

Chapter 6

Enzyme Investigations for Introductory Courses

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Notes for the Instructor

To help students “do” science, I have adapted a traditional enzyme kinetics laboratory exercise to a problem-posing format. The students investigate how changing enzyme concentrations, substrate concentrations, temperature, pH, and an inhibitor influence the reaction rate of turnip peroxidase. The peroxidase is in turnip extract prepared with a blender. The enzyme is very stable and can be kept at room temperature for hours. The students use the following skills: planning experiments, pipetting, using a spectrophotometer, graphing data, and calculating reaction rates.

Because we have 2-hour labs, I have the students use two laboratory periods for this exercise. On the first day, they hand in a flow diagram which outlines the first two problems (see Appendix A). Problem 1 is “Can you measure enzyme activity?” They plot baseline activity. Problem 2 is “Does amount of enzyme influence reaction rate?” The students should have two more lines to plot along with their baseline. The students learn the techniques that they need for the rest of the problems. The next week, each group of two students is assigned a variable to investigate. All the students must repeat the baseline protocol because the amount of enzyme varies with each extraction. Usually, one pair will look at substrate concentration, another at pH, another temperature, and the last group does both the effect of boiling and the inhibitor, hydroxylamine. These assignments equalize the work load and can be accomplished in the 2-hour period. Students then write formal laboratory reports that are due the next laboratory period (see Appendix B). During the next laboratory period, we begin by having a brief report from the different groups on what their data showed. The emphasis is in interpreting data, not “getting the right answer.” Be prepared to find that doubling the substrate concentration decreases the reaction rate and other interesting results. Because of the preparation of the turnip extract, there are times when changing enzyme or substrate concentration does not change the rate because the substrate or enzyme concentrations may be limiting. These unexpected data can lead to a discussion of experimental design and more about enzyme kinetics. I always ask the students what they would do to improve the experiment. There are some problems to outline. First, instructor's should try the protocol before they start the laboratory exercise. Purple-topped turnips have different amount of activity depending on age and storage. The amount of turnip may have to be adjusted to obtain a reasonable absorbance reading. I also found that guaiacol lost some of its activity after 3 years on the shelf. You should buy hydrogen peroxide (from a drugstore) each year because it does not store well.

Note that hydroxylamine is poisonous and should be handled with care. I leave the amount of hydroxylamine up to the students, and the effect will vary depending on the proportion of enzyme to hydroxylamine. If the glassware has become contaminated, the reaction will occur in the blank and the students can see the brown color developing. They just have to start over again.

The students have the most difficult time with plotting the data and determining the slopes of the lines. Instructor's need to point out that what is important is a comparison of the slopes not just plotting the lines. Also students need to compare their data with the baseline of the day they did the experiment. I allow the students to rewrite their reports to try and improve their grade. This exercise was adapted from Eberhard (1979). For more information see Maehly and Chance (1954).

Student Instructions

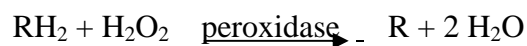
Objectives

1. Define enzyme and give the class of macromolecules to which enzymes belong.
2. Identify the substrate and products of the peroxidase-catalyzed reaction.
3. Explain why guaiacol is necessary in this experiment.
4. Design and implement experiments to describe how temperature, pH, enzyme concentration, and substrate concentration affect reaction rate.
5. Explain the use and function of the spectrophotometer.

Introduction

Enzymes are protein molecules which catalyze biochemical reactions in all living organisms. For example, the reactions in photosynthesis and respiration are catalyzed by many enzymes. Enzymes allow living organisms to carry out rapid and complex chemical activities at relatively low temperatures but cannot initiate a reaction that would not have occurred in their absence. Enzymes are highly specific, that is, they tend to accelerate only one or a group of related reactions. Therefore, there are many different enzymes in a cell, but because they are unchanged by chemical reactions, only a small amount of any given enzyme may be present. Enzymes are thought to speed up reactions by bringing reacting molecules (substrates) together to increase the chances that a reaction will occur. Each enzyme has a specific active site, a portion of the molecule where the substrates adhere. Many factors may affect enzyme activity such as temperature, pH, concentration of enzyme, concentration of substrate, concentration of products, and the presence of inhibitors or activators.

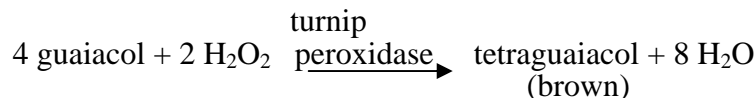
In this exercise you will use the enzyme peroxidase from turnips. Peroxidases are common in plant and animal cells. The reaction catalyzed by peroxidase can be symbolized as follows:



where RH stands for a variety of H donors and R for the form of the molecule after having donated hydrogen. What is the name for H_2O_2 ? What are the substrates in this reaction? What are the products?

Cells using molecular oxygen in their metabolism generate small amounts of H_2O_2 (hydrogen peroxide) which is very toxic. Peroxidase (and other enzymes) speeds up the conversion of H_2O_2 to water so that the cell is not damaged.

We are going to use a substance called *guaiacol* as our hydrogen donor (it can also be called a reducing agent) because it changes color after it loses hydrogens. What are *redox* reactions? In the reaction we are considering, what has been reduced? What has been oxidized?



The presence of a colored solution allows us to use a spectrophotometer to follow the above reaction. Figure 6.1 is a schematic diagram of a spectrophotometer. Basically, there is a source of light and a prism to select the particular wavelength of light needed. The slit also helps to obtain only monochromatic (one wavelength) light. The light passes through the sample (in this case the tetraguaiacol solution), and depending on the concentration of the sample, some light will be absorbed by the molecules in solution, that is, light will be absorbed. The photocell produces an electrical current proportional to the amount of light which passes through the solution, or percent transmitted light.

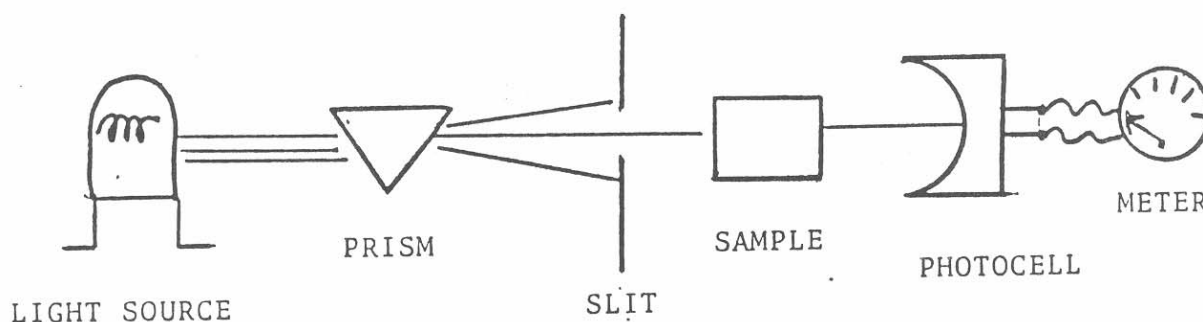


Figure 6.1. Basic components of a spectrophotometer.

A clear tube of solvent (which may or may not be water) is set to zero absorbance. A solution with a small amount of colored substance might show an absorbance of 0.1 whereas a solution with a moderate amount of colored substance might show an absorbance of 0.4. The absorbance reading is proportional to the amount of substance in solution. This relationship is called the Lambert-Beer Law and allows us to determine the concentration in a sample.

Procedure for Using the Spectronic 20 Spectrophotometer

1. Turn on by turning Power Switch/Zero Control knob (Figure 6.2) clockwise. Allow at least 15 minutes warm-up time.
2. Select desired wavelength (in this experiment, 500 nm).
3. With the sample chamber empty and closed, use the Zero Control knob to zero the meter on infinite absorbance. (When the chamber is empty, a shutter blocks all light from the phototube.)

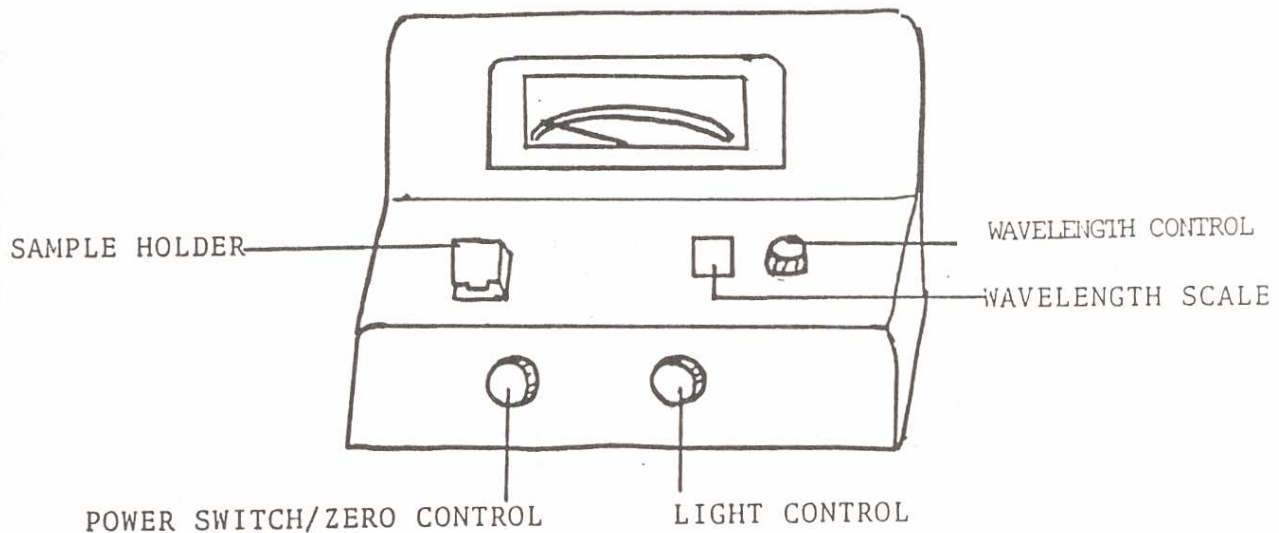


Figure 6.2. Controls for Spectronic 20.

4. Fill a spectrophotometer tube with your blank solution. Wipe it free of moisture and fingerprints with a KimWipe and insert into the sample holder. Line up the etched mark on tube with the raised line on the front of the sample holder and close the cover.
5. With the Light Control knob adjust the meter to read zero absorbance. Remove the tube, label it, and keep in a wooden test tube rack in case you need to zero the machine again.
6. Fill a sample tube with an experimental sample and place it in the holder.
7. Read the absorbance directly from the meter. Note that absorbance is a logarithmic scale that reads from right to left.
8. You should reset the machine to infinite absorbance and zero absorbance before each set of readings because the machine may drift.

Problem 1: Can you follow the activity of turnip peroxidase?

Null hypothesis: The activity of turnip peroxidase cannot be determined using a spectrophotometer.

Prediction: As the turnip peroxidase catalyzes the conversion of hydrogen peroxide to water, more tetraquaiacol will be produced and absorbance will increase.

Procedure: You will be using pipets to deliver solutions. Use a clean pipet for each solution and label the pipets with a marking pencil so each one can be reused with the proper solution. Use a pipet bulb. *Do not mouth pipet.*

Obtain a few ml (about 3 cm in a test tube) of 1% H₂O₂, a few ml of quaiacol, and a test tube filled with turnip extract. Put each into properly-labelled test tubes and use them as your stock solutions so you need not return to the front desk. Also, fill a small beaker with distilled water. Next, label three test tubes as 1, 2, and 3, and pipet in the amounts of solutions given in Table 6.1. (Use a 10-ml pipet for water, a 5-ml pipet for turnip, and 1-ml pipets for quaiacol and H₂O₂).

Table 6.1. Solutions required for baseline protocol.

Tube	Guaiacol (ml)	Turnip extract (ml)	1% H ₂ O ₂ (ml)	Distilled H ₂ O (ml)
1	0.1	1.0	–	8.9
2	0.1	–	0.2	4.7
3	–	1.0	–	4.0

Is there any peroxidase activity in Tube 1? Tube 2? Tube 3? Why?

Transfer some of the solution in Tube 1 to a spectrophotometer tube and use it as a blank to zero your Spectronic 20. This is called a reagent blank. Why do we use a reagent blank instead of distilled water? Obtain a clean spectrophotometer tube, the sample tube, and read the rest of the directions before you do anything else!

You will have 20 seconds to mix the contents of Tubes 2 and 3, pour some of the contents into the clean spectrophotometer tube, wipe the tube, and take your first reading. You will then record the absorbance every 20 seconds for 2 minutes. Make sure you can read the spectrophotometer absorbance scale. Set up a table in advance with 0-time (when you mix), 20-seconds (first reading), 40-seconds, etc. Do not try to mix Tubes 2 and 3 in the spectrophotometer tube as it only holds 5 ml. You only need two spec tubes: one for the blank and one for the reaction. It is necessary to reset the machine to infinity absorbance and zero absorbance before each set of readings. *Begin when you are ready.*

Data: Plot a graph of dependent (y -axis) versus independent variable (x -axis). This curve will represent your baseline of enzyme activity. Do your data support or reject your hypothesis? Is there a control in this experiment? Can you be absolutely sure that you are testing peroxidase?

You will compare the baseline with enzyme activity under different conditions, using the same or very similar procedures for each set of experiments. Because there is not enough time to complete all parts of this exercise, you will be assigned different parts by your instructor.

Problem 2: Does amount of enzyme influence reaction rate?

Null hypothesis: As the amount of enzyme changes, the reaction rate will not change.

Prediction: Increasing the amount of enzyme will increase the reaction rate. Decreasing the amount of enzyme will decrease the reaction rate.

Procedure: You can compare the effect of doubling and halving the enzyme concentration with your established baseline. The solutions you will need are provided in Table 6.2. Make sure you understand the procedure used in the baseline before continuing. What is the total volume in Tube 1? Tube 2 combined with Tube 3?

Table 6.2. Solutions required for doubling and halving enzyme concentration.

Tube	Guaiacol (ml)	Turnip extract (ml)	1% H ₂ O ₂ (ml)	Distilled H ₂ O (ml)
2X enzyme concentration				
1	0.1	2.0	–	7.9
2	0.1	–	0.2	4.7
3	–	2.0	–	3.9
0.5X enzyme concentration				
1	0.1	0.5	–	9.4
2	0.1	–	0.2	4.7
3	–	0.5	–	4.5

Data: Plot a graph of absorbance (y-axis) versus time (x-axis) for your baseline, 2X enzyme concentration, and 0.5X enzyme concentration. Can you support or reject your hypothesis? Explain. Can you calculate the rate of enzymatic activity?

Problem 3: Does amount of substrate influence reaction rate?

Null hypothesis:

Prediction:

Procedure: (*Hint:* Study Table 6.2 and then design and carry out an experiment to determine the effect of varying the concentration of the substrate, H₂O₂. Complete Table 6.3; make sure that the volume in your control tube is 10 ml and the volumes in Tubes 2 and 3 are 5 ml.)

Data: Plot a graph for your baseline, and varying substrate concentration. Can you support or reject your hypothesis? Explain.

Problem 4: Does the temperature of the reaction influence reaction rate?

Null hypothesis:

Prediction:

Procedure: (*Hint:* Set up an experiment to show the difference in activity found between 0° and 37°C. Use the protocol of your baseline experiment, but incubate your tubes (1, 2, 3) at the different temperatures available in the lab. Record the exact temperature of the water baths.)

Data: Plot a graph for your different temperature conditions. Can you support or reject your hypothesis? Explain.

Table 6.3. Solutions required for investigating changing substrate concentration.

Tube	Guaiacol (ml)	Turnip extract (ml)	1% H ₂ O ₂ (ml)	Distilled H ₂ O (ml)

Problem 5: Does the pH of the solutions influence reaction rate?**Null hypothesis:****Prediction:****Procedure:** (*Hint:* Substitute the different pH buffers for the distilled water in the baseline protocol.)**Data:** Plot a graph for your different pH conditions. Can you support or reject your hypothesis? Explain.**Problem 6: Does boiling the enzyme solution influence reaction rate?****Null hypothesis:****Prediction:****Procedure:****Data:** Plot a graph for your control and boiled enzyme solution. Can you support or reject your hypothesis? What has boiling done to the enzyme?

Problem 7: Does the addition of hydroxylamine to your protocol influence the reaction rate?

Hydroxylamine is *toxic*—handle with care and wash your hands immediately if you spill. Hydroxylamine is a small molecule similar enough in structure to H_2O_2 that it will bind to the peroxidase.

Null hypothesis:**Prediction:**

Procedure: (*Hint:* To test the effect of hydroxylamine, add some drops of 10% hydroxylamine to a few ml of turnip extract. Let solution stand for 1 minute, then use this modified turnip extract in the baseline protocol.)

Data: Plot a graph for your control and hydroxylamine plus enzyme solution. Can you support or reject your hypothesis? Explain the action of hydroxylamine on the enzyme's activity.

When you are finished with your work, wash and thoroughly rinse your glassware. Place the pipets tips “up” in plastic bucket provided. Hand in your graphs to your instructor. *Do not use a test tube brush on spectrophotometric tubes!*

Materials

For a class of 16–20 students:

Balance	Large container of distilled H_2O
Blender	Water bath at 37°C
Graduated cylinder (500-ml)	Parafilm
500-ml erlenmeyer flask	Spectrophotometers (4)
Funnel and filter paper	KimWipes
Boiling water bath and chips	Ice
White tape	Graph paper

For each pair of students:

Beaker for water
 Large test tubes for turnip extract, guaiacol, H_2O_2
 Additional test tubes for stock solutions (3)
 Spectronic 20 tubes in non-metal test tube holder (2)
 Test tube rack
 Test tube holder
 Pipets: 10-ml for water, 5-ml for turnip, 1-ml for guaiacol, 1-ml for H_2O_2
 Marking pencil
 Pipet bulb

Turnip: Purple-topped turnip from a grocery store. The peroxidase activity will vary with age and condition of turnip. Test activity before the laboratory. Make only one turnip extract because results will not be comparable from different batches.

Preparation of turnip extract (by the instructor): Weigh out 2–4 g of the inner-peeled portion of a turnip. Place in blender with 400 ml of distilled water. Blend at high setting for 1 minute. Filter through glass wool in a funnel. The product is turnip extract which contains turnip peroxidase.

Buffers: Use prepared buffer pellets, pH 4, pH 7, and pH 11. Make 200 ml for each laboratory (pHdriion buffers from Fisher Scientific).

Guaiacol: Mix 0.22 ml of guaiacol (Sigma #G5502) with 100 ml of distilled water. Store in brown bottles.

1% H₂O₂: Mix 3 ml of 30% H₂O₂ with 97 ml of distilled water and refrigerate. Make 200 ml. If you use 3% from a drugstore, dilute 1 part hydrogen peroxide to 2 parts distilled water.

10% hydroxylamine: Mix 1 g of hydroxylamine (Sigma 9876) with 9 ml of distilled water. Hydroxylamine may cause burns on skin! Store in dropper bottles.

Literature Cited

- Eberhard, C. 1979. Biology laboratory: A manual to accompany *Biology* by Karen Arms and Pamela S. Camp. Holt, Rinehart, and Winston, New York, 328 pages.
- Maehly, A. C., and B. Chance. 1954. The assay of catalase and peroxidase. Pages 357–424, in *Methods of biochemical analysis* (Volume 1). Interscience Publishers, New York, 521 pages.

APPENDIX A

Flow Diagrams

Flow diagrams are a quick summary of the laboratory exercise that you prepare when you are reviewing the exercise before coming to class. The purpose of these diagrams is to help you focus on the questions that you will be addressing and the procedures you will be using. Flow diagrams should make your time in the laboratory more productive because you will not have to read the entire exercise—just focus on what details that you need. Each individual could have a unique flow diagram. You decide how much detail you need. You should include the title of the exercise, the problem(s), null hypothesis, prediction, and brief outline of procedures including a data table and some idea of what the data will be like. Below is an example for Problem 3 of this exercise. You should do a flow diagram for all the parts of the laboratory exercise that you are responsible for.

Problem 3: Does amount of substrate influence reaction rate?

Null hypothesis: The amount of substrate does not influence the reaction rate.

Prediction: Increasing the amount of substrate will increase the reaction rate.

Procedure:

- Do a new baseline protocol with Tubes 1, 2, and 3.
- Record absorbance over 2 minutes at 20-second intervals.
- Create two additional sets of Tubes 1, 2, and 3: one set with twice as much hydrogen peroxide (less distilled water, so volume constant); the second set with half as much hydrogen peroxide. See Table 6.3.
- Read absorbance for two new sets of tubes.
- Graph three separate lines, one for each hydrogen peroxide concentration, and compare slopes.

APPENDIX B

Laboratory Report

Laboratory reports, like journal articles, communicate your work to others and help you to organize and analyze your data. Scientific journal articles have a particular form—IMRAD or introduction, methods, results, and discussion. We are going to shorten this format in this class because you are not expected to do an extensive literature search. I am not looking for lengthy rehashing of the laboratory manual but would like to see meaningful and thoughtful consideration of what you did.

General Requirements

1. Each student will hand in his/her own report.
2. Your report must be typed, with suitable margins and form. Please staple the upper left hand portion of the paper. We suggest that you learn a word processing package of your choice early in your college career. This skill will help you many times in your career. There are many microcomputers available on campus. Make sure to make backup files and do not wait until the last minute to write your report. “The computer ate it” is not an acceptable excuse for not submitting your report.

Specific Instructions

1. Centered on the first page, put the title of the report, your name, your partner's name, the date, and the day you have your laboratory.
2. The following clearly labelled sections should follow the above information. Do not start different sections on separate pages.

Introduction: Set the stage for your report by describing some background information concerning the problem you are writing about. You should also include a rationale for conducting this experiment and state the hypothesis or hypotheses that you are investigating.

Methods: The main types of procedures should be concisely summarized and then refer to the laboratory manual for more information. For example: “To determine the change in mass of the eggs in the different sucrose solutions, we weighed the eggs at the beginning of the experiment and then every 15 minutes for an hour. For more information see Pitkin (1990).”

Results: Describe your results in words. When appropriate, you should also include a figure or graph that displays your data so that your reader can clearly understand your results. Think of your reader as rather slow and impatient—you need to draw a picture to help him/her understand. Do not assume that the reader has any idea about the exercise that you have done. The description of the results and the figures should be able to stand alone. Do not include “raw” data tables or merely say “The results are shown in Table 1.” Table titles are printed above the tables and titles of figures are always printed under the figures. Always label the axes on figures and make sure your titles precisely describe the data.

Discussion: Evaluate the meaning of your results in terms of the original question or hypothesis. How do your data relate to the background material presented in the Introduction.

Literature Cited: You must give the proper citation for any reference that you mention. The following is an example for your laboratory manual:

Pitkin, R. B. 1990. *Introductory Biology Laboratory Manual*. Shippensburg University, Shippensburg, PA.