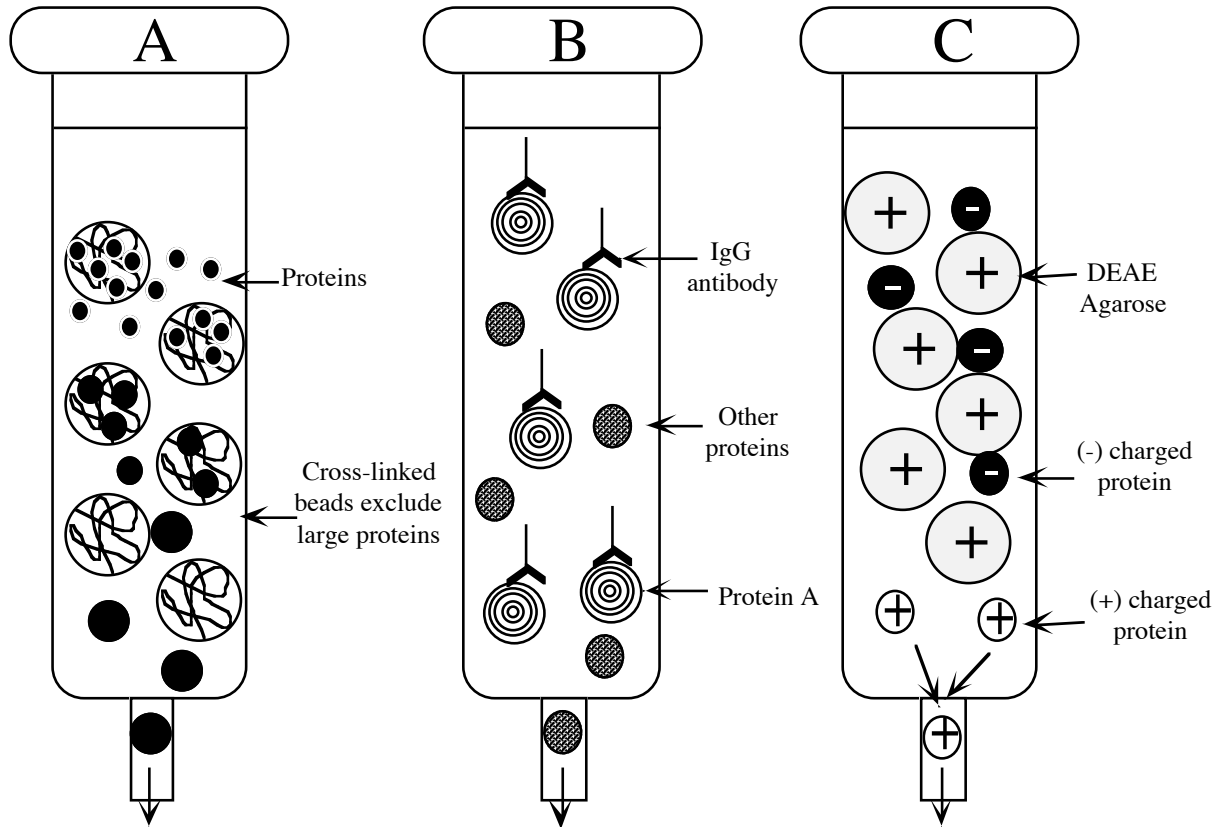


Figure 1. Separation of proteins by chromatography. A. Gel filtration, proteins are separated on the basis of size. B. Affinity, IgG is separated from other serum proteins using Protein A resin. C. Ion exchange, negatively charged proteins are separated from positively charged ones using positively charged DEAE-Agarose resin.

In affinity chromatography (Figure 1B), a molecule that will specifically bind to the protein to be purified is attached to a cross-linked agarose resin. A mixture of proteins is added to the column and everything passes through except the protein of interest, which binds to the molecule attached to the resin. Another buffer is used to get the protein to elute (come off) from the column. Often this elution buffer contains high concentrations of salt or acid. Affinity chromatography is used to purify IgG antibodies from other proteins found in serum in the following manner: Protein A (a protein isolated from *Staphylococcus aureus* that specifically binds IgG) is attached to the agarose resin and serum is added to the column. The IgG present in the serum is captured by the Protein A while other proteins freely pass through the column.

In ion exchange chromatography (Figure 1C), the resin of the column has a positively or negatively charged functional group bound to it. A mixture of proteins is added to the column and only proteins which have a charge opposite of the resin bind to the column. If the functional group has a positive charge (e.g. a diethylaminoethyl group), it will bind negatively charged molecules. This technique is called *anion* exchange. If the functional group is negatively charged (e.g. a carboxylate group), they bind positively charged molecules (*cation* exchange). (See Figure 2 below) Thus, in ion exchange chromatography, *you must choose a resin to use based on the properties of the protein of interest*. During chromatography, the protein binds to the oppositely charged resin. The bound protein on a positively charged column can be eluted from the column by running buffers with increasing concentrations of KCl over the column. The Cl^- ions compete with the negatively charged protein (anion exchange) for binding to the positively charged column. Proteins that have a low negative charge elute first and are caught in the early fractions. Later fractions contain proteins with a high negative charge.

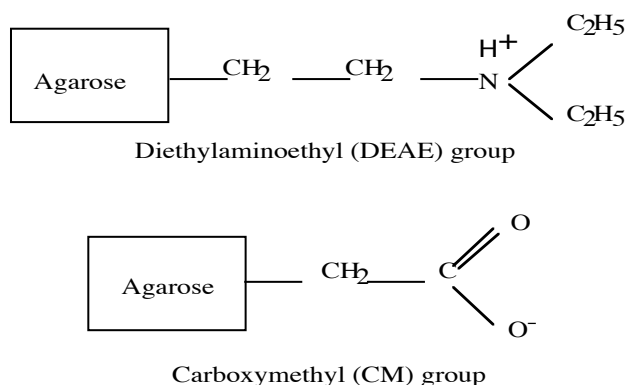


Figure 2. Positively charged (DEAE) and negatively charged (CM) functional groups used in ion exchange chromatography.

You will be isolating α -amylase enzyme (in this case, from the thermophilic bacterium *Bacillus licheniformis*) from a mixed "cocktail" of high molecular weight compounds by *gel filtration chromatography*. You will be performing a separation using gel filtration / size exclusion chromatography. You will then assay the fractions containing separated proteins for amylase activity using the starch-iodine assay that you have used previously. To determine the molecular weight of the *B. licheniformis* amylase enzyme, you will compare the molecular weight standards in your fractions to the fraction(s) containing α -amylase.

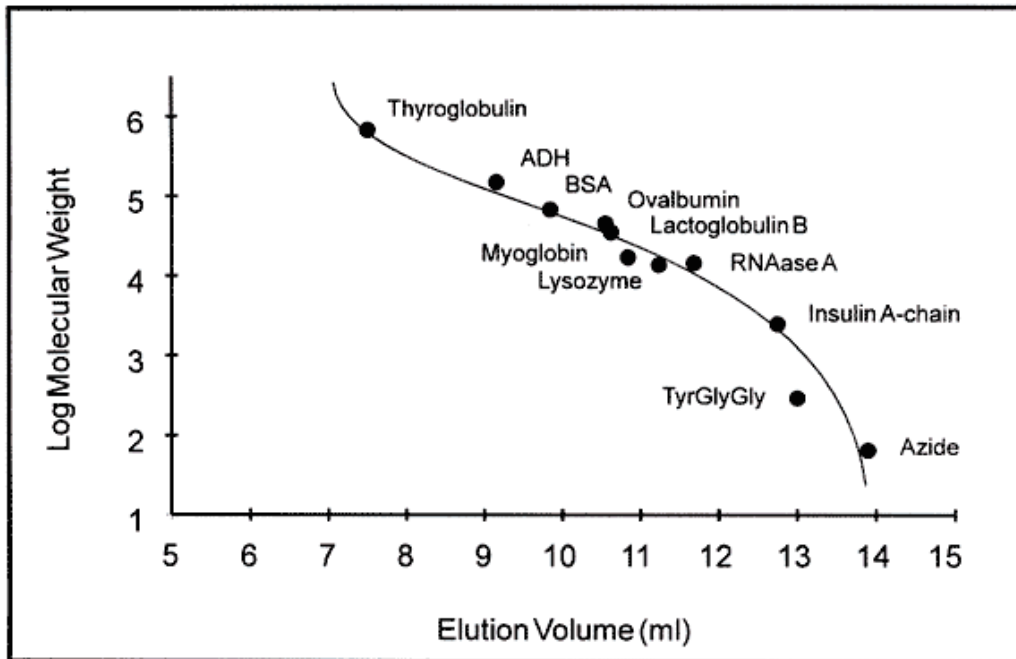
Gel Filtration Chromatography (also called Size Exclusion Chromatography) is used to separate molecules such as proteins and nucleic acids according to size. The beads (also called resins or gels) used in this type of chromatography are porous. Small molecules can penetrate the pores and enter the beads. However, larger molecules do not enter the beads and are therefore "excluded." When a mixture of molecules having different molecular weights is applied to a size exclusion column, molecules that are

larger than the pore size follow a direct path around the beads and through the column. A very large dye that cannot enter the gel, called dextran blue is used to determine the point at which fractional measurements should be taken of the eluant. (Eluant is the name for the solution that has moved through and is collected from the column.) The volume of buffer that elutes from the column before the dextran blue is called the void volume or exclusion volume. Molecules that can enter the beads take a convoluted, and therefore longer, path through the column and thus migrate more slowly. *For molecules, which can enter the beads, there is an inverse logarithmic relationship between the size of the molecule and the volume eluted from the column.* Thus, you can use a standard curve to estimate the molecular weight of the α -amylase from *B. licheniformis*. Size exclusion makes two major assumptions, 1) molecules are spherical and 2) molecules do not interact with the gel material. Nonspherical proteins elute earlier from the column than spherical molecules with the same mass. Additionally, hydrophobic proteins tend to interact with some types of gel materials and are thus retained longer by some columns. (Would this make the proteins appear smaller or larger than their actual mass?)

In this experiment you will use a polyacrylamide size exclusion resin (Sephacryl-100) to separate a cocktail containing α -amylase, cytochrome C, blue dextran, GFP, and vitamin B-12. The compounds (with the exception of α -amylase) are colored compounds and their migration, separation, and elution from the column should be easily visible. These colored proteins will be used to generate a standard curve of molecular weights from which you will determine the molecular weight of the amylase.

Determining molecular weights using size exclusion chromatography data:

After separating the cocktail into fractions based on the size of the molecules, you will measure the elution volume for each of the standards (including GFP which is visualized in the UV transilluminator). You will then determine the elution volume for amylase by assaying each fraction for amylase activity using the starch iodine assay that you have used previously. To determine the molecular weight you will graph the data from the standards (molecular weight vs. elution volume (V_e)) on semi-log paper similarly to Figure 3. Remember there is an inverse logarithmic relationship between the size of the molecule and the volume eluted. With this standard curve you will then be able to estimate the molecular weight of the amylase from *B. licheniformis*.



Zorbax® BioSeries GF-250 9.4 x 250 mm (884973.901)
 Mobile Phase: 0.2 M NaH₂PO₄, pH 7.0
 Detection: UV 230 nm

Figure 3. Semi-log graph of molecular weight standards. <http://www.mac-mod.com/an/te4-an.html>

Protocol

Variables and Controls

Independent variable

The molecular weights of the proteins in the cocktail

Dependent variable

The elution volume of the various proteins

Control variables

Column matrix (sephacryl), running buffer, starch assay for each fraction

Positive controls

Protein standards (proteins of known molecular weight).

Assay samples with amylase.

GFP sample

Negative controls

Assay samples without amylase or GFP

Materials

1.5 ml microfuge tubes (about 12)

microfuge test tube rack

test tubes to collect void volume and run amylase assays

P1000 and tips

P50-200 and tips
ring stand and clamp
ice bucket
pre-packed Sephacryl-100 column
column buffer
20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM β -mercapto-ethanol

100 μ l α -amylase/standards cocktail. (keep this on ice until use)
 β -amylase and standards cocktail: 0.1 mg/ml vitamin B-12, 0.1 mg/ml
cytochrome C, 0.1 mg/ml blue dextran in 20 mM TrisHCl, 1 mM EDTA, pH 8
acidic iodine
0.2% (w/v) starch
200 mM phosphate buffer, pH 7.0

Procedure

1. One member of each team will need to get ice.
2. Number your microfuge tubes 1 to 12 and put them in a rack. These will be for collecting your column fractions.
3. Carefully remove the top cap from the column; be sure not to disturb the gel bed. Place the column into a clamp then check that 1) the column is vertical, and 2) the test tube rack can freely be moved under the column such that the tubes can catch the drips (eluant) from the column.
4. Remove the bottom cap and allow the buffer to flow through the column into a beaker until the liquid surface is about 1 cm above the gel bed. Replace the bottom cap to stop the flow of the buffer.

Never let the meniscus touch the top of the resin bed! It is very important that the column is not allowed to become dry, contaminated with air pockets or disturbed as this will disrupt migration through the gel.

Loading and running the column:

5. **Very slowly and gently** put the tip of your pipette man through the column buffer and carefully load 100 μ ls (the entire sample) of β -amylase/standard cocktail on top of the gel bed taking care not to disturb the gel bed.
6. Place a test tube under the column and briefly remove the bottom cap just long enough to start the migration of the sample (i.e. just until the sample has completely entered the column). Replace the bottom stopper.
7. **Very slowly and gently** add 3 mls of column buffer to the column. This is best done by slowly adding the buffer (one ml at a time using your pipette man) along the side of the column just at the top of the meniscus. Now, remove the bottom cap and allow the column to "run."

8. Continue to collect the eluant in the test tube. You will begin to see the colored standards separate. Use a white piece of paper as a background to help you determine the color of the eluant drops.

The blue dextran dye does not enter the beads of the gel (i.e. is excluded). It is used to measure the void volume of the column (V_0), *which is the volume of the eluant collected from the beginning of the run until the blue dextran begins to elute*. Save this void eluate and then measure and record its volume.

9. As the blue dextran elutes, begin to collect fractions of approximately 200 μ l in volume (about 5 drops) in the microcentrifuge tubes.

Along with α -amylase and blue dextran, the cocktail contains the following molecular weight standards:

Hemoglobin	68,000 Daltons (D), brown
GFP	26,900 D fluoresces green under UV light.
Cytochrome C	12,400 D, brown/rust,
Vitamin B-12	1,350 D, pink.

10. Once the Vitamin B-12 has eluted, you may stop collecting 200 μ l fractions. Use your pipetteman to precisely measure the volume of your fractions and record those volumes.

11. Continue to run the column until the column buffer is about 1 cm above the gel bed. Replace the bottom cap and **gently** top off your column with 3 ml of column buffer as you did previously. Gently replace the top cap so as not to disturb the gel bed. Label the column with a mark using your Sharpie so that we will know it has been used and return it to the front of the lab.

12. Use your pipetteman to measure the volume in each fraction and then calculate the *cumulative volume* (i.e. void volume (V_0) plus elution volume (V_e)) for each fraction. Later you will plot the log molecular weight vs. elution volume after the void volume elutes (i.e. the volume at which the protein "comes off" the column after the blue dextran has started to come off the column). That is, the cumulative volume of all the fractions from the start of the blue dextran through the fraction that contains the particular molecular weight marker.

You will need to examine the fraction tubes over the UV light box to determine which fraction(s) contain the GFP. The other standards should be visible under normal light.

Assay for α -amylase activity

Label an empty glass test tube for each fraction, you will also need an initial tube ("I" Tube or negative control) and a positive control tube ("+").

Add 500 μ ls of 200 mM phosphate buffer (pH 7.0) to each tube.

Add 500 μ ls of 0.2% w/v starch to each tube.

Repeat the following for **each** column fraction. Each group will be provided with buffer containing amylase as a positive control and buffer alone as a negative control.

Add 50 μ ls of the fraction you are testing to one of the test tubes.

Add 50 μ ls of water to the "I" tube (negative control)

Add 50 μ ls of of buffer containing amylase to the "+" tube (positive control)

Incubate the tubes for five minutes

Add 500 μ ls of acidic iodine to each tube.

Determine the amylase-containing fractions **visually**. There is no need to use spectrophotometer (unless you want to). *Will the amylase-containing tubes be dark or light blue?*

Record all data in your lab notebook, and graph the elution volume (V_e) vs. the molecular weight of the standards. Graph your data first on *regular graph paper* and then on *semi-log graph paper*. Then use the graphs to determine the molecular weight of amylase. Good Luck

Clean Up

Rinse your glassware:

Test tubes: Starch assays can be poured down the drain. Remove all labels from the glass test tubes. Use EtOH on a Kimwipe to remove Sharpie marks. To wash, fill the tube about half full with tap water, shake and pour out a couple of times. Then rinse the tube in the same manner with distilled water twice. At this point the tube should be clear of color, powder, etc. If it is not, see your TA. Return the tubes upside down to drain and dry in the racks at your station.

Beakers: Pour eluant **down the drain in the fume hoods**. Rinse with tap water twice and distilled water twice.

Microfuge tubes, gloves: Place into a trashcan. They are **not** biohazard wastes.

Everything else: leave at your station.