

ISOLATION AND PARTIAL PURIFICATION OF EGG WHITE LYSOZYME

Lysozyme is a small enzyme (its molecular weight is about 14,600). Basic side chains dominate its surface, so it bears a net positive charge at neutral pH. This is somewhat unusual; most proteins are slightly acidic and so bear a net negative charge. Lysozyme is found in tears and (rather abundantly) in the whites of eggs.

The discovery of lysozyme was serendipitous, like many other discoveries in science. Alexander Fleming, a British microbiologist, was in the lab one day with a runny nose, and a drip accidentally fell on a plate of bacteria he was examining. Feeling rather lousy, he didn't discard the plate that day and just went home. When he returned to the lab, he discovered a clear patch in the "lawn" of bacteria on the plate. He reasoned that the drop that fell must have contained a factor that was capable of destroying these bacteria. This led to the discovery of the enzyme.

The cell walls of certain Gram-negative bacteria are built of polysaccharides composed of alternating units of N-acetylglucosamine and N-acetylmuramic acid (a modified glucosamine). Lysozyme catalyzes hydrolysis of the glycosidic bond joining these two sugars. When enough of this degradation happens, the cell wall enclosing the bacterium is weakened and the protoplast lyses due to osmotic shock.

Lysozyme can be conveniently assayed by allowing it to hydrolyze the cell walls of *Micrococcus lysodeikticus* suspended in a suitable buffer. Enzyme activity is measured as the rate of loss of turbidity in the suspension (when a cell lyses, virtually everything goes into solution, so that cell's contribution to the turbidity is lost).

In this experiment you will partially purify lysozyme from egg white, quantitate the degree of purification, and determine the effect of pH on lysozyme activity. This information can be used as a test of a proposed enzyme mechanism (which you can find in textbooks). The purification and pH dependence parts should be combined into a single report.

EXPERIMENTAL SECTION

A. ISOLATION AND PARTIAL PURIFICATION

One egg white provides sufficient material for three students. **Keep everything on ice unless not possible.**

Separate the white of an egg and put it into a bag of two layers of cheesecloth held over a 100 mL beaker in an ice bucket. Gently stroke the sides of the bag on the side of the beaker to coax the egg white through. Don't squeeze the bag. When no more comes through, discard the bag and its contents. The 100 mL beaker contains what you want.

Estimate the volume of this filtrate and add sufficient cold 0.01 M Tris buffer in 0.02 M NaCl (pH 8.0) to make a 4 fold dilution (i.e., the final volume should be about 4 times greater than the volume of the filtrate from the cheesecloth). Swirl to mix well and then pass all of the solution through a small loose plug of glass wool in a funnel. We will call this the "egg white filtrate (EWF)". Proceed individually or in pairs (instructor's choice) by dividing the filtrate from this step into as many parts as necessary.

Locate the CM-cellulose (CMC) suspension and record the composition of suspension buffer. Swirl up the suspension and take about 40 mL. Mix in 4.0 mL of the EWF (save the rest of the EWF on ice). Swirl the slurry gently and frequently on ice for about 10 minutes to allow complete binding. *The low ionic strength of the buffer favors binding to the carboxylate anions on the CMC, while the slightly alkaline pH makes most proteins carry a net negative charge and thus prevents their binding. Lysozyme, due to its high pI, retains a positive net charge under these conditions and thus binds well.*

During this time, wash a small Buchner funnel and a 500 mL vacuum flask and set up for vacuum filtration with one piece of filter paper.

Pour the slurry into the funnel and apply vacuum until the CMC is not releasing any more water. Save the filtrate (don't empty it out). Return the pad of CMC to the beaker and add enough cold 0.01 M Tris/0.02 M NaCl buffer (pH 8.0) to make a "thin mud" suspension. Swirl about two minutes, making sure that all clumps are dispersed. Filter again allowing the new filtrate to combine with the first filtrate. Remove the pad again and repeat the procedure with another portion of the cold Tris/NaCl buffer. Measure the combined volume of the filtrates and store on ice in a flask labeled "Fraction 1". *Fraction 1 should contain proteins that did not bind to CMC, and the CMC should by now have been washed mostly free of such proteins.*

Return the CMC pad to the beaker, add enough 0.5 M NaCl/0.1 M citrate, pH 4.5, to make a "thin mud" suspension. The high ionic strength releases bound lysozyme from the CMC. Swirl for five minutes, again making sure all clumps are thoroughly dispersed. Wash the filter flask and funnel to clean them from Fraction 1, then filter the suspension as before, keeping the filtrate in the vacuum flask. Repeat with a second portion of the same citrate buffer. Measure the combined volume of the citrate filtrates, label as Fraction 2, and store on ice. *Fraction 2 should contain lysozyme and any other proteins previously bound to CMC.* Put the cellulose pad in the marked beaker for recycling.

B. ASSAY OF LYSOZYME ACTIVITY AND DEGREE OF PURIFICATION

The following assays can be done in either order. **Caution:** *Whenever you change a cuvet in a colorimeter, verify visually that the cuvet has descended all the way to the bottom of the well. If it has not, your readings will be meaningless.*

1. ASSAY OF ENZYME ACTIVITY

If lysozyme is present in active form, it will hydrolyze cell walls of *Micrococcus lysodeikticus*. This can be detected as a decrease in turbidity of a cell suspension. An appropriate rate is a decrease in A_{450} of between 0.02 and 0.1 per minute. Work at room temperature.

Fractions 1 and 2 can be used without dilution, but the EWF may need dilution. Use 0.1 M NaCl/0.05 M citrate (pH 4.5) as the diluent. The suggested starting dilution is 1:10.

Generic procedure: Zero a colorimeter on water at 450 nm. Swirl the stock suspension of *M. lyso.* cells (it consists of 0.3 mg dry cells/mL of 0.1 M NaCl/0.05 M citrate buffer, pH 4.5), then pipet 2.9 mL into a cuvet. Add 0.1 mL of the sample to be tested, hold a small square of Parafilm over the opening, and mix well by inverting several times. Quickly wipe up any leakage, insert the cuvet into the colorimeter and record the absorbance at 5-second intervals for 2 minutes. The rate is computed from the line of greatest slope over any one-minute interval during this period. Fractions 1 and 2 will go quite slowly (maybe zero); EWF will go pretty fast. *It may be useful to run a blank (0.1 mL of water instead of diluted enzyme) to correct for the rate of settling of the cells. (This correction is likely to be very small.)*

Retain 3 significant figures in your slope computations. Do not present plots of these rate experiments, but do report the computed rates for the EWF, Fraction 1 and Fraction 2 in a table (see instructions for "Results").

2. PROTEIN CONCENTRATION

Several methods are available for estimating the concentration of protein in solution. Each has advantages and disadvantages. Three examples are described below.

The biuret method is fast, cheap, and easy, but not very sensitive. It relies on alkaline denaturation of proteins, which then form colored complexes with Cu^{2+} ions. Its range is 1 – 10 mg of protein per 3 mL solution.

The Lowry method is similar to the biuret except that an additional reagent (the so-called "phenol" or "Folin-Ciocalteu" reagent) is present. This turns deep blue if reducing groups like phenols (tyrosine) or SH (cysteine) are present. The method is quite sensitive (10 - 100 micrograms of protein per 3 mL), but takes about 50 minutes for full color development.

The Bradford method is very popular and will be used for this experiment. It relies on selective binding of a dye, Coomassie Brilliant Blue, to proteins in a phosphoric acid medium. It is rapid (5 – 10 minutes), sensitive (5 – 70 micrograms of protein in 3 mL of solution), and very easy to do.

As always you need to make a standard curve in order to estimate the as yet unknown concentrations of protein in the fractions. You will be given either bovine serum albumin (BSA) or immunoglobulin G (IgG) as a standard. Both can be purchased in a pure, dry form that is easily weighed. There will be available a water solution containing either ~1.0 mg of BSA or ~1.4 mg of IgG per mL. The following instructions assume that BSA is the available standard.

One drawback of the Bradford method is that the dye-protein complex tends to stick to glass and plastic. Thus it will help to do the assays in cuvetts. Find 12 unscratched plastic cuvetts and use Alconox to wash them (GENTLE brushing!). Rinse well. Number them with a grease pencil or bits of white tape.

Devise a protocol to create a standard curve for protein concentration and to assay EWF and both fractions against appropriate blanks. You'll use 50 μ L of sample or blank. Check your protocol with the instructor before proceeding.

The standard curve will be made using a set of seven samples that contain from 0 to 50 μ g of BSA (or 0 to 70 μ g IgG) in a total volume of 50 μ L (combine appropriate volumes of the protein standard solution and water to achieve a reasonably even spacing of amounts of protein). Put these in cuvetts #1-7.

Dilute 0.5 mL of the saved EWF 20-fold with Tris buffer to bring the protein concentration down to a measurable range. (What should the final volume be?) Use this dilution for your EWF assay. F1 and F2 should not be diluted.

Finally, to all cuvetts add 2.5 mL of diluted Bradford reagent and mix by inversion (use Parafilm on your thumb). Let stand at room temperature for 10 minutes, then read at 595 nm after zeroing the instrument with the contents of cuvet #1.

Construct a standard curve. The vertical axis should be A_{595} . The horizontal axis is the μ g of protein in samples 1-7 (don't worry about the 2.5 mL of Bradford reagent). From your data estimate the mass of protein (expressed as μ g of the standard you used) in each of your 50 μ l samples of diluted EWF, F1 and F2. Remember to subtract the absorbance of the pertinent blank before computing the mass. The mass of protein in a sample divided by its 50 μ L volume gives the protein concentration in the solution from which the sample was taken.

Wash your cuvetts with Alconox to get rid of the blue residue.

PRESENTATION AND ANALYSIS OF RESULTS

Your report should include the standard curve (prepared from a table *found only in your notebook*).

Summarize your results in the form of a table of 3 columns (EWF, Fraction 1, Fraction 2) plus seven rows titled as below:

- Volume (mL) [EWF volume is 4 mL]
- Protein concentration (mg/mL) [caution: *dilution corrections?*]
- Enzyme activity (U / mL ; one $U = 0.001 A_{450}$ per minute) [caution: *dilution corrections?*]
- Specific activity (U / mg protein)

- Purification factor (*specific activity of a given fraction compared to specific activity of EWF*).
- Recovery of protein }
} For each case, the value found in 4 mL EWF = 100%
- Recovery of lysozyme activity }

At the end of your report attach an appendix where you tell or show how you computed non-obvious values, and where you answer the following questions:

QUESTIONS

1. Why was CM-cellulose rather than DEAE-cellulose chosen for the separation?
2. Can you suggest another technique (recall lecture material) that you could try to further purify the lysozyme found in your most active fraction?
3. On the basis of the evidence you now have, can you compute the % purity of your most active fraction? (100% means no other proteins present.) Explain. (Caution, think carefully!)
4. What technique would offer a sensitive way to detect minor contamination by other proteins (with physical properties different from those of lysozyme) that might be present in your most active fraction?

C. pH DEPENDENCE OF LYSOZYME ACTIVITY

Many enzymes exhibit a strong dependence of their catalytic activity on the pH of the solution. Lysozyme appears to possess two essential carboxyl groups in its active site. According to the accepted mechanism of the catalyzed reaction (see any good biochem text), the cell wall polysaccharide binds in a cleft in the surface of the enzyme (the active site). One of the carboxyl groups donates a proton to the glycosidic oxygen which links neighboring sugars, making it into an oxonium ion and thereby converting one of the sugars into a good leaving group. As this leaves, a carbocation forms on the anomeric carbon of the other sugar. Water attacks this ion, forming a new oxonium ion. A proton must be removed from this to regain neutrality, and the second carboxyl group, which must be unprotonated, serves as the base.

Clearly, the pH of the reaction buffer ought to have an effect on the rate of the catalyzed reaction. If either of the two essential carboxyl groups is in the wrong state of protonation, it cannot serve its intended function.

This part of the experiment is designed to demonstrate the effect that changing the pH may have on the rate of a catalyzed reaction, and to offer a way to test the validity of a proposed mechanism that includes general acid-general base catalysis.

EXPERIMENTAL SECTION

Prepare eight test tubes according to the table below. Add 2.8 mL of the indicated buffer plus 0.1 mL of **well-swirled** 9 mg/mL *M. lysodeikticus* suspension (made in water) to each tube and mix thoroughly. Prepare one or two extra tubes containing the pH 4.5 buffer to help you find the correct lysozyme concentration (see below).

	Tube number							
	1	2	3	4	5	6	7	8
0.05M citrate/0.1 M NaCl (pH)	(3.0)	(3.5)	(4.0)	(4.5)	(5.0)	(5.5)	(6.0)	(6.5)

Zero the colorimeter at 450 nm with water. Adjust amount of enzyme in your stock solution so that addition of 0.1 mL of it to a tube containing bacteria in pH 4.5 buffer produces a loss of about 0.04 – 0.1 A₄₅₀ per minute. Immediately after adding the lysozyme, mix the sample well by inversion, pour

into a cuvet, and observe the decrease in absorbance at 450 nm as before. Once you have found the correct lysozyme concentration, use 0.1 mL of that stock solution to start the reaction in each tube when you are ready to analyze it.

Do not include plots of these rate experiments in your report, but do compute rates to 3 significant figures. Further, do not include a table of these rates in your report, since they will be evident in the plot described next.

Plot the rate of hydrolysis as a function of pH. Estimate the two pK_a values and explain your method for estimation. For each pK_a value, tell whether the responsible group needs to be protonated or unprotonated for lysozyme to be active.

QUESTIONS

1. An enzyme loses activity as the pH of the buffer in which the assay is done becomes more and more acidic. Outline an experiment that would determine whether that decrease in enzyme activity is due to irreversible denaturation or to a reversible protonation.
2. An enzyme loses activity as the pH of the solution rises (loss is reversible). Does the critical ionizable group need to be protonated or unprotonated for the enzyme to be active?