EXERCISE 8A - 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 μL 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 dH_2O.)

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 0.45μm 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 μm 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 μL 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?

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   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 <u>prepare</u> 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?

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   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 0.1mL 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.