

EXERCISE 8A

Name _____

Isolating, purifying, and characterizing proteins

Day One: How can α -lactalbumin be separated from the other molecules in milk?

Objectives

After completing this exercise, you should be able to:

- ◆ Explain why scientists are interested in purifying and characterizing the proteins and other biomolecules found in living organisms.
- ◆ Describe how size exclusion chromatography separates a mixture of biomolecules.
- ◆ Describe and use the following techniques to separate α -lactalbumin from the other biomolecules in nonfat milk: differential solubility, centrifugation, ultrafiltration, and size exclusion chromatography.
- ◆ Describe and use optical absorption at 280 nm (A_{280}) to estimate the protein concentration in an elution profile from a chromatography column.

Prelab

Before you come to lab, read this entire exercise. You must also answer all questions and complete all assignments on the first 8 pages of this exercise. Your instructor will give you directions on when and where to turn in your work.

Cells are composed of thousands of biomolecules acting and interacting in many complex ways. Most of these biomolecules are present in extremely small amounts. Therefore, in order to study the structure and function of cellular biomolecules, molecular biologists must overcome two obvious problems:

- 1) The molecules must be separated, or isolated, from all of the other biomolecules present in the cell. In other words, a single type of molecule must somehow be plucked out of the rich cellular soup made of thousands of different kinds of molecules.
- 2) Enough molecules of each type must be isolated for study. A few dozen molecules, or even a few hundred molecules, are not enough to work with. A scientist needs enough of the substance to be able to run a variety of biochemical tests in order to determine the structure and function of the biomolecule.

Often, the molecules that a biologist wants to isolate and study are proteins. This is not surprising because proteins are the most abundant and versatile organic molecules found in cells. Cells are able to make tens of thousands of different proteins, each with its own unique structure and function.

During the next four lab periods, you will study several important techniques that molecular biologists use to isolate specific proteins from a complex mixture of biomolecules. You will use these techniques to separate a protein called α -lactalbumin from the other biomolecules found in nonfat milk. At several points during the procedure, you will save samples of the purification fractions. These fractions will be analyzed in order to determine the total protein concentration of each fraction. In addition, each fraction will be analyzed using SDS-PAGE electrophoresis in order to determine the number of proteins present in each fraction and their molecular weights. Finally, you will identify the various proteins present in each purification fraction based on their molecular weights.

Schedule for Exercise 8

Part A: Carry out several purification steps in order to separate α -lactalbumin from the other biomolecules present in nonfat milk.

Part B: Determine the total protein concentration of each purification fraction.

Part C: Calculate how much of each purification fraction should be loaded onto your SDS-PAGE gel; and then load, run, and stain your gel.

Part D: Analyze your SDS-PAGE gel in order to determine which proteins are present in each purification fraction. Also, evaluate your success in isolating a sample of pure α -lactalbumin.

Main steps involved in purifying α -lactalbumin

1. Set aside a sample of nonfat milk to assay later
2. Precipitate the casein milk proteins using heat and low pH. The other milk proteins should remain in solution. Centrifuge the heat and acid treated milk to separate the precipitated casein proteins from the soluble proteins.
3. Set aside a sample of the pellet of precipitated proteins to assay later.
4. Remove any remaining precipitated proteins from the supernatant (whey) using ultrafiltration.
5. Set aside a sample of the whey to assay later.
6. Separate α -lactalbumin from the other proteins that remain in the whey using size exclusion chromatography.
7. Set aside the five chromatography fractions most likely to contain α -lactalbumin to assay later.
8. Prepare a standard curve for the Bradford assay using solutions of known protein concentration.
9. Use the Bradford assay and your standard curve to determine the protein concentrations of the 8 milk fractions that you set aside on Day 1.
10. Calculate the amount of each milk fraction that should be loaded into your SDS-PAGE gel so that each lane contains an appropriate amount of protein.
11. Load, run, and stain SDS-PAGE gels.
12. Analyze your gel to determine the number of different proteins that are present in each milk fraction and the molecular weights of these proteins.
13. Use your gel analysis to evaluate how effectively you isolated and purified α -lactalbumin from the other components of milk.

Which molecules are found in milk?

Milk is mostly water. It also contains various inorganic ions as well as many organic molecules including lipids, carbohydrates, and dozens of different proteins. Major proteins found in milk, and their molecular weights, are listed in the following table:

<u>Milk Protein</u>	<u>Molecular Weight (daltons)</u>
α -lactalbumin	14,437
β -lactoglobulin	18,000
various caseins	~19,000-30,000
blood serum albumin	68,000
lactoferrin	87,000
various immunoglobulins	~160,000-1,000,000

The **caseins** are found in large protein complexes called micelles, which contain many phosphate groups and also bind calcium ions. These protein complexes make up **about 80-90%** of the total protein found in milk and supply the newborn with calcium, phosphorus, and the amino acids needed for protein synthesis.

Another nutrient found in large quantities in milk is lactose, or milk sugar, which provides readily available energy to the newborn. Lactose is made when a glucose molecule and a galactose molecule are joined by the action of the lactose synthase enzyme complex. This complex is made up of the enzyme galactosyltransferase, which remains in the mammary cells, and **α -lactalbumin**, which is secreted into the milk.

Blood serum albumin is a protein that leaks into the milk from the bloodstream, where it helps maintain the proper osmolarity of body fluids. **Lactoferrin** has antibacterial properties, and the **immunoglobulins** are antibodies that help protect the newborn from infectious disease. The function of **β -lactoglobulin** is not known.

Although α -lactalbumin makes up only about 2-5% of total milk proteins, your goal in this lab activity is to separate it from all of the other molecules found in milk.

How can I remove the caseins from milk?

Casein molecules coagulate (stick together) when the pH of milk drops below 4.8, while α -lactalbumin and the other non-casein milk proteins do not coagulate until the pH drops below 4.5. Therefore, you can selectively coagulate only the caseins by using acid to adjust the pH of milk between 4.8 and 4.5. This process is enhanced by heating the acid-milk mixture.

Once the caseins have coagulated, the resulting “curdled” mixture will be centrifuged to force the coagulated caseins into a firm **pellet** at the bottom of the centrifuge tube. Milk minus the casein proteins is called **whey**. After centrifugation, how can you obtain a sample of milk whey?

Specifically, which of the major milk proteins will be found in the whey?

How can I separate α -lactalbumin from the other proteins in the whey?

After filtering the whey to remove any remaining particles, you will use **size exclusion chromatography** (also called **gel filtration chromatography**) to separate the proteins in the whey based on size. During size exclusion chromatography the stationary and mobile phases are packed in a narrow column, so this technique is also referred to as **column chromatography**.

The **size exclusion** column that you will use was prepared by filling a long, narrow tube with a mixture of tiny beads, which function as the stationary phase, suspended in a buffer, which functions as the mobile phase. The beads are made of dextran fibers and are marketed under the name Sephadex G-50 (other types of beads are available.) In order to separate the proteins in the whey, the whey will be loaded into the top of the column, and small amounts, or **fractions**, of the liquid that drips from the bottom of the column will be collected in separate containers. Since molecules of different sizes move through the column at different rates, different-sized molecules will end up in different fractions.

When using size exclusion chromatography, the largest molecules filter through the column fastest and collect in the early fractions, while the smallest molecules move through the column slowest and collect in the later fractions. Why does this happen? The Sephadex beads are made of a fine grained gel, with pores so small that very large molecules cannot move into the gel beads at all. Therefore, large molecules are excluded from the beads and must move around the beads in the spaces between them. Slipping past the beads, they move very quickly through the column. Smaller molecules, on the other hand, can diffuse into the gel beads, and traveling through the beads slows them down. The smallest molecules can move into the smallest spaces inside the beads, and are slowed down the most.

The ability of size exclusion chromatography to separate molecules of different size depends on a number of factors. As in thin layer chromatography (Experiment 7), the resolution depends on how much sample is used and how well the sample is loaded. If there is too much sample, or if the sample is not applied in a thin layer, the molecules will not separate well. Also, if a column is run too quickly, the smaller molecules will not have enough time to diffuse into the gel beads, and resolution will suffer. During size exclusion chromatography, the size and length of the column also has a big effect on resolution. As in thin layer chromatography, the farther the molecules are allowed to travel, the better the resolution will be.

Based on your understanding of how size exclusion chromatography works, list the whey proteins in the order in which they will emerge from the bottom of the column:

1. _____
2. _____
3. _____
4. _____
5. _____

How can I determine which column fraction will contain the highest concentration of α -lactalbumin?

You will be collecting 14 one-mL fractions from your column. How can you determine which fraction will contain the highest concentration of α -lactalbumin? First, you must calibrate your column using protein standards to make a **standard curve**. The equation of the standard curve will tell you the exact relationship between the **elution volume**, or V_e , of any molecule and its molecular weight. The elution volume is the volume of liquid, in milliliters, that has already passed through the column as the highest concentration of the molecule that interests you comes out. Therefore, if the V_e for a molecule is calculated to be 12.6 mL, and you collect one-mL fractions, then your thirteenth fraction should contain the highest concentration of your molecule.

The columns used in this course were all made in the same way, and were calibrated using four proteins of known molecular weight. Since the values obtained during the calibration of these columns were very similar, all lab groups will use the same calibration data to calculate the V_e , or elution volume, of α -lactalbumin.

Void volume

Void volume or V_0 is the volume of buffer present between the beads of a column. **The void volume for the column you will use is 7.0 mL.** In a column that has a void volume of 7.0 mL, proteins that are large enough to pass around all of the gel beads as they travel through the column will elute from the bottom once 7.0 mL of buffer has passed through the column.

Column calibration

We know that size exclusion chromatography separates proteins according to size. However, in order to determine exactly which column fraction will contain the highest concentration of a protein of known size, the column must be calibrated. To calibrate your column, 4 proteins of known size were passed through the column, 2 at a time, and then 1 mL fractions were collected from the bottom of the column. Note that these four proteins were not chosen because they occur in milk, but because they are inexpensive, readily available for purchase, and they span the size range that includes α -lactalbumin (14,437 daltons):

<u>Protein Standards</u>	<u>Molecular Weight (daltons)</u>
Bovine serum albumin (BSA)	66,000
Carbonic anhydrase	29,000
Cytochrome c	12,400
Aprotinin	6,500

In order to compare the concentrations of protein in the fractions that were collected, absorbance of the fractions at 280nm (A_{280}) was measured with a spectrophotometer. Absorption at 280nm depends mainly on the concentration of aromatic (ring) structures in the amino acids tyrosine, phenylalanine, and tryptophan. This is not a very accurate method for measuring protein concentration because different proteins contain different percentages of these amino acids. In addition, many non-protein molecules also contain aromatic rings that absorb at 280nm. Nevertheless, this method is a quick and easy way to compare the protein concentration of the different fractions that were collected, and is adequate for our purpose:

First run: Aprotinin (6,500 daltons) and Carbonic Anhydrase (29,000 daltons)			
	Fraction	A_{280}	
	1-4	(discarded)	
	5	0.431	
	6	0.420	
	7	0.489	
	8	0.619	
	9	0.559	
	10	0.489	
	11	0.503	
	12	0.550	
	13	0.508	
	14	0.501	

Study the table above. Identify the two peaks in absorbance. These are the fractions where the highest concentration of each protein emerged from the column.

During the first run, which protein came out first? _____

Which fraction number contains the highest concentration of this protein? _____

Since you collected 1 mL fractions, what is the approximate elution volume (to the nearest 1 mL) of the protein that came out first? _____

During the first run, which protein came out second? _____

Which fraction number contains the highest concentration of this protein? _____

Since you collected 1 mL fractions, what is the approximate elution volume (to the nearest 1 mL) of the protein that came out second? _____

Now, examine the results for the second calibration run and identify the 2 peaks in absorbance:

Second run: Cytochrome C (12,400 daltons) and BSA (66,000 daltons)			
	Fraction	A₂₈₀	
	1-4	(discarded)	
	5	0.412	
	6	0.463	
	7	0.878	
	8	0.539	
	9	0.603	
	10	0.665	
	11	0.526	
	12	0.463	

During the second run, which protein came out first? _____

Which fraction number contains the highest concentration of this protein? _____

Since you collected 1 mL fractions, what is the approximate elution volume (to the nearest 1 mL) of the protein that came out first? _____

During the second run, which protein came out second? _____

Which fraction number contains the highest concentration of this protein? _____

Since you collected 1 mL fractions, what is the approximate elution volume (to the nearest 1 mL) of the protein that came out second? _____

How do I make a standard curve that will allow me to determine the V_e for α -lactalbumin?

By determining when several proteins of known size elute from our size exclusion column, we can estimate when proteins of any size will elute from the same column. This is done by plotting a **standard curve**, which shows the relationship between the size of a protein and its elution volume.

In the following table, fill in the name, molecular weight, and elution volume (V_e) of the four protein standards that were used to calibrate your size exclusion column (leave the last 2 columns in the table blank for now):

Protein (name)	MW (daltons)	V_e (mL)	Log MW	V_e/V_o

Take a careful look at the data in your table. You want to use this information to determine the relationship between the size (**MW**) of a protein and its elution volume (V_e). In a general sense, you should see that as size (MW) increases, the V_e decreases. But you want something more precise. A **standard curve** will tell you the exact mathematical relationship between these 2 variables. Once you know the equation for your standard curve, you can substitute the MW of any protein into the equation and solve for its V_e .

Unfortunately, the relationship between the MW of a protein and its V_e is not linear. This means that if you prepare a scatter diagram by plotting the molecular weight of the standards on the y-axis, and the V_e of the standards on the x-axis, the points will form a curved rather than a straight line. However, if you plot the log of MW instead of MW on the y-axis, you will get a linear relationship (i.e. a straight line). Because it is easier to determine the equation of a straight line than a curved line, you will use log of MW instead of MW when constructing your standard curve.

Fill in the log of MW for each protein standard in the table above.

Another problem is that the V_e of a protein depends not only on its MW but also on the size of the column it is run through. Obviously, the same protein will have a larger V_e when it is traveling through a long column and a smaller V_e when it is traveling through a short column. To neutralize the effect of different column sizes, you have to divide the V_e of each protein by the V_o for the column (V_o is the amount of liquid in between the gel beads, so is a measure of column size.) Actually, this is only necessary when the protein standards are run through a column that has a different size than the column used for the unknown proteins, but it is a good habit to get into. Using V_e/V_o during column chromatography is similar to using R_f values (rather than migration distances) during thin layer chromatography (see Lab Exercise 7).

Given that the V_o for the column is 7.0 mL, fill in the V_e/V_o for each protein standard in the table above.

Using a sheet of graph paper or a computer with spreadsheet program, plot a scatter diagram showing the relationship between log of MW and V_e/V_o for the 4 protein standards in the table. Make sure you plot log MW on the y-axis and V_e/V_o on the x-axis. You should turn in this graph with the rest of your Prelab.

Now, using a hand-held calculator or a computer with spreadsheet program, carry out linear regression to determine the equation of the “best fit” straight line for your data points. Write this equation on your scatter diagram. This is the equation of the standard curve for your column.

Which column fraction should contain the highest concentration of α -lactalbumin?

Write the equation of the standard curve for your size exclusion column in the space below:

In this equation, “y” represents log of MW and “x” represents V_e/V_0 . This equation can be used to determine the elution volume (V_e) of any protein, provided you know the MW of the protein, and the MW lies within the size range of the protein standards used to make the standard curve (in this case, between 6,500 daltons and 66,000 daltons.)

What is the molecular weight of α -lactalbumin? _____

Does the MW of α -lactalbumin lie within the size range of the protein standards used to make the standard curve? _____

What is the log of MW for α -lactalbumin? _____

Substitute the value of “log of MW for α -lactalbumin” into your linear regression equation for “y” and calculate the value of “x”. The value of “x” equals V_e/V_0 .

(On the TI-36 you can do this by entering the y-value (log MW of α -lactalbumin) into the calculator, then press [2^{nd}] and [x']. If you have cleared your calculator before doing this step, you will need to re-enter your data.)

Based on your calculation, what is V_e/V_0 for α -lactalbumin? _____

Once you know the V_e/V_0 for α -lactalbumin, simply multiply it by V_0 to determine the V_e for α -lactalbumin.

V_e for α -lactalbumin = _____

Remember, the V_e for a protein is the volume of liquid, in milliliters, that has already passed through the column as the highest concentration of the protein that interests you comes out. Since you will be collecting 1.0 mL fractions, a V_e between 0 and 1 mL means the highest concentration of the protein will be found in the first fraction, a V_e between 1 and 2 mL means the highest concentration of the protein will be found in the second fraction, a V_e between 2 and 3 mL means the highest concentration of the protein will be found in the third fraction, etc.

In which fraction do you expect to find the highest concentration of α -lactalbumin? Write the fraction number in the space below and in the space provided on page 12 of the lab procedures:

Lab Procedures:

Important: Save all of your “leftover” samples--supernatant, pellet, clarified whey, column chromatography fractions, etc. until the end of the lab period.

Make a habit of placing these “leftovers” in your ice bucket as you finish with them. This will ensure that you don’t accidentally throw away a sample that you will need later. In addition, if something goes wrong in one step of today’s procedures, you can redo the step if you have saved your extra samples.

After your instructor has checked the samples that will be stored for next week (at the end of this lab period), you may dispose of the extra samples that you did not use.

I. Set aside a sample of nonfat milk containing all milk proteins

1. Label an Eppendorf tube with your group name and “Milk.”
2. Transfer 500 μ L of nonfat milk into the Eppendorf tube, close it, and place it in your ice bucket. At the end of the lab period, this sample will be stored at -20° C.

Next week you will determine the concentration of protein in this sample, and during weeks three and four you will analyze it using SDS-PAGE electrophoresis.

II. Precipitate the casein proteins with acid and heat, and separate them from the whey proteins by centrifugation

1. Transfer another 30 mL of nonfat milk into a 50-mL beaker that has been labeled with your group name (the calibration marks on the side of the beaker are accurate enough for this measurement). Place a small magnetic stir bar in the beaker with the milk.
2. Place the beaker on a stir plate. Turn on the stir function of the plate to begin gentle stirring of the milk. Place the pH meter electrode into the milk so that it is deep enough into the milk to accurately measure the pH, but not so deep that it is being hit by the stir bar. **Warning: the pH probe must be far enough down in the sample to submerge the electrode bulb.**
3. While stirring constantly with the magnetic stir bar, add one drop of 6.0 M HCl and wait until the pH reading has stabilized. Add more 6.0 M HCl, one drop at a time, to adjust the pH to 5.0. Once the pH has reached 5.0, use the 1.0 M HCl, one drop at a time, to adjust the pH between 4.8 and 4.5. **CAUTION: if the pH drops below 4.5, you will have to start all over again!**
4. Cover the beaker with the pH adjusted milk with some Parafilm®, and place it into the shaking water bath at 40° C for 30 minutes. While heating the solution, continue gentle stirring and adjust the heat as necessary to keep the temperature as close to 40° C as possible.

While the solution is being heated:

Place 14 test tubes in a rack and use tape to label them 1 through 14. These will be used to collect 1.0 mL fractions as they elute from your column. To calibrate these tubes, measure 1.0mL of dH_2O into Tube #1 and mark the level of the water with a marking pen. Place Tube #2 next to Tube #1 and mark the same level on Tube #2. **Always using Tube #1 as your reference**, mark all of the remaining tubes in the same way. Pour out the water in Tube #1 and turn it upside down to drain. (Alternatively, you can wait and just count drops of buffer as they elute from the column while you are running your column, and make each tube receive exactly 20 drops.)

5. After heating your pH adjusted milk for 30 minutes, pour approximately 11 mL of the acid/heated milk into a labeled 15-mL centrifuge tube. Make sure you get a sample of both the clear whey and the coagulated caseins into the tube. If another group is ready, balance your tube with theirs in the following way:
 - a) Place a small beaker on each of two electronic balances and tare the balances.
 - b) Place the two tubes in the two beakers.
 - c) Add more acid/heated milk to the lighter tube until the two tubes weigh the same.(If there is an odd number of groups in your class, one group can balance their tube of acid/heated milk with a tube of dH₂O.)
6. Place the two *balanced* tubes (either two tubes containing milk or a tube of milk and a tube of water) **directly opposite each other** in the refrigerated centrifuge.
7. Centrifuge the *balanced* tubes for 30 minutes.

While the tubes are being centrifuged:

Place the beaker with the extra acid/heated milk into your ice bucket and save it until the end of the period.

III. Set aside a sample of the pellet containing the casein proteins

1. After the centrifugation is finished, carefully pour the supernatant (whey) into a 15-mL beaker. **Be careful not to disturb the pellet.**
2. Place the beaker containing the whey in your ice bucket. The whey contains the non-casein milk proteins, including the α -lactalbumin that you are attempting to isolate.
3. Label an Eppendorf tube with your group name and "Pellet." Add 1 mL of "column buffer" to the Eppendorf tube.

WARNING: The "column buffer" contains sodium azide, a toxic, antibacterial and antifungal reagent. Therefore, you must wear gloves when handling the column buffer or any other solutions containing this reagent.

4. Using a spatula, scrape about a match head sized amount of the pellet out of the centrifuge tube and place it in the Eppendorf tube containing the column buffer. (It is not necessary to scrape out the entire pellet--just scoop out a small sample.)
5. Suspend the casein proteins (the pellet) in the column buffer by using a Pasteur pipette or a disposable transfer pipette to gently withdraw and expel (back into the Eppendorf tube) the pellet-buffer mixture several times until the contents of the tube are thoroughly mixed.
6. Close the tube and place it in your ice bucket. At the end of the lab period, this sample will be stored at -20° C.

Next week you will determine the concentration of protein in this sample, and during weeks three and four you will analyze it using SDS-PAGE electrophoresis.

IV. Remove small precipitated casein particles from the whey by ultrafiltration

1. Retrieve the beaker containing the whey from your ice bucket.

Although most of the casein proteins that were coagulated by the acid-heat treatment were removed from the whey by centrifugation, some very small particles may remain suspended because they did not have sufficient mass to be forced to the bottom of the tube. Before the whey can be loaded onto your column it must be “clarified” to get rid of the micron or submicron particles left in suspension. If this is not done, these particles will clog the column.

2. To clarify the whey, load it into a syringe and force it through the 0.45 μ m filter by pressing down on the plunger, collecting the clarified whey in a small beaker. Your instructor will demonstrate how to assemble the syringe/filter apparatus. Only particles that are 0.45 μ m or smaller will pass through the filter; the rest will be trapped on the screen. Notify your instructor if it becomes difficult to force the liquid through the screen or if you think the filter is too clogged to work properly.

V. Set aside a sample of the clarified whey containing all of the non-casein proteins

1. Label an Eppendorf tube with your group name and “Whey.”
2. Place 500 μ L of the clarified whey in the Eppendorf tube, close the tube, and place it in your ice bucket. At the end of the lab period, this sample will be stored at -20° C.

Next week you will determine the concentration of protein in this sample, and during weeks three and four you will analyze it using SDS-PAGE electrophoresis.

VI. Separate the proteins that remain in the whey using size exclusion chromatography

1. Place 5 mL of the remaining clarified whey into a small beaker. Add 0.26 mL of glycerol and mix. The glycerol is heavier than the buffer and makes the sample sink to the top of the gel when loading it on the chromatography column. Label the beaker and store it on ice until you are ready to load your column.

WARNING: The “column buffer” you will use during size exclusion chromatography contains sodium azide, a toxic antibacterial and antifungal reagent. Therefore, you must wear gloves when handling the column buffer or any other solutions containing this reagent.

2. A size exclusion chromatography column is set up at your workstation. The sample size you load onto your column should be 5 % of the total column volume (20 mL). How much sample should you load?

Using a micropipettor, transfer this amount of your whey-glycerol mixture into an Eppendorf tube.

3. Remove the top cover from the column reservoir. Check with your instructor to make sure there is enough buffer in the reservoir so that the level will not drop below the top of the gel in the column. **Never let the gel in the column dry out!**
4. Remove the nipple from the stopcock at the bottom of the column and turn the valve to allow a few drops of the buffer to drip into a waste container (beaker). To check that the column will run properly, turn the valve off to see if it will stop the drops.

Coordinate your group and prepare to run the column:

One student will load the sample into the top of the column (following the instructions below). As the sample is being loaded, another member of your group will open the valve at the bottom of the column and begin collecting the drops that elute from the column into the tubes that you labeled #1-14. Decide who will do each of these tasks and position the numbered test tubes underneath the column so that it is easy to move from one tube to the next.

5. **1st student:** Use a Pasteur pipette to withdraw your clarified whey/glycerol sample from the Eppendorf tube, being careful not to have any air bubbles mixed into your sample. Gently squeeze the bulb of the Pasteur pipette to expel any air from the tip (but without expelling any of your sample). Carefully place the Pasteur pipette tip into the top of the column, keeping the tip a cm above the interface of the gel and the column buffer (the inside rim right above the gel).

Gently expel the sample from the Pasteur pipette, being careful not to disturb the gel, slowly moving the tip of the pipette in a circle around the inside of the column at the interface in order to distribute the sample evenly. **DO NOT** disturb the gel bed by pipetting the sample too fast or by blowing air bubbles into the gel, as this will reduce the resolution of the column.

6. **2nd student:** Open the valve and begin collecting drops into tube #1 as the column is being loaded.
7. Allow drops to fall into tube #1 until the level of the liquid is at the line you drew, or count 20 drops eluting from the column. (This is Fraction 1 from your column.) Remove tube #1 and place tube #2 under the valve and collect drops until the liquid reaches the line. Continue collecting the drops in the remaining test tubes until all 14 tubes have been filled to 1.0 mL.
8. Based on the V_e for α -lactalbumin that you calculated in the prelab, which column fraction do you predict will contain the highest concentration of α -lactalbumin?

Use tape to label this fraction, along with the 2 column fractions before it and the 2 column fractions after it, with your group name. Stretch a piece of Parafilm® over each of these 5 tubes and place them in your ice bucket. At the end of the lab period, these 5 samples will be stored at -20°C .

Next week you will determine the concentration of protein in this sample, and during weeks three and four you will analyze it using SDS-PAGE electrophoresis.

9. Turn the stopcock off, and cover the column reservoir.

VII. <Optional> Protein Assay using the Scanning Spectrophotometer

The following procedure can be used to compare the total protein concentration of the various column fractions.

1. Pour the first 6 column fractions into 6 clean UV cuvettes and fill a seventh cuvette with column buffer.

IMPORTANT - handle the cuvettes on the ridged or frosted surfaces only. Fingerprints on the clear sides of the cuvette will interfere with your readings. If necessary, wipe off the clear sides of a cuvette with a Kimwipe®.

2. Using the column buffer as a blank, read and record the A_{280} of the first six fractions. When you are finished, pour each fraction back into its corresponding test tube, thoroughly shake out any excess liquid from the cuvettes, and pour the next 6 fractions into the same 6 cuvettes that you used for your first readings. Again using the column buffer as a blank, read and record the A_{280} of fractions 7 through 12. Repeat this process to read the A_{280} of fractions 13-14.

3. If any sample had an A_{280} of 1.0 or greater, you will have to dilute the sample to obtain a more accurate reading. For example, you can make a 10-fold dilution by mixing 0.1 mL of the fraction with 0.9 mL of the column buffer).

After reading the A_{280} value of the diluted sample, you will then have to calculate the A_{280} value of the undiluted fraction. To do this, multiply the A_{280} value of the diluted sample by the dilution factor. For example, if you made a 10-fold dilution, multiply the A_{280} value of the diluted sample by 10, and record this value for the fraction.

Remember, this assay measures the concentration of total protein in each fraction, not just the concentration of α -lactalbumin.

4. Check your A_{280} values to make sure that the prediction you made about which fraction should contain the highest concentration of α -lactalbumin does, in fact, contain a significant amount of protein.

VIII. Prepare your samples for Lab 8B

1. Place the following in a beaker:

- The Eppendorf tube that you set aside earlier containing the “milk” sample.
- The Eppendorf tube that you set aside earlier containing the “pellet” sample.
- The Eppendorf tube that you set aside earlier containing the “whey” sample.
- The 5 test tubes that you set aside earlier containing fractions from the size exclusion column

Ask your instructor to check the contents of your beaker to make sure that you have everything you will need for Lab 8B. **Do not dispose of anything until your instructor has okayed your beaker.**

2. Cover the beaker with a piece of plastic wrap and secure the plastic wrap with a piece of label tape that goes all the way around the beaker. On the label tape, write the following:

- **Your instructor’s name (very important!)**
- **Your lab day and time**
- **Your group name**
- **Today’s date**

Give the beaker containing your samples to your instructor, who will store it at -20°C until the next laboratory period.

Clean up

Pour the remaining column fractions, containing the toxic buffer/sodium azide solution, into your waste beaker and dispose of the contents as instructed by instructor.

Remove label tape and any marks made with a marking pen from all glassware. Wash and rinse all glassware, give it a final rinse with dH_2O , and leave it inverted at your work area in order to drain.

All disposable glassware goes into the special glass disposal receptacle.

All instruments should be turned off and unplugged.

Wipe off your workspace with a damp paper towel.

Make sure everything that you have used is clean, put away, or discarded. Leave your work area in the same order that you found it in.

Ask your instructor to check your work area before you leave.

Postlab

1. How does size exclusion chromatography separate a mixture of molecules?
2. Describe some of the factors that affect the resolution of a size exclusion chromatography column.
3. When preparing the standard curve for your size exclusion chromatography column, why did you plot the log of the molecular weight of each protein rather than the molecular weight itself? Why did you plot the V_e/V_0 of each protein rather than V_e ?
4. Use your standard curve to determine the expected V_e 's for the 6 major groups of proteins found in milk. (Note: The 6 major groups of milk proteins are listed in a table in the Prelab along with their molecular weights.)
5. During size exclusion chromatography you collected 14 one-mL fractions. At this point in the purification, do you know which proteins are in each fraction? Explain why or why not.

Answer the next 2 questions only if you measured the A₂₈₀ values for your column fractions using the Scanning Spectrophotometer.

6. On a sheet of graph paper, graph the elution profile for your run by plotting the fraction number for all 14 fractions on the x-axis and the corresponding A₂₈₀ values on the y-axis.. Make sure your graph is big enough so that it fills an entire 8.5" x 11" sheet of graph paper.
7. Examine the elution profile for your run. How many protein peaks did you obtain? Do any of the peaks correspond to the expected V_e 's for the major milk proteins, which you calculated in question 4? If so, match each of these peaks with the corresponding milk protein.