LAB #6: Enzyme Action

Objectives:

- Name the substrate and products of the peroxidase-catalyzed reaction.
- Explain the role of guaiacol in this experiment.
- To understand the terms: enzyme, activation energy, active site, pH, and denaturation.
- Distinguish between oxidation/reduction, activation energy/catalysis, substrate /product, and hydrogen peroxide/peroxidase.
- Define the term optimum with respect to peroxidase activity.
- Describe how temperature, pH, enzyme concentration, and substrate concentration can affect the reaction rate.
- Explain why peroxidase is a necessary enzyme for all aerobic or oxygen-utilizing cells.

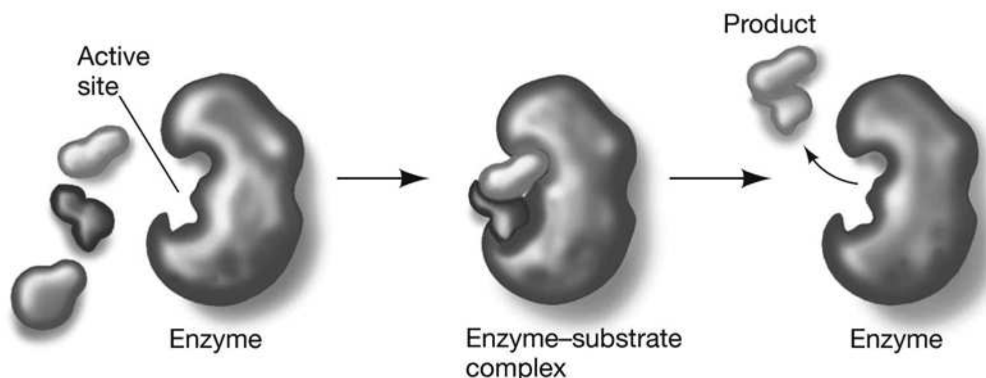
Introduction:

A catalyst speeds up a chemical reaction by lowering the activation energy required. Enzymes are biological catalysts that carry out the thousands of chemical reactions that occur in living cells. They are generally large proteins made up of several hundred amino acids. Some enzymes consist of a protein apoenzyme and a small cofactor, which might be a metal ion or a conenzyme, often a vitamin derivative.

In an enzyme-catalyzed reaction, the substance to be acted upon, or substrate, binds to the active site of the enzyme. The enzyme and substrate are held together in an enzyme-substrate complex by hydrophobic interactions, hydrogen bonds, and ionic bonds.

The enzyme then converts the substrate to the reaction products in a process that often requires several chemical steps, and may involve covalent bonds. Finally, the products are released into solution and the enzyme is ready to form another enzyme-substrate complex. As is true of any catalyst, the enzyme is not used up as it carries out the reaction but is recycled over and over. One enzyme molecule can carry out thousands of reaction cycles every minute.

Each enzyme is specific for a certain reaction because its amino acids sequence is unique and causes it to have a unique three-dimensional structure. The active site also has a specific shape so that only one of a few of the thousands of compounds present in the cell can interact with it. If there is a cofactor on the enzyme, it will form part of the active site. Any substance that blocks of changes the shape of the active site will interfere with the activity and efficiency of the enzyme.



If these changes are large enough, the enzyme can no longer act at all, and is said to be denatured. There are several factors that are especially important in determining the enzyme's shape, and these are closely regulated both in the living organism and in laboratory experiments to give the optimum or most efficient enzyme activity:

1. **Salt concentration (salinity)**. If the salt concentration is very low or zero, the charged amino acids side chains of the enzyme molecules will stick together. The enzyme will denature and form an inactive precipitate. If, on the other hand, the salt concentration is very high, abnormal interactions will occur, and again the enzyme will precipitate. An intermediate salt concentration such as that of blood (0.9%) or cytoplasm is the optimum for most enzymes.
2. **pH**. The pH scale, which is logarithmic, measures the acidity or H^+ concentration in a solution. The scale runs form 0 to 14 with 0 being highest in acidity and 14 the lowest. When the pH is in the range of 0-7, a solution is said to be acidic; if the pH is around 7, the solution is neutral; and if the pH is in the range of 7-14, the solution is basic. Amino acids side chains contain groups such as –COOH and $–NH_2$ that readily gain or lose H^+ ions. As the pH is lowered, an enzyme will tend to gain H^+ ions, and eventually enough side chains will be affected so that the enzyme's shape is disrupted. Likewise, as the pH is raised, the enzyme will lose H^+ ions and eventually lose its active shape. Many enzymes have an optimum in the neutral pH range and are denatured at either extremely high or low pH. Some enzymes, such as those that act in the human stomach where the pH is very low, will have an appropriately low pH optimum. A buffer is a compound that will gain or lose H^+ ions so that the pH changes very little.
3. **Temperature**. All chemical reactions speed up as the temperature is raised. As the temperature increases, more of the reactive molecules have enough kinetic energy to undergo the reaction. Since enzymes are catalysts for chemical reactions, enzyme reactions also tend to go faster with increasing temperature. However, if the temperature of an enzyme-catalyzed reaction is raised still further, a temperature optimum is reached: above this point, the kinetic energy of the enzyme and water molecules is so great that the structure of the enzyme molecules starts to be disrupted. The positive effect of speeding up the reaction is now more than offset by the negative effect of denaturing more and more enzyme molecules. Many proteins are denatured by temperatures around 40-50°C, but some are still active at 70-80°C and a few even withstand being boiled.
4. **Other small molecules**. Many molecules other than the substrate may interact with an enzyme. If such a molecule increases the rate of the reaction, it is an activator. If it decreases the reaction rate, then it is an inhibitor. The cell can use these molecules to regulate how fast the enzyme acts. Any substance that tends to unfold the enzyme, such as an organic solvent or detergent, will act as an inhibitor. Some inhibitors act by reducing the disulfide bridges that stabilize the enzyme's structure. Many inhibitors act by reacting with side chains in or near the active site to change or block it. Others may damage or remove the cofactor. Many well-known poisons such as potassium cyanide and curare are enzyme inhibitors that interfere with the active site of a critical enzyme.

In this experiment, you will study the enzyme peroxidase from turnips. Peroxidases are widely distributed in plant and animal cells and catalyze the oxidation or organic compounds by hydrogen peroxide as follows:

$$R\text{-}H + H_2O_2 \xrightarrow{\text{peroxidase}} R\text{-}OH + H_2O$$

Any cell using molecular oxygen in its metabolism will produce small amounts of H_2O_2 as a highly toxic by-product. It is critical that the H_2O_2 be quickly removed by enzymes such as peroxidase before it can do damage to the cell.

You will use a reducing agent, guaiacol (structure shown below), which changes color when it is oxidized. This change can be easily measured in the spectrophotometer:

CH_3O / OH

$$\underset{\text{(colorless)}}{4\ \text{guaiacol}} + 2H_2O_2 \xrightarrow{\text{peroxidase}} \underset{\text{(brown)}}{\text{tetraguaiacol}} + 8H_2O$$

A colored solution such as oxidized guaiacol solution appears that way because some of the light entering the solution is absorbed by the colored substance. A clear solution will allow almost all of the light to pass through. The amount of absorbance can be determined by using a spectrophotometer, which measures quantitatively what fraction of the light passes through a given solution, and indicates on the absorbance scale the amount of light absorbed compared to that absorbed by a clear solution. <u>The darker the solution, the greater its absorbance</u>.

Inside the machine there is a light that shines through a filter (which can be adjusted to control the color or wavelength of light), then through the sample and onto a light-sensitive phototube. The phototube produces an electric current proportional to the amount of light striking it. The absorbance meter measures how much light has been blocked by the sample and thereby prevented from striking the phototube. A clear tube of water or other solvent is the blank and has zero absorbance. A solution that contains a small amount of a colored substance might show an absorbance of 0.1, a solution with a moderate amount of substance in solution might show an absorbance of 0.4, and so forth. In fact, in the lower portion of the absorbance scale, the amount of substance in solution is directly proportional to the absorbance reading so that a graph of absorbance versus concentration will give a straight line. This very useful relationship is known as Beer's law.

<u>Procedure</u>:

Preparation of Turnip Extract

1. Weigh out 5 g of turnip (the peeled, inner portion)
2. Using a mortar and pestle, grind the turnip into pulp.
3. Combine with 200 mL of distilled water and blend at the high setting for 1 minute.

This suspension is the turnip extract, and contains the enzyme peroxidase. The activity of the turnip extract will vary from day to day, depending on the size and age of the turnip and the extent of the blending.

PART I: Enzyme Action—Baseline

Pipets will be used to measure accurately the solutions used in this experiment. BE SURE TO USE A DIFFERENT PIPET TIP FOR EACH SOLUTION SO THAT THE REAGENTS ARE NOT RUINED BY CROSS CONTAMINATION.

4. Label the pipets with a tape or marking pencil so each one can be reused with the proper solution.
5. Obtain two spectrophotometer tubes and label them B (blank) and R (reaction).
6. Obtain three test tubes and label them #1, #2, and #3. (#1 will contain a blank reaction without H_2O_2. The contents to #2 and #3 will be mixed to start the reaction.
7. Set up the three tubes as follows, and make a record of the contents of the reaction tube in **Table 1**.
   - **Tube #1:** (blank tube without H_2O_2). Add 0.1 mL of guaiacol, 1.0 mL of turnip extract, and 8.9 mL of distilled water; mix well.
   - **Tube #2:** Add 0.1 mL of guaiacol, 0.2 mL of 0.1% H_2O_2, and 4.7 mL of distilled water.
   - **Tube #3:** Add 1.0 mL of turnip extract and 4.0 mL of distilled water.
8. Adjust the spectrophotometer to zero absorbance at 470 nm (the A_{max} for tetraguaiacol), using tube B filled with solution from test tube #1, filled approximately $^2/_3$ full. Set transmittance to 100% and remove tube.

You have now set up the instrument so that any difference in the meter reading with a change in the sample will reflect a difference in oxidized guaiacol concentration.

9. Obtain a stopwatch, and be sure that you understand how to use it.
10. Prepare the sample: have ready spectrophotometer tube R, tissue/paper towel, and test tubes #2 and #3 filled with the solutions given above.

YOU WILL HAVE **30 SECONDS** TO MIX THE CONTENTS OF TUBES #2 AND #3. FILL THE SPECTROPHOTOMETER TUBE $^2/_3$ FULL, WIPE THE OUTSIDE OF THE TUBE, AND TAKE YOUR FIRST READING.

11. When you are completely ready, mix the contents of tubes #2 and #3, pour the contents back and forth two times, and then pour quickly into tube R; approximately $^2/_3$ full. Start the stopwatch when the tubes are mixed (t=0 seconds—when the tubes are mixed).
12. Wipe the outside of the tube and place it in the spectrophotometer.
13. Take your first reading 30 seconds (t=30 seconds) or as soon as possible after the tubes were mixed.
14. Continue to read the absorbance every 30 seconds for 5 minutes.
15. Record the readings in **Data Table 2** and graph the absorbance versus time.

This curve will represent the base line of enzyme activity with which the enzyme activity under varying conditions will be compared. In the following experiments you will vary one condition at a time and compare the results with the base line. Check your graph with your instructor before proceeding with the rest of the experiment.

PART II: Enzyme Action—Effect of Enzyme Concentration

16. What happens when you use:
    a. twice the amount of enzyme
    - **Tube #1:** Add 0.1 mL of guaiacol, 2.0 mL of turnip extract, and 7.9 mL of distilled water; mix well.
    - **Tube #2:** Add 0.1 mL of guaiacol, 0.2 mL of 0.1% H_2O_2, and 4.7 mL of distilled water.
    - **Tube #3:** Add 2.0 mL of turnip extract and 3.0 mL of distilled water.

    b. half the amount of enzyme
    - **Tube #1:** Add 0.1 mL of guaiacol, 0.5 mL of turnip extract, and 9.4 mL of distilled water; mix well.
    - **Tube #2:** Add 0.1 mL of guaiacol, 0.2 mL of 0.1% H_2O_2, and 4.7 mL of distilled water.
    - **Tube #3:** Add 0.5 mL of turnip extract and 4.5 mL of distilled water.

Repeat the procedure for preparing the sample (recording the contents of the reaction tube in **Table #1**, record the data in **Table #2**, and graph the results.

PART III: Enzyme Action—Effect of Substrate Concentration

17. Design and carry out an experiment to determine whether varying the concentration of the substrate H_2O_2 (using twice the concentration of peroxide), affects the rate of reaction.

Hints For Your Lab Report:

The Introduction section of your lab should have (but not be limited to) your hypotheses for the three reaction tubes that will be tested compared to the baseline. A visual hypothesis—a graph (sketched or Excel-ed)—may be included with your hypothesis.

The Results section of your lab report will include (but not be limited to):

- Table 2. (Table 1 should be included in your *Methods* section!)
- Graph of data from Table 2. (All four lines on one graph—with R^2 value.)

The Discussion section of your lab report will include the answers to the following questions (among lots of other information—see your Lab Guide):

1. What are the substrates and products in the reaction catalyzed by peroxidase?
2. In this reaction, the oxidation of guaiacol is coupled to the reduction of which substance?
3. In Part II, why was it important that Tube #1 and the combined volume of Tubes #2 and #3 equaled 10 mL?
4. In Part II, how did changing the concentration of enzyme affect the reaction rate?
5. In Part III, what would happen to the reaction rate if the concentration of substrate were increased further and further?
6. Using information from your original baseline graph, draw a curve representing the relationship of absorbance vs. time if the temperature of the reactants were 10°C instead of approximately 22°C (STP—baseline). Draw another curve for 37°C. Explain what would happen if the temperature of the reactants were increased to 80°C. This means you have four lines on a graph for the question (22°C, 10°C, 37°C, and 80°C).

Methods:

Table 1—Contents of Reaction Tube

	Baseline	2X Enzyme	½ Enzyme	2X Substrate
Contents				

Results:

Table 2—Absorbance

Time (seconds)	Baseline	2X Enzyme	½ Enzyme	2X Substrate
30				
60				
90				
120				
150				
180				
210				
240				
270				
300				