

Enzymes

1. Pre-Lab Reading

Chapter 6 Section 5 “Enzymes” Biology2e, OpenStax College

2. Purpose and Objectives

The purpose of this lab is to investigate factors affecting enzyme activity.

Upon completion of this investigation, you should be able to:

1. Describe the structure and functions of proteins.
2. Understand what enzymes are and how they work.
3. Analyze enzyme activity using peroxidase.
4. Investigate the effects of pH on enzymatic reaction rates.
5. Know how to use a spectrophotometer and micropipette.

3. Safety Guidelines

- Wear safety goggles
- Wear gloves when working with chemicals
- Be gentle with the spectrophotometers! Make sure the outside of each cuvette is dry before placing it in the instrument.

4. Introduction

Recall from weeks one and two of lab that there are four general types of macromolecules found in biological systems: lipids, carbohydrates, nucleic acids and proteins.

Proteins are the most versatile molecules in nature. Proteins are polymers of **amino acids**. All proteins, therefore, contain carbon, hydrogen, oxygen and nitrogen (CHON). As they are synthesized at ribosomes, polypeptide chains fold into characteristic three dimensional shapes. Protein function depends to a large degree on achieving the correct shape.

Proteins are very diverse in structure and function. Functions of proteins include providing structural support, movement, and immunity. Enzymes are an important type of protein that works to speed up biochemical reactions in cells.

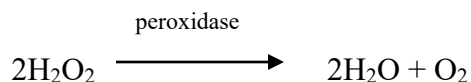
Learn more about protein structure and folding by watching the following video from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank: <http://pdb101.rcsb.org/learn/videos/what-is-a-protein-video>

Enzymes are **catalysts**, substances that speed up the rate of chemical reactions by lowering the amount of energy required to get the reaction started (i.e., the **activation energy** or E_A). Most enzymes encountered in living systems are proteins. The molecule(s) that an enzyme binds to and acts on is known as the **substrate**. Enzymes are generally substrate specific and, when the two are bonded together, form an **enzyme-substrate complex**. The part of the enzyme that interacts with the substrate is called its **active site**. Because enzymes are not consumed in the reaction, even small amounts of enzyme can have dramatic effects on the rate of a chemical reaction.

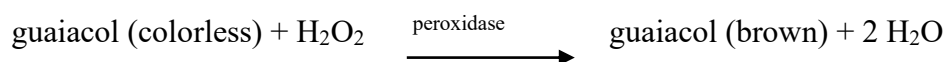
Enzymes only work under a restricted set of environmental conditions. Changes in temperature and pH, for example, can have especially strong effects on enzymes' activity because they can cause conformational changes in the protein's structure. A change in the

shape of the active site may prevent binding of the substrate. When this type of conformational change takes place, the enzyme is described as being **denatured**.

Cells obtain chemical energy from the **oxidation** of food molecules. Oxidation involves the removal of hydrogen atoms (and the high-energy electrons they contain) from organic molecules that are then used to make ATP. The oxidation of food molecules by cells is called **respiration**. During **aerobic respiration**, oxygen (O₂) serves as the final hydrogen (electron) acceptor. The transfer of hydrogen to O₂ primarily results in production of water (H₂O). Part of the time, however, hydrogen peroxide (H₂O₂) is formed instead. Hydrogen peroxide is a toxin that must be removed quickly to avoid damage to the cell. Peroxide is prevented from reaching toxic concentrations by the action of two enzymes: **catalase** and **peroxidase**. Both of these enzymes convert hydrogen peroxide to water and oxygen as illustrated in the reaction:



In this exercise, you will analyze factors affecting the activity of peroxidase isolated from turnip (*Brassica rapa*) roots with the aid of a compound called guaiacol. Guaiacol changes from colorless to brown when it reacts with oxygen:



The amount of oxidized (brown) guaiacol produced is determined using a **spectrophotometer**. As peroxidase reacts with hydrogen peroxide in the presence of guaiacol, the solution turns from colorless to orange/ brown. The spectrophotometer can measure either the amount of light the solution absorbs (absorbance) or how much light the solution allows to pass through (transmittance). In this lab, we will measure absorbance. The darker the solution turns, the more light it can absorb.

5. Spectrophotometry

Introduction

A **spectrophotometer** is an instrument that shines light of a specific wavelength into a solution, measuring how much light the solution absorbs (**absorbance**) or how much light passes through the solution (**transmittance**). The light source on the spectrophotometer we will be using emits **white light**, a combination of all possible colors of visible light. The instrument is able to shine light of a particular wavelength through a solution by using a **filter** to screen out all other wavelengths.

To measure the absorbance of a molecule at a particular wavelength, a **sample** of the solution containing the molecule is placed in a special test tube called a **cuvette**. Cuvettes are made of optically clear glass, quartz or plastic to allow the maximum amount of light to reach the solution. Light that passes through the solution is measured by a **photodetector** on the other side.

To take an accurate reading of the solution's absorbance, the instrument must compensate for light that is absorbed by imperfections in the walls of the cuvette and the solvent in which our molecule of interest is dissolved. To do this we use a **blank**, a cuvette containing only the solvent. We then **calibrate** the instrument to subtract light absorbed by the cuvette and the solvent from our sample readings. The process is analogous to zeroing a balance.

Setting up the Spectrophotometer

1. Turn on the spectrophotometer to warm up (for at least 30 minutes).
2. Set the wavelength to 470 nm using the nm + or nm - buttons.
3. Follow your instructor's directions for using the spectrophotometer properly.

6. Measuring Volume of Liquids

Measuring the volume of a liquid is a frequent task in the Biology laboratory. For some labs it will be important that you are able to do so accurately.

First, make sure that you are familiar with the metric units used to measure volume. The base unit for volume in the metric system is the **liter**, which is abbreviated “L”. Most volume measurements in the Biology lab will be expressed in milliliters. One **milliliter** is equal to one one-thousandth ($1/1000^{\text{th}}$) of a liter and is abbreviated “mL”. Occasionally we will measure volumes less than one mL. These volumes are often expressed in microliters. One **microliter** is equal to one one-millionth ($1/1,000,000^{\text{th}}$) of a liter and is abbreviated “ μL ”

Table 1: Metric Units of Volume

Milliliter (mL)	$1/1000^{\text{th}}$ liter
Microliter (μL)	$1/1,000,000^{\text{th}}$ liter

For some labs we will use disposable transfer pipettes which are simple to use but not very accurate. When volumes need to be more exact we will use one of the methods described below. Table 2 should be used as a guide to decide what type of equipment to use when measuring various volumes of liquid. It is generally preferable to use the device which has the smallest possible maximum volume because measurements will be more accurate using a smaller measuring device.

Table 2: Guide for Choosing the Appropriate Device for Measuring Liquid Volume

Volume	Device
≤ 1 mL	Micropipetman
1 to 10 mL	Graduated Pipette
> 10 mL	Graduated Cylinder (smallest size possible)

For this lab we will learn how to use the micropipetmen because we will be measuring volumes ≤ 1 mL.

Measuring Liquids: Use of Micropipettmen

The most accurate method for measuring volumes of liquid of one mL or less is to use a micropipette. These instruments can be used to measure volumes between 1 μ L and 1 mL.

There are three sizes of micropipettors or micropipettmen that are commonly used in Biology labs. They are labeled on the end based on the maximum possible volume that they can hold. The P-1000 can be used for volumes up to 1000 μ L or 1 mL. The P-200 can be used for volumes up to 200 μ L. The P-20 can be used for volumes up to 20 μ L. As with graduated pipettes or graduated cylinders it is always preferable to use the device that holds the smallest possible maximum volume to measure the volume you want.

Table 3: Guide for Choosing the Appropriate Micropipettman for Measuring Liquid Volume

Volume	Device
200 μ L to 1 mL	P-1000
20 to 200 μ L	P-200
1 to 20 μ L	P-20

The first step in using the micropipettors is to set the dial on the side to the volume you want. **NEVER SET THE DIAL TO GREATER THAN THE MAXIMUM VOLUME FOR THE PIPETTE.** This will damage the springs that control the volume and necessitate costly repairs. The numbers on the dial indicate the setting in decimal places. Where there is a decimal point the color of the numbers changes from red to black. For a P-1000, the volume is given in milliliters. The top position is the ones place, the middle is the tenths place, and the bottom is the hundredths place. For the P-200, the volume is given in microliters. The top position is the hundreds place, the middle is the tens place, and the bottom is the ones place. The volume is also given in microliters for the P-20. The top position is the tens place, the middle is the ones place, and bottom is the tenths place.¹

Next, attach the pipette firmly to a pipette tip from the box of tips on your table. The P-1000 requires a large, usually blue, tip. The P-200 and the P-20 both use smaller tips which are usually yellow. **Do not touch the pipette tip or allow it to touch any surfaces as this will contaminate the pipette and the liquid into which you place it.**

To draw up the desired volume of liquid press the plunger on the pipettor to the first point of resistance, which is called the **first stop**. While holding the plunger steady, place the end of the pipette tip into the liquid that you want to measure. Make sure that the tip is far enough below the surface that you won't fill part of it with air but not far enough that the shaft of the pipettor touches the liquid. Slowly let up the plunger. The tip should fill with the appropriate volume.

Next, move the pipette and tip to the container into which you want to dispense the liquid. Release the liquid by pressing plunger all the way down past the first stop. Remove the pipette from the container and then slowly release the plunger.

Sometimes it is convenient to mix solutions with the pipettor after you have added different components to a tube. To do this, gently pipette the mixture up and down several times to mix completely. Be careful not to jerk the plunger up and down as this will spray the liquid around in the tip and contaminate the shaft of the pipettor.

Procedure

1. Record the maximum volume settings for each type of micropipettor **in your lab book** as shown below:

P1000

P200

P20

Volume _____

Volume _____

Volume _____

2. Record in your lab book which pipettor should be used and what the dial should read for each of the following volumes: 5 μL , 200 μL , and 1 mL (1000 μL).
3. Obtain a container of dilute methylene or bromothymol blue. Pipette 5 μL , then 200 μL , and finally 1 mL (1000 μL) into a weigh boat or other container. Make a mental note of what these quantities of liquid look like both in the pipette tip and in the weigh boat.

7. Peroxidase Extraction

1. Using a blender, homogenize 4-6 g of peeled turnip in 200 mL of deionized water.
2. Filter the homogenate through 4 layers of cheesecloth. Pour the extract into a small flask or test tube. This is the enzyme extract you will use for this investigation. Dispose of the cheesecloth.

8. Peroxidase Activity: Trial Run

Procedure

1. Set the wavelength on the spectrophotometer to 470 nm using the 'nm' buttons if you have not already done so.
2. Draw a table in your lab notebook to record the data for the trial run. You will have one sample and will record absorbance readings at zero time and then every 20 seconds for a total of 3 minutes and 20 seconds (200 seconds). Don't forget to give your table a title.
3. Obtain 2 test tubes, labeling 1-2.

Add deionized water and the guaiacol/ H₂O₂ mixture provided (1 part 25 mM guaiacol: 2 parts 3% H₂O₂) to each tube as listed in Table 4. Mix. **Do NOT add enzyme yet!!** Gently mix the contents. Note the color of the solutions. *If the solutions begin to change color, stop! Either your dH₂O or guaiacol/H₂O₂ mixture is contaminated with enzyme. No color change should occur until after the enzyme is added.*

Table 4: Preparation of Trial Run Reaction Mixtures

Tube #	mL dH ₂ O	μL of guaiacol/ H ₂ O ₂ mixture	μL of Enzyme (turnip extract)	Enzyme Dilution
1	1.00	200	0.0	No enzyme
2	0.90	200	100 (add later	Undiluted

4. Transfer the solution from tube #1 to a cuvette, being careful not to touch the bottom half of the cuvette with your fingers. Place the cuvette in the spectrophotometer and press the "0 ABS/100% T" button. **This solution will serve as a blank for both experiments.**

5. Transfer the solution from tube 2 into the second cuvette. Add 0.1 mL of enzyme extract, mix quickly with the pipettor, place the cuvette in the spectrophotometer **and take your reading of absorbance immediately as the reaction will begin once the enzyme is added**. This is your time zero reading. Timing begins when you place the cuvette into the spectrophotometer.
6. Measure and record the absorbance every 20 seconds for a total of 3 minutes and 20 seconds (200 seconds). Note the final color of the solution. Rinse this cuvette with dH₂O when finished. Leave the blank cuvette as is to use for the pH experiment.
7. Graph the % absorbance (y-axis) per time in 20-second intervals (x-axis).
8. Show the graph to your instructor. If the reaction is occurring too quickly you may need to dilute the enzyme for the next experiment. To make a 1:2 dilution of the enzyme mix 1 mL of your enzyme solution with 1 mL of dH₂O in a clean test tube marked "1:2 Enzyme".

9. Effect of pH on Enzyme Activity

In this exercise, you will investigate how pH affects the activity of turnip peroxidase.

Procedure

1. Write a hypothesis concerning the effect of pH on turnip peroxidase activity. Write a prediction that describes the expected results.
2. Draw a table in your lab notebook **to** record the data for the pH experiment. You will have four samples and will record absorbance readings at zero time and then every 20 seconds for a total of 3 minutes and 20 seconds (200 seconds). Label the samples in the table with the pH used **not** just the tube number. Don't forget to give your table a title.
3. Label four test tubes 4, 6, 7, and 8.

4. Your instructor will tell you which enzyme dilution to use in this experiment based on your results in Part 7. ***Be sure to record which dilution you used.***

5. Set up tubes with the pH buffers and guaiacol/H₂O₂ **but no enzyme** with the volumes listed in Table 5.

6. Transfer the contents of tube 4 into a clean cuvette. Add 0.1 mL of enzyme dilution to the cuvette, mix gently and place in the spectrophotometer, recording % absorbance every 20 seconds for 3 minutes and 20 seconds (200 seconds).

7. Repeat step 5 for the remaining tubes. **Remember to use the blank and zero the spectrophotometer between trials.**

Table 5. Preparation of Reaction Mixtures for pH Experiment

Tube #	mL buffer solution	μL of guaiacol/ H ₂ O ₂ mixture	μL of Enzyme
4	0.9 of pH 4	200	100
6	0.9 of pH 6	200	100
7	0.9 of pH 7	200	100
8	0.9 of pH 8	200	100

8. Graph the % absorbance per unit time with all data from this experiment plotted on the same graph.

10. Conclusion and Summary

1. Do your results support your hypothesis for the pH experiment? Explain.
2. What appears to be the pH optimum for peroxidase from *Brassica rapa* roots?
3. Would you expect an enzyme from the human stomach to have the same pH optimum? Why or why not?
4. What most likely happened to the enzyme at pHs where it had little or no activity?
5. Were any of the results unexpected? Explain. Are you confident in the validity of your results? Explain.
6. What are at least two possible sources of error that could have affected your results? Please don't just say "human error"; be specific in describing sources of error.
7. Describe three ways in which the investigation could be extended to increase your understanding of enzymes.

References:

1. "The Micropipette" at <http://www.sigmaldrich.com/life-science/cell-culture/learning-center/cell-culture-videos/the-micropipette.html>