Chapter 11

Two Reliable and Inexpensive Lysozyme Assays for Teaching Enzymology and Microbiology

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Introduction

A simple and reliable enzyme assay is a valuable teaching aid in an introductory biology course. Of even greater value is one that is also useful in upper-level courses and for research. We present two assays for the measurement of the antibacterial enzyme lysozyme. Each satisfies a number of criteria for being a good teaching assay at any level of undergraduate instruction.

The Ideal Assay

The enzyme assay of choice would demonstrate both didactic and practical qualities. For the purpose of demonstrating principles, the enzyme to be displayed would have readily understandable functions and would occur in those organisms that immediately capture the interest of students. Any enzyme important in human medicine fits this description. Then, to broaden the level of interest, the chosen enzyme would have qualities that make it a subject of study in other areas, such as protein chemistry, genetics, molecular evolution, or microbiology.

A good assay for such an enzyme is one that would serve as an introduction to the proper use of scientific tools and methods. It would require that students measure out and mix chemicals, and gather data for analysis. It would provide to students realistic activity that accurately teaches the research experience. The assay would be one that scientists actually use and that students could use later in projects for advanced courses. The ideal assay would also be adaptable to many situations. One could easily modify it to demonstrate the importance of temperature, ionic environment, and other factors. It could be a model of a class of assays for several different enzymes.

Finally, the assay of choice would be easy to set up, reliable in its results, undemanding of manual dexterity by inexperienced workers, and inexpensive.

Lysozyme Assays

Lysozyme is one of several enzymes that can hydrolyze components of the walls of certain bacteria. The cell lysis, or bursting, that usually follows is the basis for its bacteriocidal activity *in vivo*. Most assays for lysozyme are turbidimetric, measuring the clearing of a suspension of dead bacterial cells as their walls break down (Klass et al. 1977). Both of the assays described here are based on this model.

The first method is for a radial diffusion assay somewhat similar in principle to widely used antibiotic assays. In these, a test compound diffuses out from a filter paper disc placed on a nutrient agar surface seeded with bacteria, producing a visibly clear circle within which bacterial growth has been inhibited. As modified for lysozyme, non-nutritive agar contains an opaque suspension of dead bacteria of a species susceptible to cell wall disruption by lysozyme. Wells, punched in the agar sheet, are filled with solutions to be as-

sayed. Any bacteriolytic enzyme diffusing out of a well shows its presence by producing a ring that is cleared of intact cell walls. After a standard incubation period, the diameter of each cleared ring is proportional to the concentration of lysozyme in the solution that diffused out of the well. This assay has been variously labeled the "intra-agar lysis assay" (Nace et al, 1965) or the "lysoplate assay" (Osserman and Lawlor 1966).

Our second method of lysozyme detection involves spectrophotometric measurement of light transmission through a suspension of bacteria in buffer. As lysozyme destroys cell walls the suspension clears, causing a gradual increase in measured "percent transmittance." Although one can perform this assay using dead cells (as with the first assay), the method presented here involves harvesting and using living bacteria as the substrate.

The antibacterial activity of lysozyme immediately captures student interest, especially when demonstrated in their own body fluids. The chance manner in which Sir Alexander Fleming discovered its antiseptic property, followed by the recognition and careful analysis of its potential medical value, is a classical example of a prepared mind responding appropriately to a fortuitous event (Baumel et al. 1976). Medically oriented students will also be interested in lysozyme's potential roles in cartilage formation (Kuettner et al. 1974) the immune mechanism (Kokoshis et al. 1978) and cancer therapy (Osserman et al. 1973). High lysozyme levels in human serum or urine are diagnostic of certain diseases, including leukemia (Pruzanski and Platts 1970). A lysozyme assay also becomes a point of entry for discussion of protein structure, since chicken egg-white lysozyme was the first enzyme whose three-dimensional shape was accurately shown by X-ray crystallography (Blake et al. 1965). This, is turn, led to its extensive use as a reference protein in the field of molecular evolution (Wilson and Prager 1974).

Students can easily modify the assays described here in order to test the optimum pH of a particular lysozyme and to show the effects of various ions or of ambient temperature. The intra-agar assay is also the basis for determining the location of lysozyme isozymes in polyacrylamide gels after electrophoresis (Ostrovsky et al. 1976). Variations upon the theme of a radial diffusion assay exist to test for several other enzymes that catalyze the breakdown of opaque substrates (Davis 1977).

The intra-agar assay described here is a very reliable test. Since all standards and samples of unknown enzyme concentration can be tested simultaneously on a single plate, small measuring errors in the preparation of the plate do not significantly affect results. However, two cautions must be issued for both this assay and the spectrophotometric method. First, neither of them measures initial reaction rates. Therefore, an instructor cannot use them to teach enzyme kinetics. The spectrophotometric assay, if modified to operate at 37°, will run fast enough to obtain measurements in the first minute, but

the complexity of the bacterial cell wall and the possibility of several reactions occurring simultaneously make evaluation of these measurements difficult. The second caution, applicable to both assays, is that a positive reaction is not sufficient proof of lysozyme's presence. One must also investigate physical and chemical properties, such as molecular weight and isoelectric point, before assigning the name "lysozyme" to the enzyme in question (Jollès 1967).

The reagents and equipment needed for either assay are neither exotic nor expensive. Reagents can be stored for over a year. Although a student cannot obtain quantitative results with the intra-agar assay until several hours have elapsed, the time required to prepare plates and start the assay does not exceed one hour, exclusive of the time used in preparing the standards and other samples. An instructor may choose to provide samples ready for assay, or plates ready to receive samples, or to use a complete laboratory period to instruct students in obtaining, preparing, and analyzing their own samples. Hen's egg-white, human saliva, and human nasal secretion all produce lysis rings within five minutes after application. (See Appendix A.) A bonus associated with the assay is that students can take home with them a valuable reminder of their experiment—the developed plate.

The spectrophotometric assay, while requiring a more sophisticated piece of equipment, the spectrophotometer, does provide the student with results within minutes. As required by the instructor or time constraints per lab period, students may do none or all of the simple set-up procedures.

Instructors' Materials

The following instructions are for single 100 × 15 mm petri dish sufficient for the assay of up to 30 samples. One can easily prepare up to four such plates in a single batch by appropriate multiplication of reagent quantities. Many more are possible if smaller petri dishes are used.

1. Place 6.0 g of agar cubes and 6.0 ml of phosphate-NaCl buffer into a 125 ml erlenmeyer flask. Seal the flask with aluminum foil, attach a test tube holder to its neck, and place it in a boiling water bath to melt the agar. Swirl occasionally.
2. While waiting for complete melting of the agar, weigh out 15 mg of dried *Micrococcus* cells and place them in a grinding device such as a glass tissue grinder or a very small mortar and pestle. Add 0.5 ml of buffer and mix thoroughly to suspend the cells. Transfer the suspension to a 25 ml graduated cylinder.
3. When the agar cubes are thoroughly melted, pour the resultant liquid into the graduated cylinder, up to a total volume of 11 ml. Mix thoroughly to produce a uniformly opaque suspension of *Micrococcus*.

4. Immediately remove 10 ml of the suspension with a serological or mohr pipet. (Careful! Use a safety pipet filler to avoid burning your mouth.)

The Intra-Agar Lysozyme Assay

Materials

1. Agar (such as Difco "Bacto Agar") rehydrated in distilled water at 4% concentration (w/v) to yield 500 ml. Cut the gel into cubes of approximately 0.5 cm and wash them in running tap water for several days to remove colored impurities. Soak washed cubes for at least 24 hours in several changes of distilled water. Store cubes indefinitely at 5° to 10°, covered with distilled water. This amount is sufficient for preparing approximately 80 of the plates described below.

2. Buffer, pH 6.6, 0.067 M. Optimum pH varies according to the source of the lysozyme being tested, but this buffer is good for most animal tissues.

Na_2HPO_4	3.56 g
KH_2PO_4	5.65 g
H_2O to make 1000 ml	

Add 22.67 g NaCl to this buffer, since these ions enhance the lytic activity of many animal lysozymes (Metcalf and Deibel 1969).

3. Bacterial cells. Obtain this material in dried form from a biochemical supply company, ordering it as *Micrococcus lysodeikticus (M. luteus)*. One gram, suitable for over 50 assays, costs only a few dollars. Store below 0° indefinitely.

4. Lysozyme standard, purified from chicken egg-white. Obtain it from a biochemical supply company in lyophilized form. One gram costs a few dollars and is sufficient for over 1000 assays. For use, make a series of 2 ml solutions in distilled water, ranging from 0.1 to 10.0 mg/ml. One can store powder or solutions below 0° indefinitely with little loss of enzymatic activity. Transfer this volume to a 100 × 15 mm petri dish placed on a perfectly horizontal surface, to form a uniform layer over the bottom of the plate. Cover, and allow the agar to gel undisturbed for about 20 minutes.

5. With either a gel puncher (such as is used for immunodiffusion assays) or a cork borer of 4 mm or smaller diameter, punch the desired number of sample wells into the agar. Remove agar plugs by suction through a pasteur pipet connected to a water aspirator or other weak vacuum source.

6. Using a 22 to 26 gauge needle attached to a 1 ml syringe, fill wells with standards or test solutions. You can use the same needle and syringe for all samples if you rinse them between samples by drawing up and squirting out water five to ten times. Fill each well exactly to its top.

7. Cover the plate, seal it in clear plastic wrap to avoid dessication, and place it in an incubator set between 37° and 45°. For full development at 45°, incubate at least 3 hours. At 37°, increase total incubation time to 10 hours. Lysis activity also occurs visibly at room temperature, but at least 24 hours must be allowed for full development. A solution very rich in lysozyme will produce an observable ring of lysis at the well margin within 15 minutes, even at room temperature.

8. To obtain quantitative results, measure lysis diameters made by standards and "unknowns", using vernier calipers or a ruler graduated in millimeters. For best visibility during measuring, hold the plate toward a light source and provide a darkfield background (see Fig. 11.1). A bacterial colony counter is ideal. To construct a standard curve, plot the concentrations of chicken egg-white lysozyme standards vs. their lysis diameters. Use three-cycle semilog graph paper, placing concentrations on the logarithmic axis.

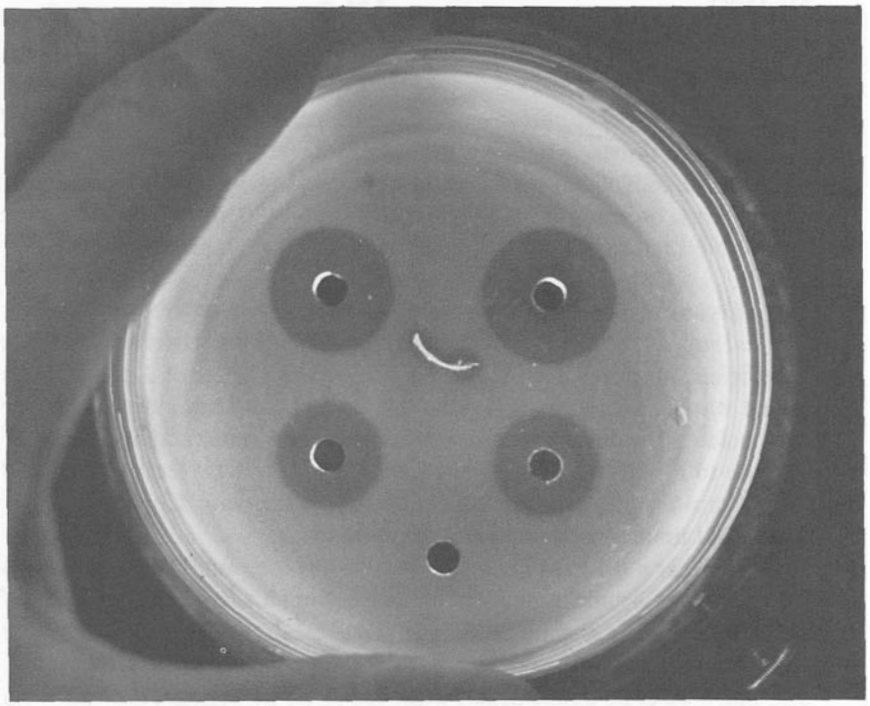


Figure 11.1. An intra-agar lysis assay plate after 5 hour incubation at 37°. Upper left: purified chicken egg-white lysozyme, 1.0 mg/ml. Upper right: chicken egg-white, whole. Lower left: human saliva. Lower right: human nasal secretion. Center: human fingernail clipping. Bottom: distilled water (control).

9. To stop further lysis, cover the agar surface with a 0.1 N NaOH solution for at least 15 minutes. This treatment may also make the border of the lysis zone around each well more distinct.

The Spectrophotometric Assay for Lysozyme

Medium preparation

Materials: 1 liter flask
Aluminum foil—to cover flask
20 100 × 15 mm petri dishes
Agar—7.5 grams
Nutrient broth—4 grams
500 ml of distilled water

Prepare nutrient agar petri dishes as follows:

1. Place 7.5 grams of agar and 4 grams of nutrient broth in a 1 liter flask.
2. To this flask add 500 ml of distilled water and swirl the contents in the flask for a few seconds.
3. Cover the mouth of the flask with aluminum foil or an inverted beaker.
4. Autoclave at 15 to 20 psi for 20 minutes.
5. Pour the medium into 100 × 15 mm petri dishes to a depth of 1/2 to 3/4 full. *Be sure to swirl the flask of medium before pouring it into the petri dishes.* This procedure will thoroughly mix the contents in the flask which will result in agar of uniform consistency in all petri dishes.
6. After the plates have solidifed they may be used to culture the bacteria.

Culture techniques

Materials: 1 inoculating loop
1 Bunsen burner
Petri dishes containing nutrient agar
Stock culture of *Micrococcus lysodeikticus*

Culture Micrococcus lysodeikticus (M. luteus) *as follows:*

1. Using a Bunsen burner, flame the inoculating loop until it is "red hot."
2. Remove the lid from the stock petri dish of *Micrococcus lysodeikticus* and cool the inoculating loop by touching it to the agar where no bacterial colonies are present.
3. After a few seconds slide the inoculating loop through a colony of bacteria. Remove the inoculating loop and replace the cover on the stock petri dish.

4. Remove the lid from a sterile uncontaminated petri dish containing nutrient agar. Streak the inoculating loop back and forth on the agar at one end of the petri dish.
5. Using a back and forth "zig-zag" movement streak the tip of the inoculating loop through the bacteria streak. Close the petri dish.
6. Invert and incubate the nutrient agar petri dishes in a 37° incubator for 36 to 48 hours. Petri dishes may be incubated at room temperature for 3 days if an incubator is not available.

Harvesting Micrococcus lysodeikticus

Materials: Petri dish containing *Micrococcus lysodeikticus*
Inoculating loop or cotton swabs
Distilled water
1 funnel
125 ml flask

Harvest the *Micrococcus lysodeikticus* as follows:

1. Open a petri dish containing the *Micrococcus lysodeikticus* and add enough distilled water to the dish to cover the bacteria and agar.
2. Move an inoculating loop or cotton swab over the surface of the agar in a "zig-zag" motion which will loosen and suspend the bacteria in the distilled water.
3. Using a funnel pour the bacteria solution into the flask.
4. Repeat procedures 2 and 3.
5. The harvested bacteria are now ready to use. However, the bacteria solution can be stored in the refrigerator for several days.

Phosphate buffer solutions

Materials: Monobasic sodium phosphate
Dibasic sodium phosphate
1 liter storage containers for solutions
3 50 or 125 ml flasks
10 ml graduated cylinder

Prepare phosphate buffer solutions as follows:

1. Using Table 11.1 prepare 1 liter of stock solutions as indicated.
2. For this experiment prepare buffer solutions for pH 5.7, 6.9, and 8.0. Divide "x" and "y" on Table 11.1 by 10). *Do Not* dilute the buffer solutions to 20 ml (200 ml on Table). This dilution will occur automatically later in the experiment.

Preparation of lysozyme

Materials: 1 250 ml beaker
Lysozyme crystalline
200 ml pH 6.9 buffer
analytical balance
100 test tubes
10 ml syringe

TABLE 11.1. Phosphate Buffer.

Stock Solutions:
A: **0.2M solut**ion of monobasic sodium phosphate (27.8 g. in 1000 ml.).
B: **0.2M solut**ion of dibasic sodium phosphate (53.65 g. of $Na_2HPO_4 \cdot 7H_2O$ or 71.7 g. of $Na_2HPO_4 \cdot 12H_20$ in 1000 ml.).
x ml. of A + y ml. of B, diluted to a total of 200 ml.

x	y	pH	x	y	pH
93.5	6.5	5.7	45.0	55.0	6.9
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.0	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	90.5	7.7
62.5	37.5	6.6	8.5	91.5	7.8
56.5	43.5	6.7	7.0	93.0	7.9
51.0	49.0	6.8	5.3	94.7	8.0

Prepare lysozyme solution as follows:

1. Using Table 11.1 make up 200 ml of phosphate buffer pH 6.7 and place this solution into a 250 ml flask.
2. Add 0.2 g of lysozyme to the buffer solution and stir until the enzyme is completely dissolved. Always do a trial run with the enzyme solution to determine if it is concentrated enough to produce results needed for the period you have selected.
3. Using a 10 ml syringe or pipette place 2 ml of the enzyme solution into each of the 100 test tubes.
4. Place the test tubes in the freezer until needed.

Sample Experiment—The Effect of pH on Lysozyme in Relation to the Lysis of *Micrococcus lysodeikticus*

Materials: 1 spectrophotometer
5 cuvettes (test tubes)
1 grease pencil

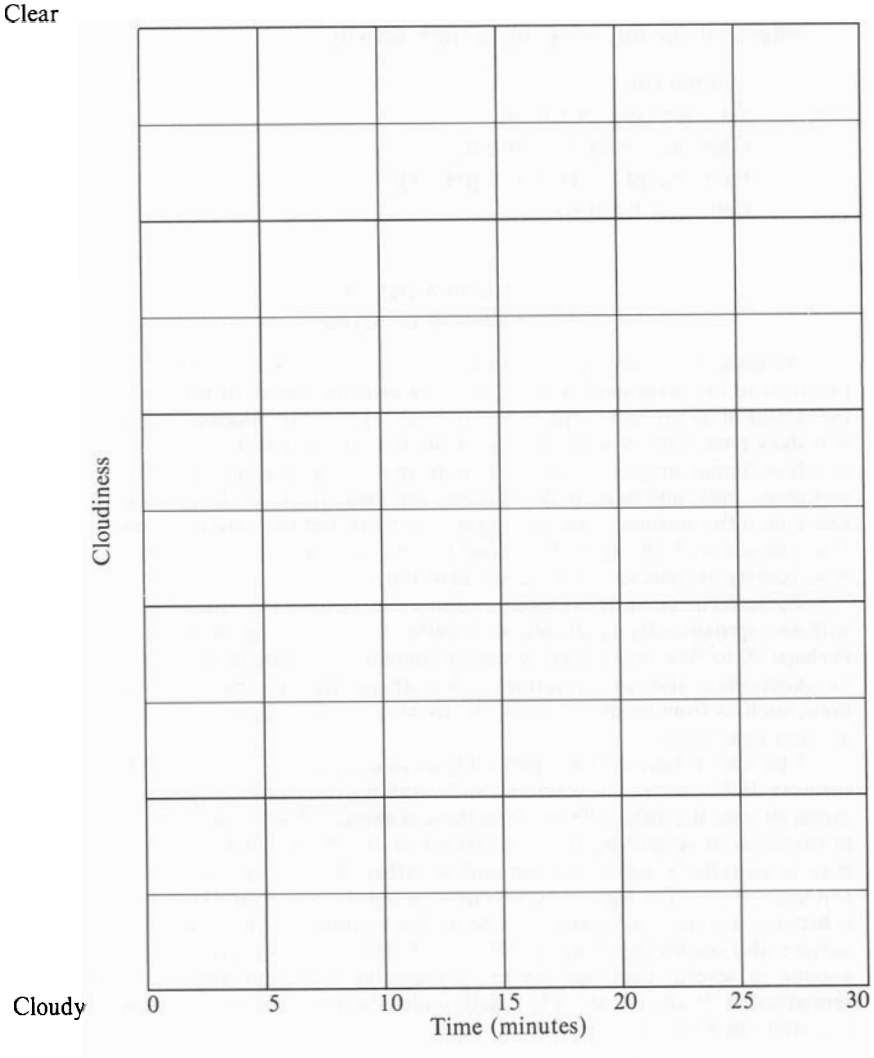
2 ml lysozyme
Micrococcus lysodeikticus stock solution in distilled water
Phosphate Buffer—pH 5.7
Phosphate Buffer—pH 6.9
Phosphate Buffer—pH 8.0
1 1 ml pipette
4 5 ml pipettes

Procedure

1. Label the 5 cuvettes in a sequence of A, B, C, D, E.
2. Place 1 ml of buffer pH 6.9 in tube E and add 1 ml of water. This tube will serve as a "blank" for calibrating the spectrophotometer. The spectrophotometer should be set at 525 nm.
3. Using distilled water dilute the bacterial solution to 10–15% T.
4. Using a 5 ml pipette place 1 ml of the bacteria solution in tubes A, B, C, and D.
5. Add 1 ml of buffer pH 5.7 to cuvette A.
6. Add 1 ml of buffer pH 6.9 to cuvettes B and D.
7. Add 1 ml of buffer pH 8 to cuvette C.
8. Take a % T reading on cuvettes, A, B, C, and D.
9. Add 0.1 ml lysozyme solution to cuvettes A, B, and C.
10. Take a % T reading at 5 minute intervals on tubes A, B, C, and D for 30 minutes.
11. Record results on the table below.

	Minutes						
Tube	0	5	10	15	20	25	30
A							
B							
C							
D							

12. Plot results on the graph below.



13. Students can be asked to design other types of experiments using this system as a model. For example, they can design experiments to study the effects of the following on enzyme activity:

    Temperature
    Enzyme concentration
    Chemical enzyme inhibitors
    Extreme pH (pH 2 and pH 13)
    Different bacteria

APPENDIX A
Source of Lysozyme

Animals of so many species are known to contain lysozymes that it would be impractical to list them here. Historically, the primary source of this enzyme has been the whites of avian eggs. This material from a hen's egg obtained in a grocery store will show lytic activity without need of preliminary preparation. Lysozyme-rich materials of human origin that do not require special preparation are saliva, tears, nasal secretions, milk, and fingernails. Students can assay the latter by placing clippings directly onto the surface of an intra-agar lysis plate and pressing them down to ensure close contact with the agar. The blood plasma or serum of many vertebrates exhibit lytic activity. Rabbit serum is particularly active.

To perform quantitative assays upon other tissues, one must homogenize them with an approximately equal volume of buffer, then centrifuge to obtain a supernatant. Perhaps 80 to 90% of the total lysozyme content of a tissue or organ will be found in the supernatant after such treatment. One can qualitatively test very small tissue portions, such as from embryos or insects, by placing them directly upon the surface of an intra-agar plate.

There are relatively few reports of lysozyme in plants. The turnip (Hara and Matsushima 1972), papaya (Heneime 1972), and fig (Glazer et al. 1969) contain lysozymes structurally quite different from those of animals. There is a lysozyme-like activity in the seeds of several legumes (Lawrence et al. 1978), but the responsible enzyme may be an endo-N-acetylglucosaminidase rather than a true lysozyme (Powning and Davidson 1979). The legume lytic activity is highest when the pH of the assay buffer is between 4.0 and 5.5, significantly below the optimum pH for animal lysozymes. Lysozyme also occurs in a fungus (Felch et al. 1975). Among prokaryotes, there is lysozyme in several bacterial species, presumably acting to alter cell walls during reproduction. It also occurs in host cells under the genetic direction of bacteriophages to enable their lysis from within the hosts.

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