

Lysozyme Experiment Manual

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Introduction

Lysozyme is a low molecular weight enzyme with anti-microbial activity. Lysozyme is found in a number of animal secretions such as tears, saliva, milk, and mucus because it destroys certain types of bacteria (gram positive) that try to enter the body through these passageways. It is also found in some plants. A ready source of lysozyme is found in the white of eggs. This enzyme is known as a glycoside hydrolase. It kills the bacteria by cleaving bonds in their cell wall and makes them susceptible to osmotic pressure. Lysozyme breaks down bacterial cell walls by catalyzing hydrolysis of 1, 4 beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine.

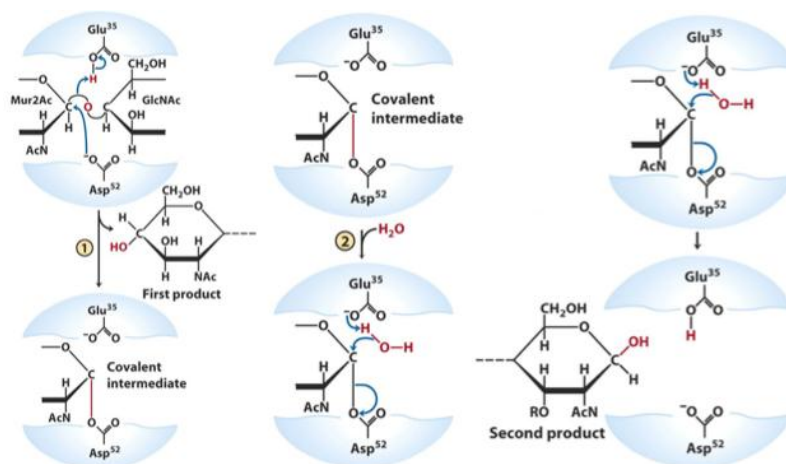


Figure 2. Proposed lysozyme mechanism (SN2). <https://www.google.com/search?q=lysozyme+mechanism>

Lysozyme is an excellent protein for studying enzyme activity. The antibacterial activities of Lysozyme have been of considerable interest to the medical, pharmaceutical, and food industries (Coleman 2013). The protein is very accessible and stable, which makes it a great resource for students to manipulate and learn about enzymatic function. Most proteins are aggregated and denature unless kept at a specific temperature, pH, or osmolality. Lysozyme is stable at room temperature in water, easy to obtain, and there is a simple non-toxic assay for measuring its activity.

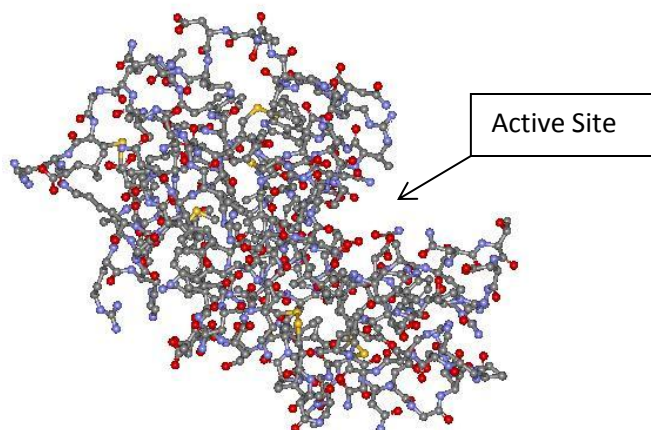


Figure 1. Ball and stick model of chicken egg white lysozyme. <http://lysozyme.co.uk/results/lysozyme-ball-stick.jpg>

The assay for lysozyme involves using a spectrophotometer to measure the amount of light scattering in a suspension of bacterial cell walls after mixing it with the enzyme. Before Lysozyme is added to the cell wall solution, light hits the cell wall material and scatters. When the enzyme is added, the bonds in the cell wall begin to break, and light scattering decreases, resulting in an increase of light passing through the suspension. Light scattering can be measured using a spectrophotometer which reads the change in absorbance (light scattered) within a solution. As the reaction proceeds there will be a decrease in absorbance.

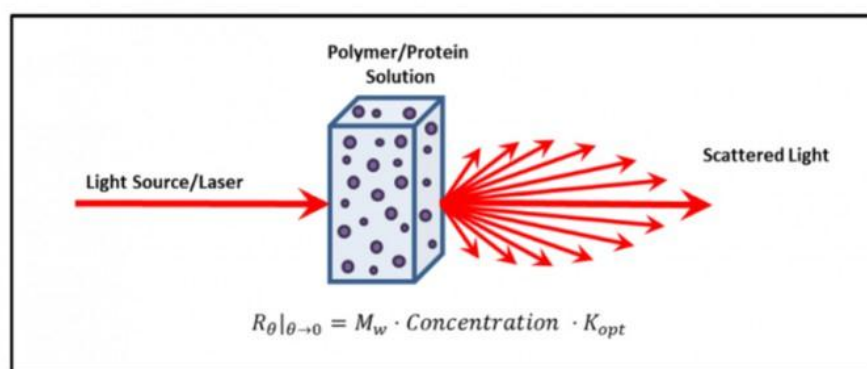


Figure 3. Diagram of light scattering after hitting particles in a solution. <http://polyanalytik.com/v2/wp-content/uploads/2015/11/gpc-light-scattering-1-700x334.jpg>

Basic Assay

Standard Materials:

- Spectrophotometer
- 300 mg bacterial cell prep (M3770, Sigma Chemical Company) in 1L phosphate buffer at pH7 (see appendix for mixing phosphate buffer)
- Mortar and Pestle (for grinding cell walls)
- 20mg of Lysozyme (Lysozyme, L6876, 50,000 units per mg, Sigma Catalogue) dissolved in 200 mL tap H₂O or phosphate buffer (pH 7)
- 1 Plastic cuvette (1cm*1cm, 3mL)
- Stop watch
- Parafilm
- 200 μ L pipette
- Pipette tips

Alternative Materials (cheaper):

- Colorimeter (OPN Colorimeter) <http://pages.stolaf.edu/opn-lab/equipment/>
- 300 mg bacterial cell prep (M3770, Sigma Chemical Company) in 1L tap H₂O
- Mortar and Pestle (for grinding cell walls)
- Lysozyme (egg white) 1mL egg white in 30 mL tap H₂O or phosphate buffer (pH 7)
- 1 Plastic cuvette (1cm*1cm, 3 mL)
- Stop watch
- Water source and sink
- Parafilm
- 200 μ L pipette

Methods

- A. Turn on spectrophotometer and set the wavelength to 450 nm
- B. Shake up the cell wall suspension which should be at room temperature (microwave for 10-30 seconds if the cell wall suspension was refrigerated), and add 2.5 mL to a cuvette.
- C. Place the cuvette in the spectrophotometer and record the absorbance (don't zero the spectrophotometer). This is the zero time reading.
- D. Add 100 μ L of the lysozyme solution and start the stop watch. Mix the solutions by placing parafilm over the cuvette and shaking it gently. Place the cuvette back into the spectrophotometer and take the absorbance reading every 30 seconds for 5 minutes.

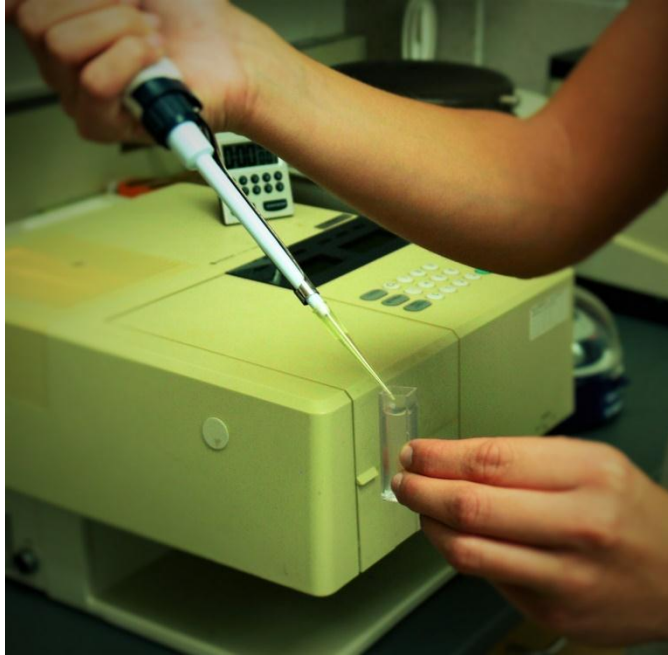


Figure 4. Addition of Lysozyme to bacterial cell wall solution.

Graphing Procedure

- A. Type time intervals in the column and a μL in a row on an excel spreadsheet
- B. Subtract the zero time absorbance from the other absorbance readings.
- C. Record all the absorbance readings at each time interval (the numbers should be negative values)
- D. Graph the data, the time intervals should be on the x-axis and absorbance readings on the y-axis of the scatter plot graph

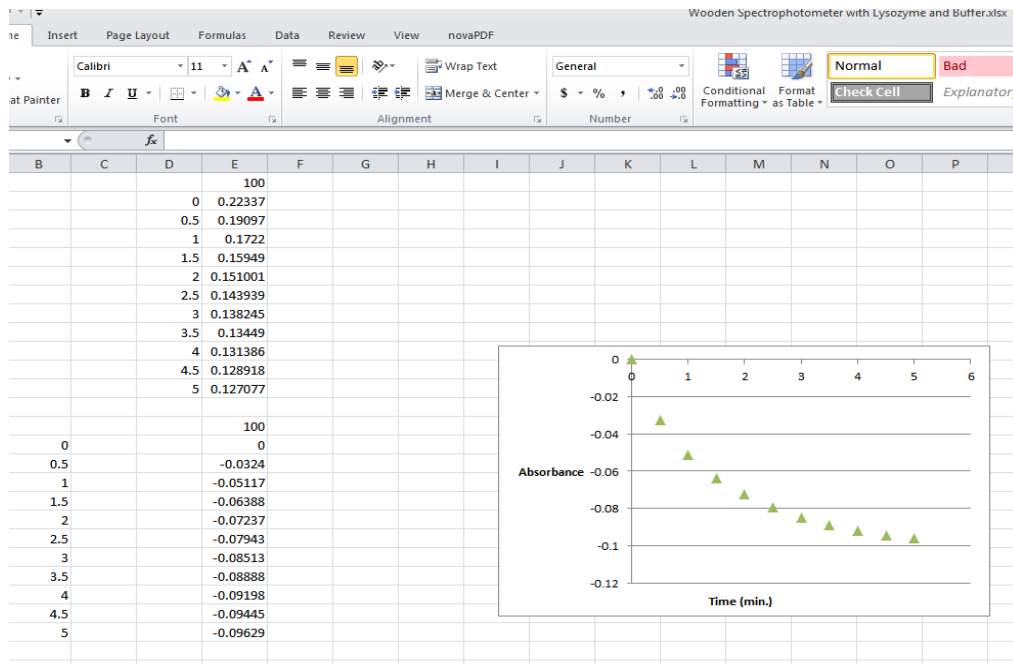


Figure 5. The top data set is the actual absorbance result, and the bottom data set is the absorbance readings subtracted from the zero time reading. (Example: the E16 value in the second data set was calculated from E3-E2 in the first data set)

Alternative Experiments (things you can do)

1. You can measure the effect of different amounts of enzyme on the rate of the reaction. Run the reaction with 25, 50, 100, 200 μl of enzyme preparation. Have the students graph all the data on one graph (Fig,6)

Materials:

- Same materials used in the basic assay

Methods:

- Follow the methods for the basic assay, and run the reaction 4 times using 25, 50, 100, 200 μl of lysozyme dissolved in phosphate buffer (pH 7) or tap water.

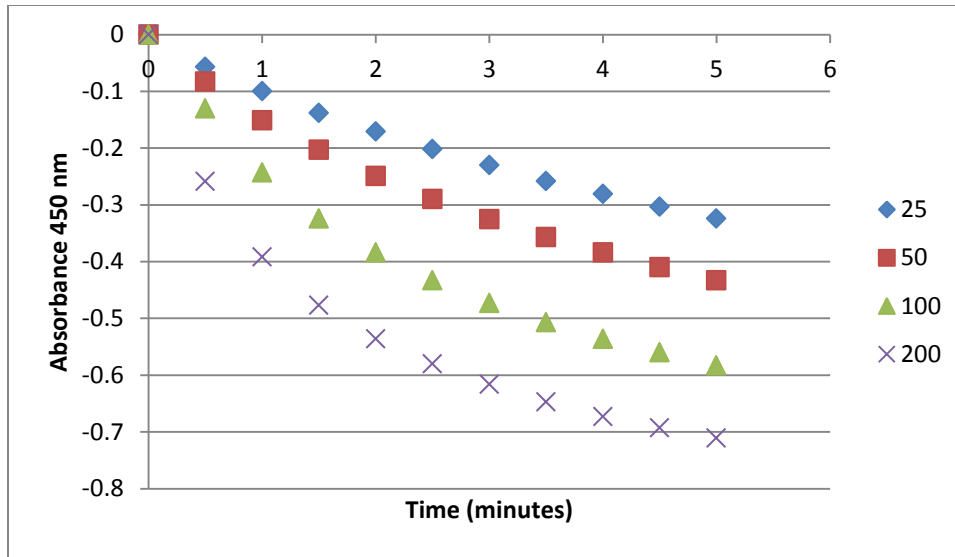


Figure.6. Effect of Lysozyme on Bacterial Cell Wall Breakdown at Various Concentrations of enzyme. This figure illustrates that the greater the amount of enzyme in solution, the larger the probability of an enzyme bouncing into a substrate and a reaction taking place.

Question for students: Why are the lines not linear on the graph?

Answer: The cell wall material is limiting reactant, therefore, it can only get broken down to a certain amount. The more enzyme added, the faster the reaction rate will be, but theoretically, all the lines will eventually meet once all the bacterial cell wall is broken down.

- A. You can also introduce the idea of the initial rate of the reaction when the substrate is at its highest concentration. This is the rate of the reaction before the consumption of the substrate limits the rate of the reaction. This is a very important concept in biochemistry. (Fig, 7)

Initial rate of the lysozyme reaction.

- A. Run a lysozyme assay with 100µl of your sample.
- B. Plot your data using Excel or similar program.
- C. Select the first 4 data points and do a linear curve fit. This should be your initial rate. ($\Delta A/\text{time}$)
- D. If the R^2 squared value is less than 0.97 repeat the assay with less sample. The closer the R^2 value is to 1.0, the better the fit of the regression line. Meaning, the closer the line is to passing through all the points on the graph.
- E. Convert your rate into units of activity. One unit of activity is defined as 0.001 absorbance change per minute.
Example: rate: $-0.015 / (0.001/\text{min}) = 15$ units of activity/100µl protein
- F. Now calculate number of units per µl by dividing the number of µl you used in the assay.
Example: Units of activity= $15\text{units}/100\mu\text{l} = 0.075/\mu\text{l}$

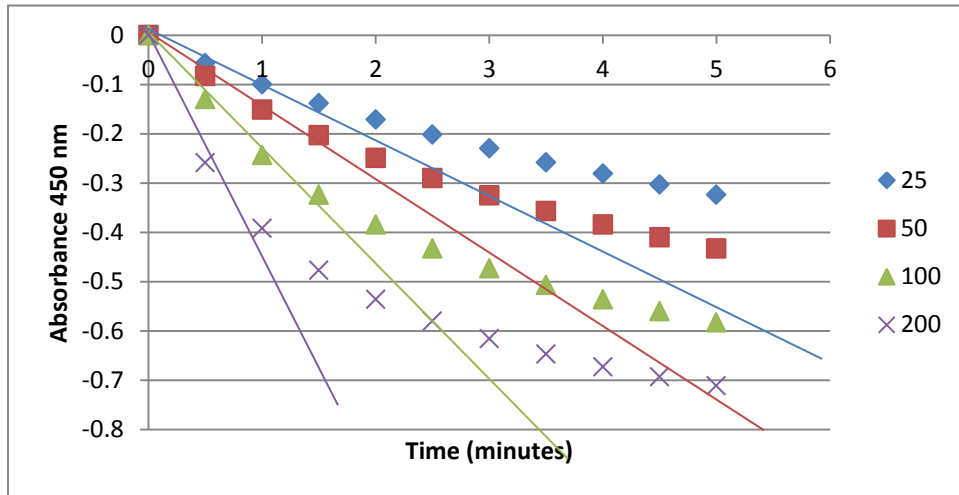


Figure.7. Initial Rate of Reactions at Various Concentrations of Lysozyme. This graph illustrates the initial rate of the reaction changing as the substrate (lysozyme) goes from low concentration of 25 μ l to its highest concentration of 200 μ l.

2. You can measure the effect of human saliva on the breakdown of bacterial cell walls. Incorporate initial rate into the graph and compare the initial rate before and after looking at a candy bar.

Materials:

- Same materials listed above

Methods

- A. Repeat steps A-C from the methods on page 4.
- B. Have the students drool into a beaker until they fill the beaker with 5-10 ml of saliva.
- C. Add 200 μ L of the saliva to the cuvette and start the stop watch. Mix the solutions by placing parafilm over the cuvette and shaking it gently. Place the cuvette back into the spectrophotometer and take the absorbance reading every 30 seconds for 5 minutes.
- D. Repeat the experiment, but modify step B. Have the students smell/look at a candy bar and envision eating it while they drool into the beaker. Again, obtain 5-10 ml of saliva, and then repeat the rest of the experiment.
- E. Graph the data collected from both the plain saliva experiment and the saliva after looking at a candy bar experiment and analyze the results.

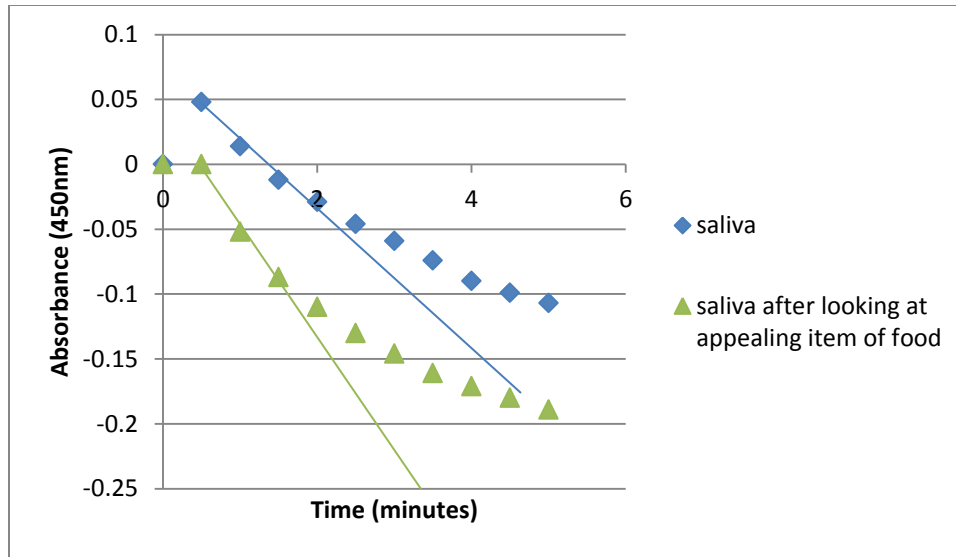


Figure.8. Human Saliva as Alternative Source of Lysozyme and the Effects of Saliva before and after looking at a candy bar/ appealing item of food. This figure illustrates that the lysozyme in saliva is capable of breaking down bacterial cell walls. The slight increase in absorbance after initially adding the lysozyme represents the change in absorbance in the solution because our saliva absorbs some light. The green triangles on the graph represent the breakdown of bacterial cell walls by saliva after looking at a candy bar. This shows an increase in lysozyme production in our salivary glands of our mouth after looking at an appealing item of food.

Question for Students: What are possible reasons for our salivary glands to secrete more lysozyme in saliva after looking at a candy bar?

Possible Answer: The body may be preparing for food to enter our system. The primary foods sources for cariogenic bacteria (cavity-causing) include sucrose, fructose, lactose, and starches. The body might be preparing to breakdown the cariogenic bacteria by secreting more lysozyme in saliva.

3. **You can measure the effect of pH on the rate of the lysosome reaction. Mix up phosphate buffers at different pH2 (3,4,5,6,7,8,9). Add cell wall to the buffers and run the assays. See the appendix for easy instructions of how to mix up the phosphate buffers. You should find a pH optimum of 6 with the rate dropping off on either side.**
 - You can ask the students to explain this result. Although the precise answer is a bit more complicated, remind them that extremes of pH denature enzymes.
4. **You can measure the effect of temperature on the rate of the lysosome reaction. Incubate the cell wall solution at the temperature you want to test (4°, 10°, 20°, 30°, 40°, 60°, 70°). You can use a water bath, refrigerator and ice baths to get the temps you want. Let the**

cell wall suspension equilibrate at the desired temperature for 10 minutes. Then run the reaction.

- You should see an increase in the rate of reaction until you get to $\approx 40^\circ$ and then a drop. Have the students explain why this is the case. You can remind them that with increasing temperature there is an increase in the number of collisions between substrate and enzyme thus increasing the rate, also, when you reach a high enough temperature, the enzyme denatures and loses activity.
- 5. Human tears are a great source of lysozyme. If your students can collect tears you can measure the activity. We used a cut onion to induce tearing.**
 - 6. You can measure the effect of adding acetone to the reaction. Using 100% acetone, run the reaction with increasing amounts of acetone. Begin with 20 μ l then work your way up.**
 - Ask the students why acetone inhibits the reaction (denatured protein). The above experiment can be tried with lots of organic molecules (ethanol, methanol, etc.)
 - 7. You can measure the effect of heating the enzyme prior to the reaction. Place 250 μ l of enzyme in a test tube or microfuge tube. Heat in a water bath for 10 minutes, then allow to return to room temp. Then run the reaction. Start at 30 $^\circ$ c and work your way up to 90 $^\circ$ c.**
 - 8. You can compare lysozyme activity in different types of egg white. (chicken, duck, etc).**
 - 9. You could compare lysozyme content in egg white –vs- yoke. Use a similar dilution of each and measure the activity.**
 - 10. You can measure the effect of various salts on lysozyme activity. Start with a 1M solution of the salt to be tested. Add small amounts till you see an effect.**
 - 11. You can test the effect of detergents to a lysozyme reaction. Start with a 10%v/v stock solution and add small amounts till you see an effect. Remember detergents are agents that cause proteins to denature.**
 - 12. You could use urea in the reaction. Urea also causes proteins to denature.**

Simple Purification

This simple exercise is to help students understand the principles of protein purification. Depending on the pH of the solution a protein can have a net negative or positive charge based its amino acid composition. Lysozyme (in a pH9.2 buffer) has a net positive charge and will readily bind to a column of negatively charged Sephadex particles. Proteins without such a negative net charge will pass right through the column. To release the lysozyme you simply have to elute the column with concentrated salt solutions (NaCl). A low concentration of NaCl will wash away the weakly positive proteins in the egg white solution. A high concentration of NaCl will wash away the strongly positive proteins left in solution. The lysozyme is a strongly positive protein, and will elute with the high concentration of salt solution.

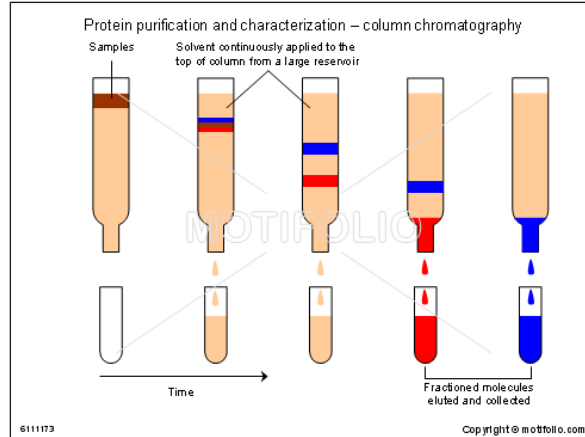


Figure 9. Diagram of protein purification- column chromatography.

Materials:

- 3 Small beakers (50ml)
- 1 L glycine buffer (pH 9.2)
- 100mL glycine buffer (pH 9.2) ,100mM NaCl (low salt)
- 100mL glycine buffer (pH 9.2) ,750 mM NaCl (high salt)
- 3g SP sephadex
- 10mL syringe
- Small amount of glass wool
- Parafilm

Methods

- A. Place 1g of Sephadex in 15mL of glycine buffer (pH 9.2). Let sit over night to hydrate.
- B. Prepare the 10 mL syringe as a column by removing the pressure pump and placing a small amount of glass wool at the bottom. Attach the syringe to a ring stand. (Fig. 10)



Figure 10. Syringe column with removed pressure pump and glass wool and hydrated Sephadex.

- C. Mix and pour the hydrated Sephadex into the column. You want a packed volume of 4mls so pour the slurry in slowly. You will have extra Sephadex left over. Cover the extra Sephadex slurry and refrigerate.
- D. Rinse the column with 4 ml of glycine buffer.
- E. Prepare the egg white by first separating the white from the yoke. Take 10ml of egg white and dilute to 60ml in glycine buffer. Stir gently with a glass rod until the egg white and glycine buffer are mixed well. Filter through 4 layers of cheese cloth then through filter paper.

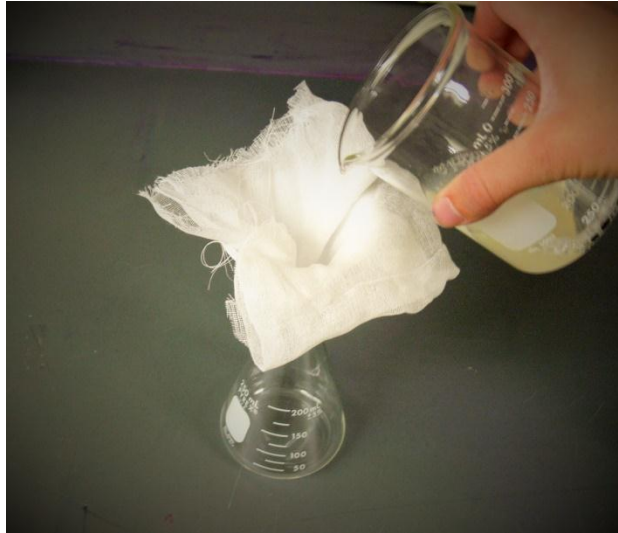


Figure 11. Filter dilute egg white and glycine buffer through 4 layers of cheese cloth

- F. Run the column by pouring 4ml of the filtered egg white slurry onto the column. Catch and save the effluent.
- G. Wash the column with 4 ml of 100mM NaCl glycine buffer. Catch and save the effluent (weakly positively charged proteins).
- H. Now wash the column with 4ml of 750mM NaCl glycine buffer. Catch and save the effluent (strong positively charged proteins).
- I. Measure protein and enzyme activity (initial rate) in the diluted and filtered egg white, egg white effluent, 100mM NaCl effluent and the 750mM NaCl effluent.
- J. You can recharge the column by washing it with 8ml 100mM NaCl glycine buffer followed by 8ml of glycine buffer (pH 9.2).

Calculations

Initial rate of the lysozyme reaction.

- A. Run a lysozyme assay with 100 μ l of your sample.
- B. Plot your data using Excel or similar program.
- C. Select the first 4 data points and do a linear curve fit. This should be your initial rate. ($\Delta A/\text{time}$)

- D. If the R^2 squared value is less than 0.97 repeat the assay with less sample. The closer the R^2 value is to 1.0, the better the fit of the regression line. Meaning, the closer the line is to passing through all the points on the graph.
- E. Convert your rate into units of activity. One unit of activity is defined as 0.001 absorbance change per minute.
Example: rate: $-0.015 / (0.001/\text{min}) = 15$ units of activity/100 μl protein
- F. Now calculate number of units per μl by dividing the number of μl you used in the assay.
Example: Units of activity= $15\text{units}/100\mu\text{l} = 0.075/\mu\text{l}$

Total Activity Per Fraction

- A. Calculate the total activity by multiplying the number of μL in each effluent by the number of units per μl for that fraction.
- B. Total activity= total units

Example: total units= $(0.075 \text{ U}/\mu\text{l}) * (4,800 \mu\text{l} \text{ solution in beaker}) = 360 \text{ U}$

Amount of protein in each fraction

- A. Follow the directions for using the Bradford assay.
- B. Begin by using 40 μl of each sample. You may have to use more sample if the protein content of the sample is low, or less if the numbers are off your standard curve.
- C. Calculate the total amount of protein in each effluent.

Specific Activity

- A. Divide the total amount of units of enzyme activity in each effluent by the total amount of protein in each fraction. (units/ μg protein)

Example:

total protein= $(0.168 \text{ protein}/\mu\text{l}) * (4800 \mu\text{l} \text{ solution in beaker}) = 806.4 \mu\text{g} \text{ protein}$

specific activity= $360 \text{ U}/806.4 \mu\text{g} \text{ protein} = 0.4464 \text{ U}/\mu\text{g} \text{ protein}$

Purification of Human Saliva Lysozyme

Materials:

- Same materials as above for simple purification

Methods

- A. Repeat steps A-D for simple purification
- B. Collect 15 ml of saliva by spitting into a beaker and dilute with 15 ml of glycine buffer. Stir with a glass rod until the saliva and glycine buffer are mixed well. Filter the saliva through 4 layers of cheese cloth. The filtered saliva is now a saliva slurry.
- C. Repeat steps F-I for simple purification. Wherever it mentions egg white slurry in the directions, substitute that with the saliva slurry.

Alternative Experiments (things you can do)

- A. You can try different types of eggs.
- B. You could try collecting fractions with different concentrations of NaCl.
- C. You could try other salt in the elution buffer.

Bradford Protein Assay

It is often important to know the amount of protein in a biological sample. If you are measuring the amount of enzyme activity for example, you need to know how much protein is in your preparation. We measure the amount of protein in a sample using the Bradford assay. By using BSA as our standard, we can graph the absorbance at 0, 10, 20, 30, and 40 μL . This graph is our standard curve. The equation for the line is $y(\text{absorbance}) = \text{slope} * x(\text{amount of protein})$. Since we knew how much protein we used for our standard, we can measure the amount of protein in our sample by measuring the absorbance and finding where our sample falls on the curve. BSA is a great protein to use as a standard because it is 1mg/1mL BSA standard. The Bradford reagent is used because the protein changes the absorbance of the Bradford reagent at 595 nm.

Materials:

- Bio Rad quick start Bradford reagent
- 10 Test tubes
- 5ml pipette
- 200 μL pipette
- Spectrophotometer
- 1 cm cuvette
- 1 mg/ml BSA (bovine serum albumin) standard

Methods

- A. Add 0, 10, 20, 30, 40 μL of BSA standard to 5 test tubes.
- B. Add H_2O so that the total volume is 100 μL in the test tube.
- C. Add 5 mL Bradford reagent to each test tube (mix well).
- D. Wait 10 minutes.



Figure 12. This figure shows the effect of the BSA protein on the absorbance of the Bradford reagent. The left test tube has zero protein added, and appears light brown. Each test tube from left to right increases in BSA protein by 10 μl . The far right test tube contains 40 μl of BSA protein, and appears dark blue. The absorbance of each test tube will be used to create the standard curve.

- E. Read OD at 595 nm.
- F. Create a standard curve on excel with the equation displayed on the graph. The line should be linear.

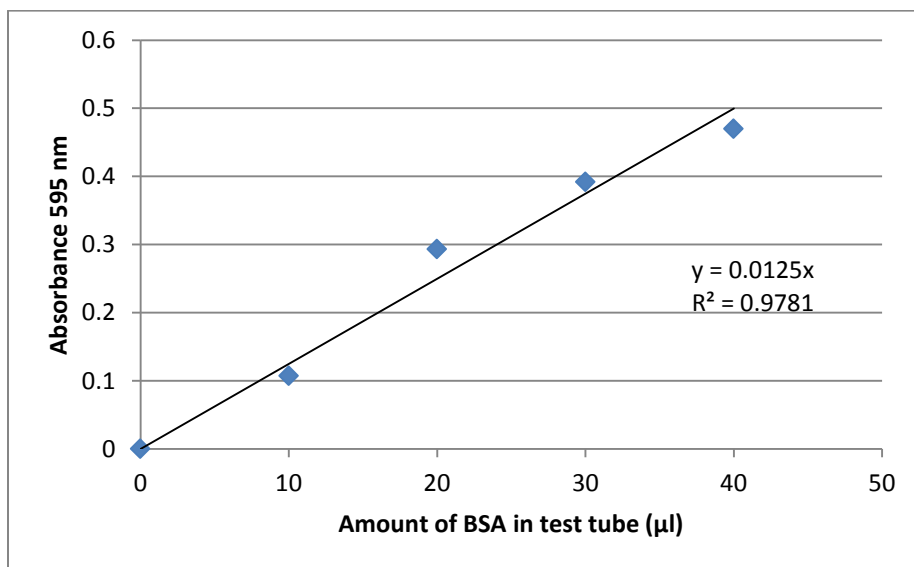


Figure.12. Standard Curve Based on Measured Absorbance of Various Amounts of Bovine Serum Albumin (BSA). This figure shows how the absorbance of light changes at a linear rate as the amount of bovine serum albumin (cow blood protein) increases in each test tube.

- G. Test your unknown by placing 10 μL of low salt effluent protein to a test tube and 10 μL of high salt effluent protein to another test tube. Repeat steps B-E for each test tube of the sample protein (high salt and low salt). You may have to adjust the amount of protein you add to the test tube to make sure that the absorbance of the high and low salt protein samples are within the standard curve.
- H. Solve for the amount of protein in each test tube by using the equation for the standard curve.
- Remember: the amount of protein you solve for is $\mu\text{g}/10 \mu\text{L}$ of solution.
 - You can solve for the amount of protein in your 50 mL beaker using conversions.

Application of Enzyme-Catalyzed Reactions (Lysozyme)

Station Set-Up: 12 stations, 6 per bench, pH experiment

Materials:

- 2 small beakers
- 1, 3 ml cuvette
- 1 spectrophotometer
- 1 stop watch
- 1, 200 μ l pipette
- KIM WIPES
- Pieces of parafilm
- Water bottle
- 200 μ l pipette tips
- 1, 5 ml pipette
- 1 pipette bulb

Refrigerator:

pH3 buffer with cell walls
pH4 buffer with cell walls
pH5 buffer with cell walls
pH6 buffer with cell walls
pH7 buffer with cell walls
pH7 buffer with cell walls
pH8 buffer with cell walls
pH9 buffer with cell walls

Methods

Prior to Lab

- Allow buffer solutions to come to room temperature

Enzyme Preparation

- Prepare 200 ml of enzyme prep at 1 mg per 10 ml. (Lysozyme, L6876, pg 1221, 50,000 units per mg)
- Aliquot the prep into 1 ml epindorf tubes and place in the freezer. These should be reused from lab to lab. (50,000 U/10 ml)
- There are 20 mg enzyme per 200 ml

APPENDIX

Buffers

- Prepare 3 stock solutions (1M H₂PO₄, 1M K₂HPO₄, 1M KH₂PO₄)
- Use the chart below to mix the desired pHs
- At each pH, there is 100mL of solution that should be diluted to 1 L
- 300 mg of cell wall prep (M3770, page 1337 Sigma Catalogue) should be added to each liter
- Store solutions in refrigerator

pH values	1M H ₂ PO ₄	1M K ₂ HPO ₄	1M KH ₂ PO ₄
pH 3	12 mL		88 mL
pH 4	1.3 mL		98.7 mL
pH 5		2.0 mL	98.0 mL
pH 6		13.2 mL	86.8 mL
pH 7		61.5 mL	38.5 mL
pH 8		94.0 mL	6.0 mL
pH 9 (bring to pH 9 w/ NaOH)		100 mL	

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