LABORATORY 2. ENZYME CATALYSIS

OVERVIEW

In this laboratory you will observe the conversion of hydrogen peroxide (H_2O_2) to water and oxygen gas by the enzyme catalase. You will then measure the amount of oxygen generated and calculate the rate of the enzyme-catalized reaction.

OBJECTIVES

Before doing this laboratory you should understand:

● the general functions and activities of enzymes;

● the relationship between the structure and function of enzymes;

● the concept of initial reaction rates of enzymes;

● how the concept of free energy relates to enzyme activity; and

● that changes in temperature, pH, enzyme concentration, and substrate concentration can affect the initial reaction rates of enzyme-catalyzed reactions.

After doing this laboratory you should be able to:

● measure the effects of changes in temperature, pH, enzyme concentration, and substrate concentration on reaction rates of an enzyme-catalyzed reaction in a controlled experiment; and

● explain how environmental factors affect the rate of enzyme-catalyzed reactions.

INTRODUCTION

In general, **enzymes** are proteins produced by living cells; they act as catalysts in biochemical reactions. A **catalyst** affects the rate of a chemical reaction. One consequence of enzyme activity is that cells can carry out complex chemical activities at relatively low temperatures.

In an enzyme-catalyzed reaction, the substance to be acted upon (the **substrate = S**) binds reversibly to the **active site** of the enzyme (**E**). One result of this temporary union is a reduction in the energy required to activate the reaction of the substrate molecule so that the **products (P)** of the reaction are formed.

In summary:
$$E + S \rightarrow ES \rightarrow E + P$$

Note that the enzyme is not changed in the reaction and can be recycled to break down additional substrate molecules. Each enzyme is specific for a particular reaction because its amino acid sequence is unique and causes it to have a unique three-dimensional structure. The **active site** is the portion of the enzyme that interacts with the substrate, so that any substance that blocks or changes the shape of the active site affects the activity of the enzyme. A description of several ways enzyme action may be affected follows.

1. **Salt concentration.** If the salt concentration is close to zero, the charged amino acid side chains of the enzyme molecules will attract each other. The enzyme will denature and form an inactive precipitate. If, on the other hand, the salt concentration is very high, normal interaction of charged groups will be blocked, new interactions will occur, and again the enzyme will precipitate. An intermediate salt concentration such as that of human blood (0.9%) or cytoplasm is the optimum for many enzymes.

2. **pH.** pH is a logarithmic scale that measures the acidity or H^+ concentration in a solution. The scale runs from 0 to 14 with 0 being highest in acidity and 14 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 acid 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 perform optimumly in the neutral pH range and are denatured at either an extremely high or low pH. Some enzymes, such as pepsin, which acts in the human stomach where the pH is very low, have a low pH optimum.

3. **Temperature.** Generally, chemical reactions speed up as the temperature is raised. As the temperature increases, more of the reacting 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 value the kinetic energy of the enzyme and water molecules is so great that the conformation of the enzyme molecules is disrupted. The positive effect of speeding up the reaction is now more than offset by the negative effect of changing the conformation of 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 boiling.

4. **Activations and Inhibitors.** 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**, and if it decreases the reaction rate it is an **inhibitor**. These molecules can 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 -S-S- bridges that stabilize the enzyme's structure. Many inhibitors act by reacting with side chains in or near the active site to change its shape or block it. Many well-known poisons such as potassium cyanide and curare are enzyme inhibitors that interfere with the active site of critical enzymes.

The enzyme used in this lab, catalase, has four polypeptide chains, each composed of more than 500 amino acids. This enzyme is ubiquitous in aerobic organisms. One function of catalase within cells is to prevent the accumulation of toxic levels of hydrogen peroxide formed as a by-product of metabolic processes. Catalase might also take part in some of the many oxidation reactions that occur in all cells.

The primary reaction catalyzed by catalase is the decomposition of H_2O_2 to form water and oxygen.

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2\ (gas)$$

In the absence of catalase, this reaction occurs spontaneously, but very slowly. Catalase speeds up the reaction considerably. In this experiment, a rate for this reaction will be determined.

Much can be learned about enzymes by studying the kinetics (particularly the changes in rate) of enzyme-catalyzed reactions. For example, it is possible to measure the amount of product formed, or the amount of substrate used, from the moment the reactants are brought together until the reaction has stopped.

If the amount of product formed is measured at regular intervals and this quantity is plotted on a graph, a curve like the one that follows is obtained.

Figure 2.1: Enzyme Activity
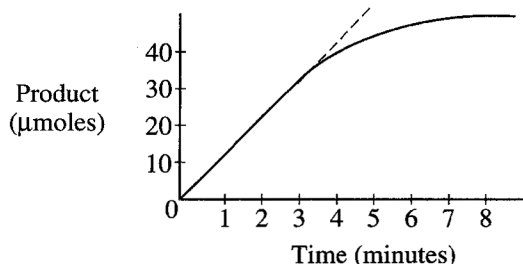
Study the solid line on the graph of this reaction. At time 0 there is no product. After 30 seconds, 5 micromoles (μmoles) have been formed; after one minute, 10 μmoles; after two minutes, 20 μmoles. The rate of this reaction could be given as 10 μmoles of product formed per minute for this initial period. Note, however, that by the third and fourth minutes, only about 5 additional μmoles of product have been formed. During the first three minutes, the rate is constant. From the third minute through the eighth minute, the rate is changing; it is slowing down. For each successive minute after the first three minutes, the amount of product formed in that interval is less than in the preceding minute. From the seventh minute onward, the reaction rate is very slow.

In the comparison of the kinetics of one reaction with another, a common reference point is needed. For example, suppose you wanted to compare the effectiveness of catalase obtained from potato with that of catalase obtained from liver. It is best to compare the reactions when the rates are constant. In the first few minutes of an enzymatic reaction such as this, the number of substrate molecules is usually so large compared with the number of enzyme molecules that changing the substrate concentration does not (for a short period at least) affect the number of successful collisions between substrate and enzyme. During this early period, the enzyme is acting on substrate molecules at a nearly constant rate. The slope of the graph line during this early period is called the **initial rate** of the reaction. The initial rate of any enzyme-catalyzed reaction is determined by the characteristics of the enzyme molecule. It is always the same for any enzyme and its substrate at a given temperature and pH. This also assumes that the substrate is present in excess.

The rate of the reaction is the slope of the linear portion of the curve. To determine a rate, pick any two points on the straight-line portion of the curve. Divide the difference in the amount of product formed between these two points by the difference in time between them. The result will be the rate of the reaction which, if properly calculated, can be expressed as μmoles product/sec. The rate then is:

$$\frac{\mu moles_2 - \mu moles_1}{t_2 - t_1}$$

or from the graph,

$$\frac{\Delta y}{\Delta x}$$

In the illustration of Figure 2.1, the rate between 2 and 3 minutes is calculated:

$$\frac{30-20}{180-120} = \frac{10}{60} = 0.17 \text{ } \mu\text{moles/sec}$$

The rate of a chemical reaction may be studied in a number of ways, including the following:

1. Measuring the rate of disappearance of substrate (in this example, H_2O_2);

2. measuring the rate of appearance of product (in this case, O_2, which is given off as a gas); and

3. measuring the heat released (or absorbed) during the reaction.

General Procedure

In this experiment the disappearance of the substrate, H_2O_2, is measured as follows:

1. A purified catalase extract is mixed with substrate (H_2O_2) in a beaker. The enzyme catalyzes the conversion of H_2O_2 to H_2O and O_2 (gas).

2. Before all of the H_2O_2 is converted to H_2O and O_2, the reaction is stopped by adding sulfuric acid (H_2SO_4). The H_2SO_4 lowers the pH, denatures the enzyme, and thereby stops the enzyme's catalytic activity.

3. After the reaction is stopped, the amount of substrate (H_2O_2) remaining in the beaker is measured. To assay (measure) this quantity, potassium permanganate is used. Potassium permanganate ($KMnO_4$), in the presence of H_2O_2 and H_2SO_4 reacts as follows:

$$5 \text{ } H_2O_2 + 2 \text{ } KMnO_4 + 3 \text{ } H_2SO_4 \rightarrow K_2SO_4 + 2 \text{ } MnSO_4 + 8 \text{ } H_2O + 5 \text{ } O_2$$
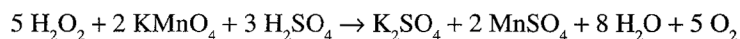
Note that H_2O_2 is a reactant for this reaction. Once all the H_2O_2 has reacted, any more $KMnO_4$ added will be in excess and will not be decomposed. The addition of excess $KMnO_4$ causes the solution to have a permanent pink or brown color. Therefore, the amount of H_2O_2 remaining is determined by adding $KMnO_4$ until the whole mixture stays a faint pink or brown, permanently. Add no more $KMnO_4$ after this point. The amount of $KMnO_4$ added is a proportional measure of the amount of H_2O_2 remaining (2 molecules of $KMnO_4$ reacts with 5 molecules of H_2O_2 as shown in the equation).

Figure 2.2: The General Procedure

Catalytic Reaction   Reaction Stopped   Assay

O_2 ⌐—Catalase   ⌐—H_2SO_4

H_2O_2 Catalase H_2O_2 H_2SO_4 Catalase H_2O_2 Measurement of Remaining H_2O_2
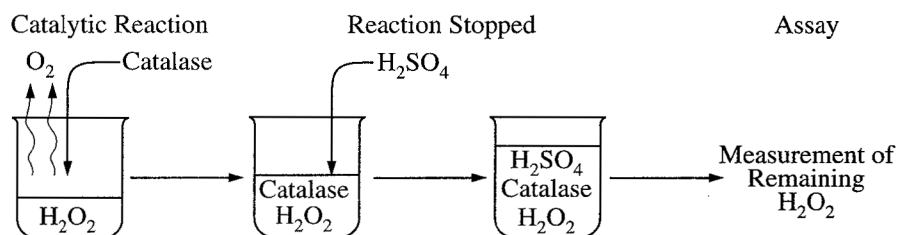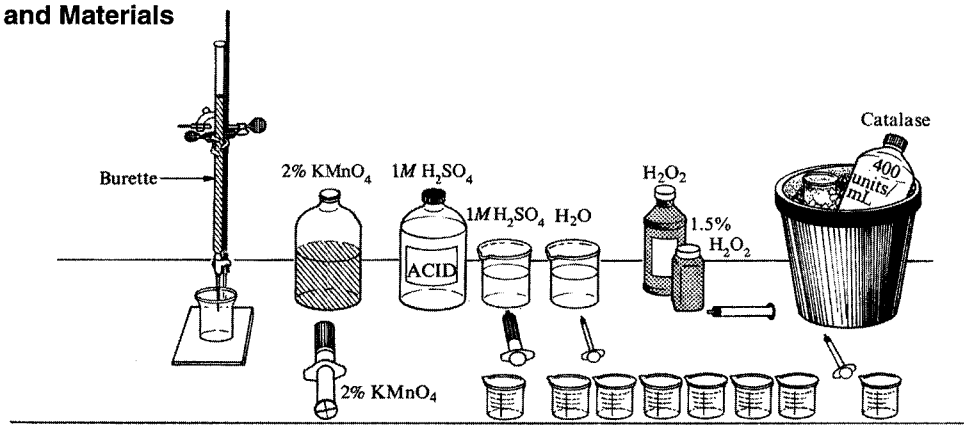
Figure 2.3: The Apparatus and Materials

EXERCISE 2A: Test of Catalase Activity

1. To observe the reaction to be studied, transfer 10 mL of 1.5% (0.44 M)H_2O_2 into a 50-mL glass beaker and add 1 mL of the freshly made catalase solution. The bubbles coming from the reaction mixture are O_2, which results from the breakdown of H_2O_2 by catalase. Be sure to keep the freshly made catalase solution on ice at all times.

   a. What is the enzyme in this reaction? _____

   b. What is the substrate in this reaction? _____

   c. What is the product in this reaction? _____

   d. How could you show that the gas evolved is O_2? _____

2. To demonstrate the effect of boiling on enzymatic activity, transfer 5 mL of purified catalase extract to a test tube and place it in a boiling water bath for five minutes. Transfer 10 mL of 1.5% H_2O_2 into a 50-mL glass beaker and add 1 mL of the cooled, boiled catalase solution. How does the reaction compare to the one using the unboiled catalase? Explain the reason for this difference.

   _____

   _____

3. To demonstrate the presence of catalase in living tissue, cut 1 cm³ of potato or liver, macerate it, and transfer it into a 50-mL glass beaker containing 10 mL of 1.5% H_2O_2. What do you observe? What do you think would happen if the potato or liver was boiled before being added to the H_2O_2?

   _____

   _____

EXERCISE 2B: The Baseline Assay

To determine the amount of H_2O_2 initially present in a 1.5% solution, one needs to perform all the steps of the procedure without adding catalase (enzyme) to the reaction mixture. This amount is known as the baseline and is an index of the initial concentration of H_2O_2 in solution. In any series of experiments, a baseline should be established first.

Procedure for Establishing Baseline

1. Put 10 mL of 1.5% H_2O_2 into a clean glass beaker.

2. Add 1 mL of H_2O (instead of enzyme solution).

3. Add 10 mL of H_2SO_4 (1.0 M). USE EXTREME CARE IN HANDLING ACIDS.
   Your teacher will instruct you about the proper safety procedures for handling hazardous materials.

4. Mix well.

5. **Remove a 5 mL sample.** Place this 5 mL in another beaker, and assay for the amount of H_2O_2 as follows. Place the beaker containing the sample over a white paper. Use a burette or 5 mL pipette to add $KMnO_4$ a drop at a time to the solution until a persistent pink or brown color is obtained. Remember to gently swirl the solution after adding each drop. Check to be sure that you understand the calibrations on the burette or syringe. Record below.

Baseline calculation

Final reading of burette _____ mL

Initial reading of burette _____ mL

Baseline (Final - Initial) _____ mL $KMnO_4$

Figure 2.4: Proper Reading of a Burette
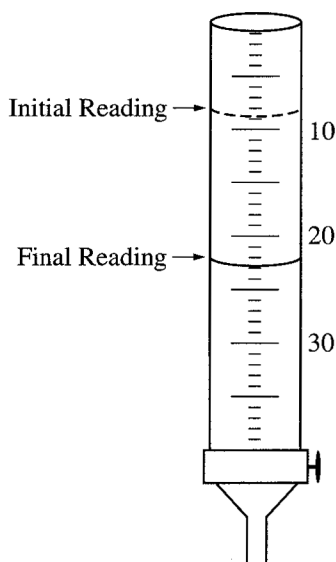
The baseline assay value should be nearly the same for all groups. Compare your results to another team's before proceeding.

Remember, the amount of $KMnO_4$ used is proportional to the amount of H_2O_2 that was in the solution.

Note: Handle $KMnO_4$ with care. Avoid contact with skin and eyes.

EXERCISE 2C: The Uncatalyzed Rate of H_2O_2 Decomposition

To determine the rate of spontaneous conversion of H_2O_2 to H_2O and O_2 in an uncatalyzed reaction, put a small quantity of 1.5% H_2O_2 (about 15mL) in a beaker. Store it uncovered at room temperature for approximately 24 hours. Repeat steps 2-5 from Exercise 2B to determine the proportional amount of H_2O_2 remaining (for ease of calculation assume that 1 mL of $KMnO_4$ used in the titration represents the presence of 1 mL H_2O_2 in the solution). Record below.

Uncatalyzed H_2O_2 decomposition

Final reading of burette   _____ mL

Initial reading of burette   _____ mL

Amount of $KMnO_4$ titrant   _____ mL

Amount of H_2O_2 spontaneously decomposed
(mL baseline – mL $KMnO_4$)   _____ mL

What percent of the H_2O_2 spontaneously decomposes
in 24 hours? [(mL baseline – mL 24 hours)
/mL baseline] x 100   _____ %

EXERCISE 2D: An Enzyme-Catalyzed Rate of H_2O_2 Decomposition

In this experiment, you will determine the rate at which a 1.5% H_2O_2 solution decomposes when catalyzed by the purified catalase extract. To do this, you should determine how much H_2O_2 has been consumed after 10, 30, 60, 120, 180, and 360 seconds.

If a day or more has passed since you did Exercise 2B, you must reestablish the baseline by determining the amount of H_2O_2 present in your 1.5% solution. Repeat the assay procedure (steps 1 through 5, page 24) and record the results below. The baseline assay should be approximately the same value for all groups. Check with another team before proceeding.

Baseline Calculation

Final reading of burette_____ mL

Initial reading of burette_____ mL

Baseline (Final - Initial)_____ mL $KMnO_4$

Procedure for a Time-Course Determination

To determine the course of an enzymatic reaction, you will need to measure how much substrate is disappearing over time. You will measure the amount of substrate decomposed after 10, 30, 60, 120, 180, and 360 seconds. To use lab time more efficiently, set up all five of these at the same time, and do them together. Stop each reaction at the proper time.

10 seconds

1. Put 10 mL of 1.5% H_2O_2 in a clean 50-mL glass beaker.

2. Add 1 mL of catalase extract.

3. Swirl gently for 10 seconds.

4. At 10 seconds, add 10 mL of H_2SO_4 (1.0 M).

30, 60, 120, 180, and 360 seconds

Each time, repeat steps 1 through 4 as above, except allow the reactions to proceed for 30, 60, 120, 180, and 360 seconds, respectively.

Note: Each time, remove a 5 mL sample and assay for the amount of H_2O_2 in the sample. Use a burette to add $KMnO_4$, a drop at a time, to the solution until a persistent pink or brown color is obtained. Should the end point be overshot, remove another 5 mL sample and repeat the titration. **Do not discard any solutions until the entire lab is completed.** Record your results in Table 2.1 and graph.

Table 2.1

KMnO4 (ml)	TIME (seconds)					
	10	30	60	120	180	360
A. Baseline*						
B. Final Reading						
C. Initial Reading						
D. Amount of KMnO₄ consumed (B minus C)						
E. Amount of H₂O₂ used (A minus D)						

Record baseline value, obtained in 2D, in all boxes on line A above.

*Remember that the baseline tells how much H_2O_2 is in the initial 5-mL sample. The difference between the initial and final readings tells how much H_2O_2 is left after the enzyme-catalyzed reaction. The shorter the time, the more H_2O_2 remains and therefore the more $KMnO_4$ necessary to titrate to the endpoint.

Graph the data for enzyme-catalyzed H_2O_2 decomposition.

For this graph you will need to determine the following:
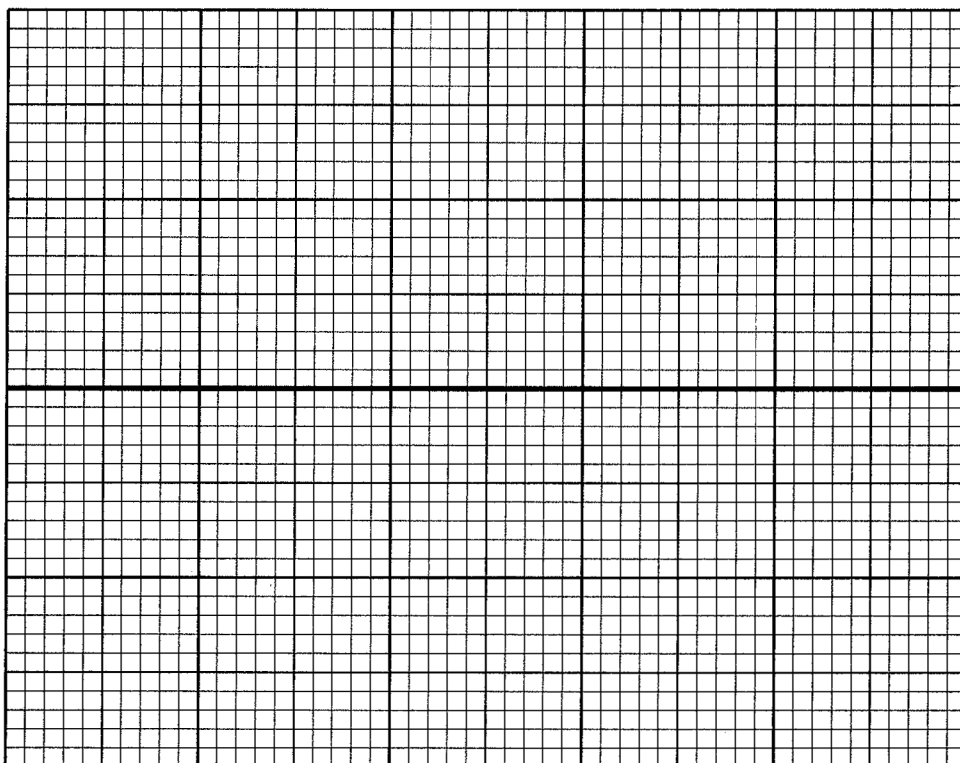
a. the *independent* variable _____
Use this to label the horizontal (X) axis.

b. the *dependent* variable _____
Use this to label the vertical (Y) axis.

Graph Title: _____

Graph 2.1

Analysis of Results

1. From the formula described earlier recall that rate $= \dfrac{\triangle y}{\triangle x}$

   Determine the initial rate of the reaction and the rates between each of the time points. Record the rates in the table below.

Time Intervals (seconds)						
	Initial 0 to 10	10 to 30	30 to 60	60 to 120	120 to 180	180 to 360
Rates*						

*Reaction rate (mL H_2O_2/sec)

2. When is the rate the highest? Explain why.

   _____

   _____

   _____

3. When is the rate the lowest? For what reasons is the rate low?

   _____

   _____

   _____

4. Explain the inhibiting effect of sulfuric acid on the function of catalase. Relate this to enzyme structure and chemistry.

   _____

   _____

   _____

5. Predict the effect lowering the temperature would have on the rate of enzyme activity. Explain your prediction.

   _____

   _____

   _____

6. Design a controlled experiment to test the effect of varying pH, temperature, or enzyme concentration.

   _____

   _____

   _____

   _____

   _____