BC 367 Experiment 3
Purification and Characterization of the Enzyme Lysozyme

Introduction

Enzymes are truly remarkable catalysts. For example, catalase can carry out the decomposition of up to $5x10^6$ moles of H_2O_2 per minute per mole of enzyme (the turnover number). Even though the turnover numbers of most other enzymes are substantially lower than this, most of the reactions they catalyze occur at rates at least a million times faster under physiological conditions in the presence of the enzyme than in its absence.

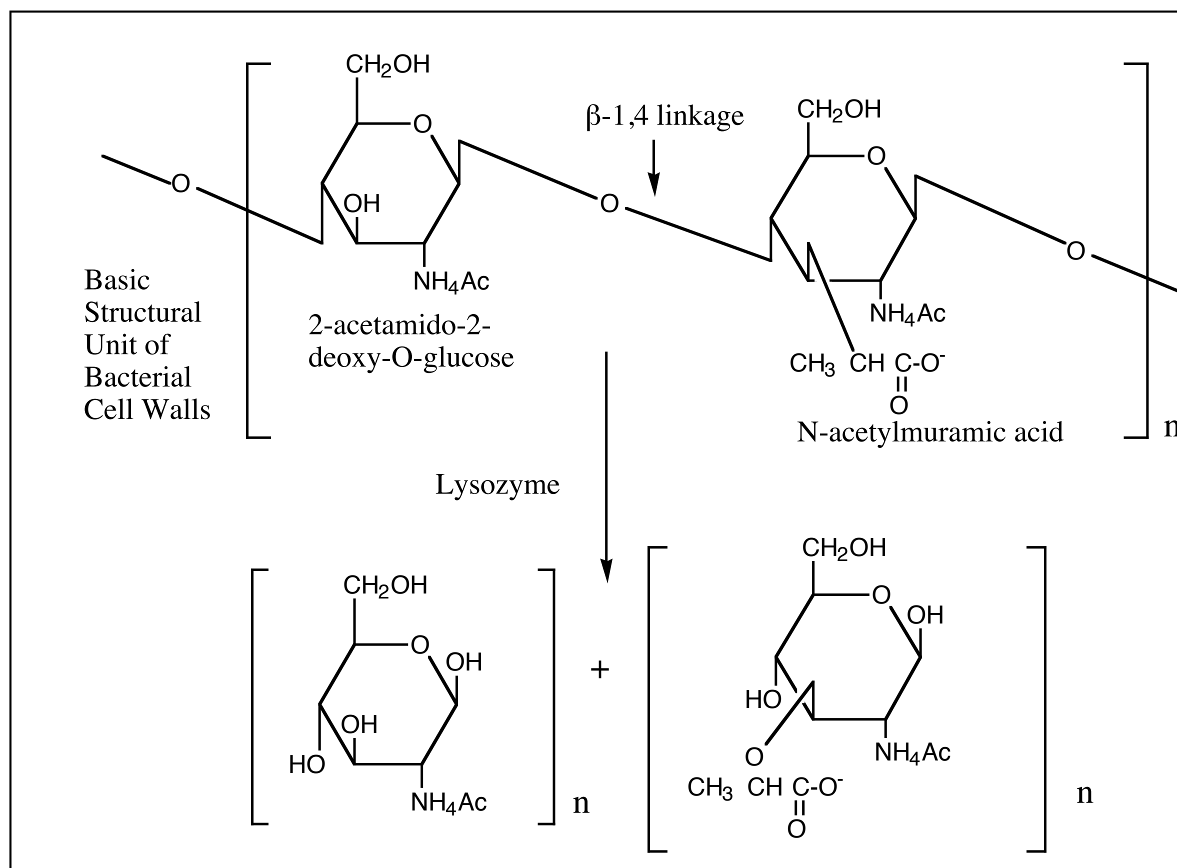
In this experiment you will purify an enzyme, using its known activity to monitor the process. The isolation and purification of a specific protein or enzyme is generally a difficult task. First, the enzyme must be liberated from its source tissue in an active form. Fortunately, a wide variety of tissue disruption techniques have been developed. However, the enzyme to be purified is usually only a small percentage of the total protein in the crude extract of the tissue. The object of protein purification is to remove nonprotein contaminants as well as to isolate the protein in question from other proteins. The first objective is relatively easy to obtain, whereas the latter is more difficult. For example, it is not unusual for an enzyme to be 0.1% of the total protein in a crude tissue extract. To purify this enzyme to homogeneity, 99.9% of the protein must be removed, preferably with as little loss as possible of the desired enzymatic activity. This can be a difficult task for two reasons:

1. Enzymes are relatively labile molecules. Most enzymes are denatured by foaming, by heating, by organic solvents (particularly at room temperature), by drying at room temperature, and by concentrated acids or bases. Furthermore, proteins in aqueous solutions are excellent nutrient systems for microorganisms, and therefore, cleanliness of equipment and avoidance of unnecessary contamination are vital for successful purification attempts.

2. Differences between some of the various protein molecules in the tissue extract may be subtle. A single purification step is seldom adequate to purify an enzyme completely. Typically, several different procedures that exploit different properties of proteins must be used. Generally, either salt precipitation, organic solvent precipitation, or isoelectric precipitation is used at an early point in the procedure. Chromatographic procedures, such as ion-exchange, gel-filtration, or adsorption chromatography, are employed after the enzyme has been partially purified by one of the precipitation techniques. Unfortunately, the establishment of an enzyme purification procedure must be done largely by trial and error.

Determination of the progress of a purification process is important. Specific activity and total activity are the critical parameters in enzyme purification. The activity of an enzyme is defined in some unit, usually micromoles of product formed per minute. The specific activity, then, is defined as activity units per milligram of total protein. As the enzyme is purified, the specific activity increases because inactive protein is removed. However, the yield of the desired enzyme is also important. For example, a purification step which yields a 100-fold increase in specific activity and a recovery of units (activity) of only 5% is not as useful as a step that yields a 10-fold purification with a recovery of 90%.

Despite the many difficulties associated with enzyme purification, numerous enzymes from many sources have been purified to homogeneity or close to it. The enzyme you will purify in this experiment is lysozyme, one of the first enzymes for which the complete three-dimensional structure was determined by X-ray diffraction. Egg white, human milk, tears, spleen, and many other tissues, including plant sources, contain this enzyme. The systematic name for lysozyme is "mucopeptide N-acetylmuramylhydrolyase." Lysozyme catalyzes the hydrolysis of β-1,4-linkages between N-acetylmuramic acid and 2-acetamide-2-deoxy-D-glucose residues in mucopolysaccharides or mucopeptides of a variety of microorganisms. The reaction catalyzed is illustrated below:



Lysozyme performs an antibiotic function for the human body. This action is attributed to the ability to destroy invading bacteria by hydrolyzing the mucopolysaccharides of the cell wall. Egg white is particularly rich in lysozyme and thus will be the starting material for your purification. Lysozyme has a lower molecular mass (14.3 kDa from chicken egg white) than most other proteins, and therefore considerable purification can be achieved by simple gel-filtration, which separates proteins on the basis of size. Furthermore, the unique charge characteristics of lysozyme, which has an unusually high pI of 10.5, can also be exploited through ion exchange chromatography. Moreover, lysozyme is a remarkably stable enzyme and retains catalytic activity even after storage for several days at room temperature. These unique characteristics coupled with the fact that a major source of the protein is cheaply obtained at the local grocery store or farm make it an excellent choice for an introduction to enzyme purification techniques.

Each group will use one of the two chromatographic techniques mentioned above in this experiment, allowing a comparison of their relative powers to purify lysozyme. In both cases, the purification will begin with straining and centrifugation to remove viscous and insoluble material from the egg white. This will be followed by column chromatography, which should lead to a several-fold purification of the protein. One column procedure will use a cation exchange resin (CM-Sephadex) that has negatively charged carboxylic acid groups. This resin will bind positively charged proteins, such as lysozyme at neutral pH. The majority of proteins will be negatively charged and pass through the column. The lysozyme can be eluted by raising the pH. (Why does a pH change elute the protein?) Two different size beads will be used for cation exchange: C-25 with an exclusion limit of ~30 kDa and C-50 with an exclusion limit of ~200 kDa. The other column procedure involves a gel filtration resin (Sephadex G-75) consisting of inert porous beads. Proteins larger than the diameter of the pores will flow through the column whereas proteins smaller than the pores will be retained and thus elute later. Fractions will be monitored for lysozyme activity, as well as total protein content, to monitor the purification procedure.

Experimental Procedure

This experiment will consist of seven parts:

1. Preparing buffers and solutions (Week 1).
2. Packing the gel-filtration or ion exchange column (Week 1).
3. Preparing the starting material (egg white) for the enzyme purification (Week 2).
4. Performing column chromatography on the egg white (Week 2).
5. Assaying the egg white and the fractions from the column for enzyme activity and protein (Week 2).
6. Analyzing the egg white and the purified enzyme preparation by polyacrylamide gel electrophoresis to determine the extent of purification (Week 3).
7. Electrospray Ionization Mass Spectrometry of a lysozyme standard (Week 3).

I. Preparation of Buffers and Solutions- Week 1

Calculations should be done prior to lab. Formula weights are included here for your convenience.

Tris[hydroxymethyl]aminomethane (Tris base)- FW 121.1
Sodium chloride- FW 58.44
Potassium phosphate monobasic (KH_2PO_4)- FW 136.1
Potassium phosphate dibasic (HK_2PO_4)- FW 174.2
Sodium carbonate (Na_2CO_3)- FW 106.0

General notes for good lab practice:

- When weighing out chemicals, never return excess to reagent bottle because of possible contamination.
- Hot plates should not be turned up past about half-way.
- When making solutions for which you need to adjust the pH, it is a good idea to add less than the final desired volume at first, adjust the pH, and then bring the final volume up to the desired amount with water.

- For this experiment, adjustment of volumes with graduated cylinders should provide enough accuracy.

Operating the pH meter

For a pH electrode to work correctly, **the filling hole must be open**. Check that it is prior to calibrating. Calibrate your pH electrode with standard buffers at pH 4.0 (usually a pink solution), 7.0 (usually a yellow solution), and pH 10.0 (usually a blue solution).

Solutions to be made:

A. For those doing gel filtration (one lab table):

Sephadex G-75:

Start with ~2 g dry sephadex G-75. Swell the dry sephadex in ~200 mL distilled H_2O by boiling for 1 h, using the "double-boiler" method. After cooling, equilibrate with column buffer (see below: 0.05 M Tris, 0.05 M NaCl [pH 8.2]) by decanting H_2O and replacing it with ~100 mL buffer. Stir gently and allow gel to settle. Decant buffer. Repeat buffer addition, and then decant, this time leaving approximately a volume of buffer equal to the volume of the gel slurry.

0.05 M Tris Base, 0.05 M NaCl (pH 8.2)- Tris-NaCl Column Buffer:

Calculate the masses of Tris and NaCl needed *before coming to lab*. Prepare 350 mL of this column buffer to equilibrate and run your column. Adjust the pH with HCl before adjusting the volume to 350 mL.

Blue Dextran:

Prepare 2 mL of a solution in H_2O containing 2 mg blue dextran/mL.

0.1 M potassium phosphate buffer (pH 7.0):

Prepare 100 mL of 0.1 M potassium monobasic phosphate and 100 mL of 0.1 M potassium dibasic phosphate. Calculate the masses of each needed *before coming to lab*. Use the Henderson-Hasselbach equation to determine approximately how much of the monobasic to add to 100 mL of the dibasic to give a pH of 7.0 (pK_a= 6.86). Put 100 mL of the dibasic solution into a beaker and add a few mL less than calculated amount of the monobasic solution. Check the pH with the pH meter and continue to add the monobasic solution until you reach a pH=7.0. Store the remainder of this buffer in a labeled bottle in the cold room when you are done today.

B. For those doing ion exchange with CM-Sephadex C25 (another lab table):

CM-Sephadex C25:

Each group should start with 0.6 g dry CM-sephadex. Swell the dry sephadex in ~200 mL distilled H_2O by boiling for 1 h, using the "double-boiler" method. After cooling, equilibrate with Tris-NaCl column buffer (see below: 0.05 M Tris, 0.05 M NaCl [pH 8.2]) by decanting H_2O and replacing it with ~100 mL buffer. Stir gently and allow gel to settle. Decant buffer. Repeat buffer addition, and decant leaving approximately a volume of buffer equal to the volume of the gel slurry.

0.05 M Tris Base, 0.05 M NaCl (pH 8.2)- Tris-NaCl Column Buffer:

Calculate the masses of Tris and NaCl needed *before coming to lab*. Prepare 300 mL of this column buffer to equilibrate and run your column. Adjust the pH with HCl before adjusting the volume to 300 mL.

0.2 M carbonate buffer (pH 10.5):

Prepare 50 mL of 0.2 M sodium carbonate (Na_2CO_3) buffer to run your column. Calculate the mass of Na_2CO_3 needed before lab. Adjust the pH. Note that the pH of this buffer is critical to the success of your experiment.

0.1 M potassium phosphate buffer (pH 7.0):

Prepare 100 mL of 0.1 M potassium monobasic phosphate and 100 mL of 0.1 M potassium dibasic phosphate. Use the Henderson-Hasselbach equation to determine approximately how much of the monobasic to add to 100 mL of the dibasic to give a pH of 7.0 (pK_a= 6.86). Put 100 mL of the dibasic solution into a beaker and add a few mL less than calculated amount of the monobasic solution. Check the pH with the pH meter and continue to add the monobasic solution until you reach a pH=7.0. Store the remainder of this buffer in a labeled bottle in the cold room when you are done today.

C. For those doing ion exchange with CM-Sephadex C50 (the final lab table):

CM-Sephadex C50:

Start with 0.4 g dry CM-sephadex C50. Swell the dry sephadex in ~200 mL distilled H_2O by boiling for 1 h, using the “double-boiler” method. After cooling, equilibrate with Tris-NaCl column buffer (see below: 0.05 M Tris, 0.05 M NaCl [pH 8.2]) by decanting H_2O and replacing it with ~100 mL buffer. Stir gently and allow gel to settle. Decant buffer. Repeat buffer addition, and decant leaving approximately a volume of buffer equal to the volume of the gel slurry.

0.05 M Tris Base, 0.05 M NaCl (pH 8.2)- Tris-NaCl Column Buffer:

Calculate the masses of Tris and NaCl needed *before coming to lab*. Prepare 300 mL of this column buffer to equilibrate and run your column. Adjust the pH with HCl before adjusting the volume to 300 mL.

All other solutions should be identical to those prepared in part **B** above for CM-Sephadex C25.

II. Preparation of Columns-Week 1

A. Gel Filtration (to be done by one-third of the groups)

Wash your column and then rinse with deionized water. Check to make sure water flows through column. Place a plastic funnel in the top of the column to serve as a packing reservoir and a buffer reservoir. You may wish to secure your funnel with parafilm. Place a plastic stopcock on the tip at the bottom of the column and make sure it is in the closed position. You will use the sephadex G-75 prepared above, which excludes proteins with a molecular weight of about 75 kD. Fill the column about 1/4 full of the Tris-NaCl column buffer. Add enough of the sephadex to fill the column. Turn the stopcock to "on" and allow to drip. Continue to add slurry until the column is filled to about one inch from the top of the column. **DO NOT allow the level**

of buffer to go below the level of the gel at any time or your column will crack and be ruined.

The homogeneity of the column packing can be checked by watching the passage of blue dextran through the column. If the column is properly packed, the blue-dextran band will broaden as it passes through the column, but the band will not become skewed. Skewing of the band indicates that irregularities exist in the packed column of gel and that the column should be repacked. Blue dextran is an extremely large polysaccharide with a molecular weight of approximately 2,000,000. It cannot enter the pores of the sephadex gel beads and simply percolates down the column in the buffer exterior to the beads. Thus, in addition to permitting a test of the homogeneity of the packed column, passage of blue dextran allows the calculation of the void volume, Vo, of the column. The void volume of the column is defined as the volume of solvent which must pass through the column before any solute molecule will be eluted.

Test the homogeneity of your column and calculate its void volume by applying l mL of a 2 mg/mL solution of blue dextran to the top of the column and eluting it from the column with the Tris-NaCl column buffer. Do this in the same manner as you will do the buffered egg white (see Part IV below). Turn off the stopcock at the bottom of the column, label your column, and carefully store it in the cold room until next week.

B. Ion Exchange Chromatography (to be done by the other groups)

Wash your column and then rinse with distilled water. Check to make sure water flows through column. Place a plastic funnel in the top of the column to serve as a packing reservoir and a buffer reservoir. You may wish to secure your funnel with parafilm. Place a stopcock on the tip at the bottom of the column and make sure it is closed. Fill the column about 1/4 full of 0.05 M Tris-NaCl column buffer. Add enough of your prepared CM-sephadex to fill the column about halfway. Turn the stopcock to "on" and allow to drip. Continue to add slurry until the column is filled about 3 inches or until you have used up all your slurry. **DO NOT allow the level of buffer to go below the level of the gel at any time or your column will crack and be ruined.** If necessary, remove excess CM-sephadex with a long pasteur pipet, being careful not to break the pipet. Fill the column with buffer and allow to drip for about 15 minutes, then turn off the column, label it, and carefully store it in the cold room until next week.

III. Preparing the Enzyme Source (egg white)- Week 2

Your starting material will be a chicken egg. Begin by separating the white from the yolk. Place a 10 x10 cm square of a double layer of cheese cloth over a 100 mL beaker. Gently filter the egg white by stroking the balled cheesecloth against the side of the beaker. Do not force the egg white through. Transfer 4 mL of the filtered egg white into two 2-mL microcentrifuge tubes and spin for 4 min at 12,000 rpm, 4°C in the microcentrifuge. Remove the supernatant into a fresh plastic culture tube, add 8 mL of the Tris-NaCl buffer, mix thoroughly by inverting the tube until homogeneous, and place the egg white-buffer mixture (“buffered egg white”) on ice. This will be the starting material for the purification.

IV. Chromatography of the Buffered Egg White Solution- Week 2

IMPORTANT NOTES:

- Throughout the entire procedure, store the buffered egg white solution and all column fractions on ice. Be sure to save the remaining buffered egg white for activity and protein determinations. Be sure to keep all column fractions and the remaining buffered egg white for PAGE analysis next week.
- You will assay each column fraction and the buffered egg white for protein via the Warburg-Christian assay. However, the protein concentrations of the egg white and some of the column fractions may be too great to assay quantitatively with this assay. **Dilutions of samples that give an A_{280} > 1.5 must be prepared for quantitative protein assay.** For column fractions that are off-scale, try pipeting 0.2 mL of the column fraction and 0.8 mL of Tris buffer into test tubes and mixing. (What fold dilution is this?) Try a 50-fold dilution of the buffered egg white. **Make sure that you account for any dilutions later on in your calculations.**

A. Gel Filtration

1. Set up the Ocean Optics to determine A_{280} for column fractions. Use Tris-NaCl buffer as your blank.
2. Label 15 UV-cuvettes for collecting column fractions. Add 2 mL of water to one cuvette and mark the other cuvettes with a line at the 2-mL volume.
3. Allow the Tris-NaCl buffer to run out of the column until the buffer first reaches the top of the packed slurry. **Stop the flow of the column!**
4. Pipette 1 mL of the buffered egg white *carefully* into the column. Avoid disturbing the top of the column; put the pipette tip against the side of the column. Allow the 1 mL of buffered egg white to run into the column. As soon as the solution reaches the top of the slurry, add 1 mL of the Tris-NaCl column buffer to the column. When the buffer again reaches the top of the slurry, again add 1 mL of the Tris-NaCl column buffer and then *slowly* fill the column with the column buffer.
5. Begin collecting 15 sequential 2.0 mL quantities of eluent in separate UV-cuvettes. **Do not at anytime let the column go dry**. Immediately measure the A_{280} for fractions as they come off the column and then place the fractions on ice.
6. Label microcentrifuge tubes with fraction numbers for each fraction collected from the column. Add ~1 mL from each fraction to the corresponding microcentrifuge tube and store in the freezer.
7. Begin assaying the enzymatic activity for each fraction that has an $A_{280} \geq 0.1$ (see below for activity measurements).

B. Ion Exchange

1. Set up the Ocean Optics to determine A_{280} for column fractions. Use Tris-NaCl buffer as your blank for the early samples and then switch to carbonate buffer for the later samples.
2. Label 20 UV-cuvettes for collecting column fractions. Add 2 mL of water to one cuvette and mark the other cuvettes with a line at the 2-mL volume.
3. Take 2 mL of the prepared egg white and add 2 mL of cold Tris-NaCl buffer to it, mixing gently. **Make sure that this solution is homogeneous or you will clog your column!**
4. Allow the Tris-NaCl buffer to run out of the column until the buffer first reaches the top of the packed slurry. **Stop the flow of the column!**

5. Pipette all 4 mL of the diluted egg white *carefully* into the column. Avoid disturbing the top of the column; put the pipette tip against the side of the column. Allow the egg white to run into the column. As soon as the solution reaches the top of the slurry, add 1 mL of the Tris-NaCl column buffer to the column. When the buffer again reaches the top of the slurry, again add 1 mL of the Tris-NaCl column buffer and then *slowly* fill the column with the column buffer.
6. Begin collecting 10 sequential 2.0 mL quantities of eluent in separate UV-cuvettes. **Do not at anytime let the column go dry**. Immediately measure the A_{280} for fractions as they come off the column and then place the fractions on ice.
7. You may not need to collect all 10 fractions. If the A_{280} drops to less than 0.1, you can move onto the second buffer. Once the right number of fractions has been collected, stop the column and slowly pipet off the remaining buffer from the top, trying not to disturb the column. SLOWLY add 0.2 M carbonate buffer (pH 10.5) to fill the column up the funnel and begin collecting 2 mL fractions. Again, begin measuring A_{280} for column fractions as soon as they come off the column. Once it looks like all your protein has come off the column, you can stop collecting fractions.
8. Label microcentrifuge tubes with fraction numbers for each fraction collected from the column. Add ~1 mL from each fraction to the corresponding microcentrifuge tube and store in the freezer.
9. Begin assaying the enzymatic activity for each fraction that has an $A_{280} \geq 0.1$ (see below for activity measurements).

V. Assay of Starting Material (buffered egg white) and Fractions from Chromatography for Enzyme Activity and Determining Protein Content- Week 2

A. Enzyme Activity Assays

Lysozyme of egg white will hydrolyze bacterial cell walls. The assay method is based on observing changes in turbidity of a suspension of dried cell walls of *Micrococcus lysodeikticus*. This is a spectrophotometric assay in which the digestion of the cell wall suspension can be measured at 450 nm. As digestion takes place, the turbidity of the cell wall suspension decreases. This decrease is used to measure enzyme activity. An absorbance change (ΔA) of about 0.020 to 0.040 per minute is considered to be a good rate for measuring activity. The first minute of recorded change in absorbance is used to determine the rate of catalysis. In the actual assay itself, the substrate is a uniform suspension of 0.3 mg/mL of dried *Micrococcus lysodeikticus* in 0.1 M phosphate buffer (pH 7.0), kept on ice. The buffered egg white and column fractions are kept on ice, and dilutions are made with 0.1 M phosphate buffer at room temperature. You may need to dilute your buffered egg white to obtain good activity data. First try running the sample as is, then dilute accordingly with Tris-NaCl buffer and run the kinetics again if it is too active. The column fractions should not require dilution.

1. Prepare 100 mL of substrate in 0.1 M potassium phosphate buffer (pH 7.0) by adding ~30 mg of cells to 5 mL of phosphate buffer and homogenizing with a mortar and pestle until a uniform suspension is obtained.
2. Transfer the cells to a flask and dilute with phosphate buffer to a final volume of 100 mL.
3. Set the Ocean Optics to monitor at 450 nm using phosphate buffer as the reference. Follow the Ocean Optics instructions for acquiring kinetic data. Make sure that you bring the correct instructions to laboratory with you (the ones for Experiment 3), as well as the ones used in Experiment 2 if you can't remember how to initialize the spec.

4. A standard assay consists of transferring 2 mL of the substrate solution to a dry cuvette with a magnetic stirrer and adding 100 μL of protein solution. MAKE SURE THAT YOU MIX THE SUSPENSION OF SUBSTRATE WELL BY INVERSION JUST BEFORE TAKING THE 2 ML FOR THE ASSAY. IT IS NOT A TRUE SOLUTION AND IS NOT UNIFORM THROUGHOUT.
5. Check that the absorbance of your cells alone is on scale ($A_{450} \leq 1.0$) before adding your protein solution, then acquire kinetic data for 1 minute. Record the rate (absolute value of the slope, ΔA/min) for each fraction in your notebook. Print out one kinetic graph as an example for your notebook.
6. Collect kinetic data for the egg white (which may need to be diluted) and for all column fractions that showed $A_{280} \geq 0.1$.

B. Protein Determinations

You have already determined the A_{280} for all column fractions. Make sure that you also determine the A_{280} for the buffered egg white, diluting if necessary as described above. Use the lysozyme standard curve for the Warburg-Christian method generated during Experiment 2 to determine the protein concentration for each fraction and for the buffered egg white. Make sure that you correct your protein concentrations for dilution if necessary.

VI. Calculations and Completion of the Purification Table- post-lab analysis after Week 2

In order to compare the different purification methods, each lab group will load samples from each type of column on their gel during Week 3. You must post your RATES and PROTEIN CONCENTRATIONS for each fraction on the group spread sheet in the File Server BC367 folder within 24 hours following lab. You will post this data on the spreadsheet provided for you on the File Server. This information allow each lab group to determine which fractions are the most pure and what volume should be loaded onto the gel to obtain 25 μg of protein.

Additionally, before coming to lab next week, the purification table below should be completed in EXCEL and recorded in your lab notebook. The instructions below are to help in your completion of this table.

PURIFICATION TABLE

Fraction	[Protein] mg/mL	Units of Activity	Specific Activity units/mg	volume of fraction	total protein mg	total activity units	% recovery	fold pure
egg white								
Fraction 1								
Fraction 2								
etc.								

Note: Analyze the data in this table carefully and critically. Do the values in this table make sense in respect to the purpose of the experiment? For example, a “fold-pure” of less than 1 indicates that lyzoyme was <u>contaminated</u> during the course of the experiment rather than purified! Such a result is highly unlikely and suggests a calculational error.

l. Calculation of protein concentration.

This comes from the A_{280} data used to determine protein concentrations from your lysozyme Warburg-Christian standard curve. Be sure to correct for any dilutions that you made.

Sample Calculation:

Assume that the absorbance value for a particular fraction corresponds to a protein concentration of 0.5 mg/mL as determined from the standard curve. This fraction was diluted 5-fold for the protein assay.

$$\textbf{Protein concentration} = \frac{0.5 \text{ mg}}{\text{mL}} \times 5 \text{ (dilution factor)} = \mathbf{2.5\ mg/mL}$$

2. Calculation of enzyme activity units and specific activity:

You determined the initial activity of the starting material (the buffered egg white) and the column fractions in terms of ΔA_{450}/min. The activity of most enzymes is defined in terms of activity units. For lysozyme one unit of activity is **defined** as being equal to an absorbance decrease at 450 nm of 0.001 per minute at pH 7.0 and 25°C ($-\Delta A_{450}$/min =0.001; note that enzyme activities MUST BE positive numbers). The specific activity is defined as enzyme units per milligram of protein in the assay. The calculation of specific activity is essential for determining the effectiveness of the purification. As the enzyme is purified, the specific activity will increase since inactive protein is removed.

Sample Calculations:

Suppose, for example, that your buffered egg white had a protein concentration of 12 mg/mL (after correcting for dilution). It was diluted 50-fold for the enzyme activity assay and had an activity of 0.030 ΔA/min. The original, undiluted egg white therefore has the following:

$$\textbf{Units of activity} = \frac{\Delta A/\text{min}}{0.001\ \Delta A/\text{min/unit}} = \frac{0.030\Delta A/\text{min}}{0.001\ \Delta A/\text{min/unit}} = 30 \text{ units}$$

Correcting for 50-fold dilution: 30 units x 50 = **1500 units**

Specific activity = Units/mg protein

mg protein = *12 mg/mL* x 0.1 mL (used in activity assay) = 1.2 mg
from standard curve and corrected for dilution already

$$\text{Specific activity} = \frac{1500 \text{ units}}{1.2 \text{ mg}} = \frac{1250 \text{ units}}{\text{mg protein}}$$

Similar calculations should be made for each column fraction that shows activity. Column fractions that do not show a gradual and continual change in absorbance during the assay interval do not possess enzyme activity.

3. Calculation of Total Protein, Total Activity, % Recovery, and Fold Purification.

Remember: Total volume of buffered egg white applied to the gel-filtration column was 1 mL, and the total volume of each gel-filtration fraction was 2 mL. Total volume of buffered egg white applied to the ion-exchange column was 2 mL, and the total volume of each fraction was 2 mL.

Total Protein = Total volume x protein concentration
(mg) (mL) (mg/mL)

Total activity = Specific activity x total protein
(units/mg) (mg)

$$\text{\% Recovery} = \frac{\text{Total activity of Fraction x 100}}{\text{Total activity of egg white}}$$

$$\text{Fold Purification} = \frac{\text{Specific activity of fraction}}{\text{Specific activity of egg white}}$$

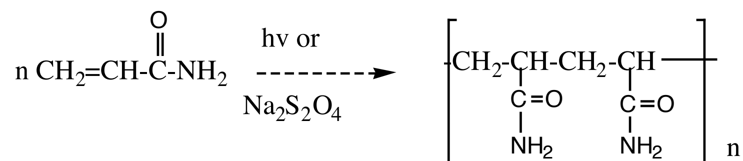
4. Additional Analysis

In your notebook, also include a plot of both protein concentration and total activity versus fraction number (plotted on the same graph). Do not include egg white on the graph. Include this plot in your report.

NOTE: you may plot these parameters as "percent of total" (the sum of the fractions) to achieve similar scales for the two graphs.

VII. PAGE on the Crude Buffered Egg White and the Purified Lysozyme- Week 3

Polyacrylamide gel electrophoresis (PAGE) is a form of zone electrophoresis used to separate proteins or nucleic acids. The support medium is a polymer of acrylamide cross-linked with N,N dimethyl bis-acrylamide.



PAGE is usually carried out in slabs of the gel thus combining electrophoresis with molecular sieving. By varying the concentration of the gel and its degree of cross-linking, its molecular-sieving properties can be controlled over a wide range, and it can be used very effectively to separate mixtures of proteins. Charged proteins are separated during PAGE into discrete bands. The separated zones of proteins can be made visible with dyes that bind to proteins and scanned photometrically with densitometers that have the capacity to integrate the resulting curves. By this means, a simultaneously qualitative and quantitative analysis of the mobile charged species is obtained upon separation. PAGE gives an almost unparalleled separation of protein components in a sample and is thus a very important analytical tool as a

criterion of purity. Even though partial purification of lysozyme is achieved by the column chromatography, the substance obtained is by no means a pure protein, as you will see through PAGE.

Experimental Procedure

A. Assembly of the Mighty Small Slab Gel Electrophoresis Unit

You will pour your gels in the Hoefer Dual Gel Caster, and then move the polymerized gel and support plates to the double-unit electrophoresis pod. Clean plates, spacers, comb, and casting unit thoroughly with water and then carefully dry them prior to assembly.

CAUTION: Acrylamide is a neurotoxin. Use gloves when handling!

1. Place the alumina plate on the bench top with the notched edge of the plate at the top.
2. Lay the two spacers on the edges of the short side of the alumina plate so that the thick sides of the spacers overhang the plate.
3. Place the clean glass plate on top of the spacers. Again, the thick side of the spacers should stick out beyond the edge of the glass slide.
4. Holding the sandwich assembly firmly, slide the entire assembly into the Gel Caster. The alumina plate should be against the thick, clear plastic of the gel caster. Gently tighten the screws of the Gel Caster to hold the sandwich assembly firmly in place. <u>**Check to be sure the spacers are still correctly in place between the alumina and glass plates, and that the sandwich assembly is flush on the bottom gasket</u>.
5. Place the black cams into the sides of the Gel Caster with the short side of the cams facing up. Simultaneously twist both cams so the short side of the cams faces down. This squeezes the entire gel casting assembly into the bottom gasket, theoretically sealing the bottom.

   If your final assembly leaks after adding acrylamide, you can try the following. Pipet a small amount of melted agarose around the bottom of the sandwich assembly so that the agarose is drawn up into the assembly by capillary action. The agarose will act like caulking. Allow the agarose to set so that the space between the glass plate and the alumina plate is sealed. If you feel unlucky, you may try this method first.

6. Using a ruler, make a mark with a Sharpie pen approximately 2 cm down from the top of the alumina plate (measure from the grooved area). The resolving gel should be added to this mark. This will allow room for the stacking gel to be added.
7. The acrylamide resolving gel (see below) may now be added to the unit from the top with a pasteur pipet. Make sure you add the APS and TEMED immediately prior to this step, and work quickly. Be sure to pipet the acrylamide between the glass plate and the alumina plate.
8. Overlay the resolving gel with isopropanol carefully to inhibit the formation of a meniscus and to exclude O_2, which inhibits the polymerization reaction. Wait for the gel to polymerize.
9. Once the gel has polymerized, pour off the isopropanol. The gel unit may be turned upside down over the sink to remove all isopropanol.
10. Make up the stacking gel (see below) and pipet it on top of the resolving gel until it is just at the top of the alumina plate. Carefully insert the comb into the stacking gel and be sure the gel unit is completely filled with stacking gel to create wells.
11. Once the stacking gel has polymerized, the sandwich assembly must be very carefully removed from the Gel Caster and placed into the electrophoresis pod. Before doing this,

grease the silicon rubber gasket of the pod lightly with celloseal. The gasket will seal the pod against the alumina (white) plate of the sandwich.

12. To remove the gel sandwich, turn the cams so the short side of each cam is facing up. Remove the cams. While holding the sandwich assembly together, remove it from the Gel Caster. Remove any obvious chunks of polyacrylamide.
13. Place the alumina plate of the sandwich assembly squarely on the pod with the notched edge of the plate at the top of the pod.
14. Holding the sandwich assembly firmly, attach the two red clamps so that the gel sandwich is secure in the electrophoresis pod.
15. Loosen the comb from the wells and gently pull the comb out. Outline the wells with a Sharpie pen- they will be invisible once running buffer is added.
16. Fill the top chamber with buffer above the level of the gel and check for leaks into the lower chamber. If the unit leaks significantly repeat steps 11-14, checking for cracks in plates, "goobers", etc. If the unit does not leak, then fill the lower chamber with buffer.
17. Load your samples and electrophorese as described below.

B. Preparing the Gel

Prepare a 14% polyacrylamide resolving gel using the recipe below, adding all ingredients except for the polymerization catalysts (APS and TEMED) until immediately before pouring the gel. Mix well. Prepare the stacking gel likewise.

	14% Resolving Gel	Stacking Gel
ACRYLAMIDE (30%)	4.66 mL	0.67 mL
RESOLVING BUFFER	2.50 mL	----
STACKING BUFFER	----	1.25 mL
10% SDS	100 μL	50 μL
DISTILLED WATER	2.63 mL	3.00 mL
10% APS	100 μL	50 μL
TEMED	10 μL	4 μL
FINAL VOLUME	10.00 mL	5.00 mL

C. Preparing the Samples

1. While your polyacrylamide gel is polymerizing, set up a boiling water bath with a floating rack inside.
2. Once the water is heating, you may prepare your samples. There are 10 lanes on each gel. Two lanes will be used for standards, one is for crude egg white, and the other seven are for column fractions. These fractions should be selected as follows:
   - your fractions that contain high lysozyme activity
   - your fractions that contain high total protein
   - high-activity fractions from other groups using different chromatography

   Other samples that should be included on the gel include the buffered egg white and lysozyme standard (provided at 1.0 mg/mL).

   Plan on loading ~25 μg of protein in each well. Use the concentrations from the protein assay data to determine what volume of each fraction needed to obtain 25 μg. (Do not exceed 25 μL.) **This calculation should be done for each sample prior to lab.**

3. Place the appropriate volume of each sample in a separate microcentrifuge tube. For samples containing less than 25 μL, add enough water to bring the samples to a total volume of 25 μL.
4. Add 25 μL of "loading buffer" to each sample. This buffer serves both to reduce the disulfide bonds in the proteins as well as to "sink" the samples in the wells. Spin all samples briefly in the microcentrifuge to mix and then heat the samples for 4 min in the boiling water bath, centrifuge again, and place each sample on ice.

D. Running the Gel

Note: make sure to load your gel asymmetrically as you will lose orientation during the staining process. With a pipettor, carefully layer each protein-dye mixture into a separate well. Also load 10 μL of a protein standard ladder, a commercial mixture of proteins that serve as molecular weight markers on the gel. Place the safety lid on the unit and attach the leads to the power supply. The power supply will be run at 120 volts. This will allow the run to be complete within ~1.5 hours. At the pH of the buffer system (about 8.9) most proteins are negatively charged and will migrate toward the anode.

E. Staining the Gel

1. When the tracking dye reaches the bottom of the gel, turn off the power supply, pour off the tank buffer, and disassemble the gel unit.
2. Remove the spacers and use one of the spacers to break the seal of the sandwich.
3. Remove the gel gently from the plate and put it into a labeled plastic container. Add enough dH_2O to cover the gel and microwave for 45 sec or until the solution starts to boil. Let the container mix by placing on a shaker for 1 min. Remove the water.
4. Add enough ProtoBlue Safe stain solution to cover the gel and microwave for two 20-sec bursts. Be careful not to let the solution boil.
5. Allow the gel to shake for 5 min, and then decant the stain into the waste container.
6. Rinse the gel three times with dH_2O (no microwaving) and let the gel shake until the desired contrast is achieved.
7. Gels can be wrapped in Saran Wrap or placed in a zip-lock bag, viewed on the light box, and scanned for your report (please keep the gel wrapped while scanning).
8. Measure the distance traveled for standard proteins, unknown proteins, and the lysozyme standard. Make a plot of $\log(M_w)$ versus distance traveled to determine the M_w of any unknown proteins from your column fractions. This plot should be in your notebook. Based on their molecular weights, identify at least two contaminating proteins in egg white.

VIII. Mass Spectrometry of Lysozyme- Week 3

Mass spectrometry (MS) is widely recognized as the most viable analytical tool for routine analysis of proteins and other biopolymers. It is the method of choice because of its high sensitivity (low femtomole to attomole), vast amount of information content in the data, and ability to be automated. MS identifies molecules based upon their unique isotope-dependent molecular mass. When the molecule is either too large to be studied, or too redundant in sequence, the "parent" molecule is fragmented to produce a series of unique "daughter" ions that can be reconstructed into the parent molecule.

The two most common methods for generating gas-phase ions of a biomolecule for MS analysis are electrospray ionization (ESI) of a solution, and matrix-assisted laser desorption

ionization (MALDI) of a solid. While both methods are widely used, each technique has potential benefits and detriments. MALDI is the more forgiving and most frequently used for automated rapid screening of protein samples, while ESI is often directly coupled to a chromatography separation and can be used to selectively fragment and identify daughter ions from a parent molecule.

The ESI MS of a single protein in acidic solution will generally be observed as a series of peaks consisting of different charge states for the protein. Each peak is a protein molecule that has added or lost a single proton and a single charge. By comparing the differences in mass/charge ratio (*m/z*) between peaks, the "true" mass of the protein can be determined.

The *m/z* of a peak is determined by Equation 1 where M is the mass of the protein, n_2 is the number of charges, and X is the mass of the added group(s).

$$\left(\frac{m}{z}\right)_2 = \frac{M + n_2 X}{n_2}$$

Equation 1

For most experiments, the added group will be a proton (mass = 1.00794). When the added group is a proton, the neighboring peak at a smaller *m/z* ratio will follow Equation 2. By combining Equations 1 and 2, we obtain two additional equations (Equations 3 and 4) that allow determination of M and n_2 from any two peaks that differ by one X group. Note that Equation 3 has a typo in Lehninger (pg. 99).

$$\left(\frac{m}{z}\right)_1 = \frac{M + (n_2 + 1)X}{n_2 + 1}$$

Equation 2

$$n_2 = \frac{\left(\frac{m}{z}\right)_1 - X}{\left(\frac{m}{z}\right)_2 - \left(\frac{m}{z}\right)_1}$$

Equation 3

$$M = n_2\left[\left(\frac{m}{z}\right)_2 - X\right]$$

Equation 4

Experimental Procedure

While your gel is running, you should obtain electrospray ionization mass spectrometry (ESI MS) data on a sample of pure lysozyme.

1. Follow the instructions at the ESI MS to obtain your spectrum.
2. Use Equations 3 and 4 in EXCEL to calculate the average mass of lysozyme. How does this compare to the calculated mass from the amino acid sequence? Be sure to include a copy of your ESI MS and EXCEL data analysis in your notebook.

Analysis

Compare your purification to those of your classmates who ran the other types of purification. You can either use their purification tables, your PAGE results, or both for this assessment. Which purification protocol seemed to work best? How might you improve your particular purification scheme? What evidence do you have that you actually purified lysozyme?

References

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