RECENTLY UPDATED!

Edvo-Kit # 282

Edvo-Kit #282

Principles of Enzyme Catalysis

Experiment Objective:

The objective of this experiment is to understand enzyme catalysis. Students will perform an enzyme assay and determine the rate of a biochemical reaction.

See page 3 for storage instructions.

Version 282.111117

EDVOTEK®

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Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Experiment Components

Components		Storage	Check (✓)
A	Catalase solution	Freezer	❑
B	Hydrogen peroxide (stabilized)	Refrigerator	❑
C	Phosphate buffer, pH 7.2 (conc.)	Refrigerator	❑
D	Assay reagent, potassium iodide (conc.)	Refrigerator	❑
E	Acidification solution 0.1M HCl	Refrigerator	❑
F	Color enhancer (conc.)	Refrigerator	❑
G	Color developer (conc.)	Refrigerator	❑
•	One 15 mL plastic tube	---	❑

Experiment #282 contains material for up to 10 lab groups.

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Requirements *(NOT included in this experiment)*

- Visible wavelength spectrophotometer
- Test tube racks
- Timers or clock with second hand
- Lab permanent markers
- Test tubes (13 x 100 mm, 10 mL)
- Beakers
- Distilled water
- 5 and 10 mL pipets
- Pipet pumps or bulbs
- Linear graph paper
- 20 - 1 mL pipets, reusable glass or plastic with 0.1 mL divisions, or an automatic pipette (200-1000 µL) and tips
- Ice

Background Information

PROTEINS AND ENZYMES

Proteins are large biological compounds responsible for many functions within a cell. They can act as a messenger, provide structure, balance fluids, transport nutrients, and maintain pH, just to name a few. Proteins are built of amino acids, which link together through peptide bonds. Enzymes are one class of proteins critical to cellular function.

Enzymes function to facilitate biochemical reactions. As such, enzymes are a part of a larger group of molecules known as catalysts. Catalysts are able to speed up reactions. However, catalysts only affect the rate of approach affects the rate of approach to equilibrium but not the equilibrium constant itself. Enzymes are used in trace amounts and accelerate the rate of a biochemical reaction without being consumed or transformed during the reaction; an enzyme can accelerate reactions by as much as 10^{14} or 10^{20} times. Some enzymes are physiologically regulated, meaning they require a certain temperature or pH range. Most enzymes function best under mild physiological conditions of neutral pH, and temperatures of 37 °C, however there are some exceptions to this. For example, Taq DNA polymerase which is commonly used in the polymerase chain reaction (PCR) to amplify DNA functions optimally at a whopping 72 °C!

Enzymes are generally very specific for the reactions that they catalyze. Certain enzymes are regulated by metabolites that are not directly involved with the reaction. For example, the enzyme may be bound by an effector molecule which regulates when and where it is active. This type of enzyme regulation is termed allosteric.

The discovery of enzymes dates back to 1897, when Eduord Buchner demonstrated that cell-free yeast extracts could catalyze the fermentation of sugar to produce alcohol. However, it wasn't until 1926 that J.B. Sumner demonstrated that enzymes were actually a class of proteins. With the exception of specialized RNA molecules involved in RNA self-splicing and certain cyclodextrins, all naturally occurring enzymes to date are proteins. Thousands of different enzymes are known to have diverse and complex structures. There are, however, certain common structural features that are shared by all proteins.

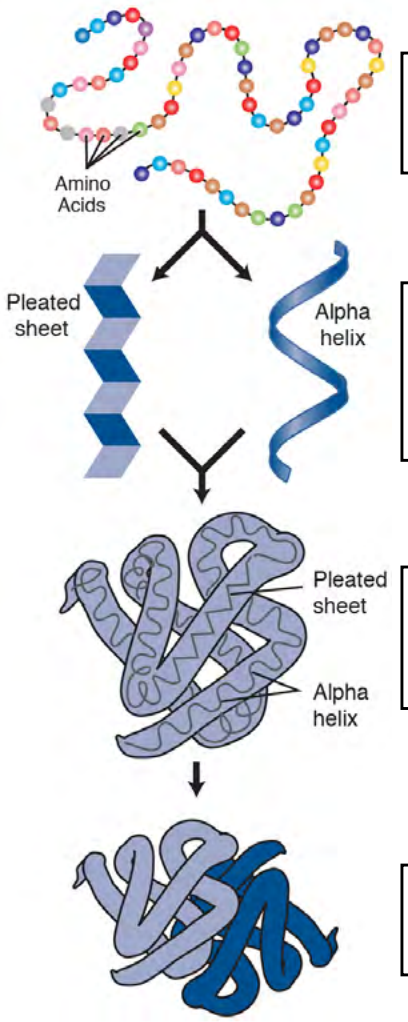


Figure 1

Image Courtesy: National Human Genome Research Institute. www.genome.gov

CONFORMATION OF PROTEINS

Proteins consist of specific sequences of amino acid residues linked to each other by peptide bonds (Figure 1). The sequence of residues in a polypeptide chain

is called **primary structure**. The unique amino acid sequences is the most important feature of a protein. The sequence and distribution of amino acids have profound effects on the solubility, the three dimensional shape (**conformation**) and biological activity of a protein. Chemical varieties of amino acid side chain functional groups such as hydroxyl, carboxylic acid, amino, guanido, phenolic, sulfhydryl are largely responsible for the chemical activity, binding specificities, and electrical properties of proteins. For example the non-polar hydrocarbon groups of amino acids such as valine and alanine are important in maintaining the overall structure of a protein and creating the appropriate chemical environments within that is not in contact with the aqueous environment.

The backbone of the polypeptide chain consists of peptide bonds. The folding path of the backbone through space is called **secondary structure** of a protein. The folding patterns are complex, having bends, twists and spirals. Secondary structures are mainly determined by hydrogen bonds between backbone oxygens, nitrogens and hydrogens. Well known examples of secondary structure include **α-helices** and **β-pleated** sheets. Protein secondary structure is influenced by the type of amino acids present in that part of the polypeptide chain.

The complete three dimensional folding pattern of a polypeptide chain, including the positioning of the amino acid functional groups relative to each other is called the **tertiary structure**. Examples of bonds which stabilize the tertiary structure of proteins are ionic bonds, hydrogen bonds, disulfide linkages, Van der Waals interactions, and hydrophobic interactions. The tertiary structure creates the three-dimensional crevices and pockets which enable the protein to bind and react with a substrate and other protein molecules. It also gives proteins unique conformation and affect solubility. Most importantly, the precise tertiary structure is absolutely necessary for the biological activity of proteins.

Many proteins consist of several polypeptide chains that are specifically associated with each other by non-covalent and covalent bonds. The three dimensional arrangement of polypeptide chains to each other in a protein is called **quaternary structure**. The individual polypeptide chains that make up the protein are often called subunits. The subunits of a protein can be identical, similar, or completely different from one another. Different subunits can be responsible for different functions within a protein.

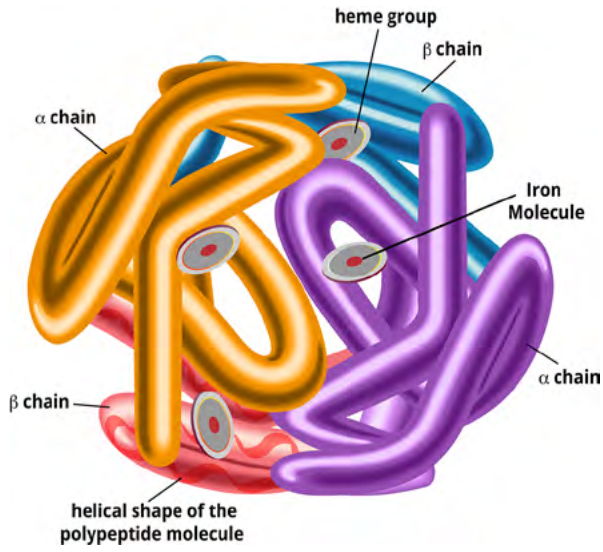


Figure 2

BIOLOGICAL ACTIVITY OF ENZYMES

Certain proteins contain, as integral part of their structure, chemical groups that are not amino acid residues, but are absolutely required for biological activity. These groups include small organic molecules, such as certain vitamin derivatives, and certain metal ions. Such moieties are called **prosthetic groups**. A well known prosthetic group is **heme** which consists of an iron atom coordinated to active nitrogen moieties (Figure 2).

A protein that contains all its natural structural elements and possesses biological activity is called **native**. When a protein is unfolded, it no longer possesses biological activity even though the backbone and the amino acid groups remain intact. Unfolding can also cause subunit dissociation if there are no inter-subunit covalent links between them. Unfolded or inactive proteins are called **denatured**. Agents or conditions that denature enzyme structure or function will destroy their biological activity. Enzyme denaturation can be caused by high temperatures, extremes in pH, organic solvents, and repetitive cycles of freezing and thawing. Ionic detergents, such as (SDS) sodium decyl sulfate, are potent protein denaturants that will bind to proteins and unfold their native forms. Other agents such as heavy metals, free radicals and peroxides disrupt protein structure by direct chemical reaction with the amino acid residues.

ENZYME ASSAYS

An enzymatic reaction measurement is referred to as an **assay**. The probability of forming enzyme substrate complex increases with more substrate molecules present. Generally, the substrate concentration is thousands of times greater than the enzyme concentration for when the experiment is done in a lab to determine the rate, known as *in vitro* kinetic studies. At the early stages of such a reaction, the substrate concentration is in great excess and the rate is approximately linear per unit of time and is termed the **initial velocity (v) or initial rate** of the reaction. The characteristics of the enzyme molecule determine the initial velocity. It will always remain the same for an enzyme as long as the substrate is present in excess, the products are not inhibitory and the pH and temperature remain constant.

$$V = \frac{[S]_1 - [S]_2}{T_1 - T_2}$$

In the above equation $[S]_1$ is the molar concentration of substrate at some initial time T_1, and $[S]_2$ is the substrate concentration at a later time T_2. Note that the concentration of substrate decreases with time and the concentration of product increases with time. Graphically, this can be represented with the substrate concentration plotted on the y-axis and time on the x-axis. The decrease in the substrate concentration with time will generate a curve. The rate of decrease is fastest at the earliest time points of the reaction since the substrate concentration is comparatively higher. The rate of decrease diminishes at later times because the substrate concentration is lower and the reaction is slower **(Figure 3)**. Within short time intervals there will be sections of the curve that are approximately linear and the rate of the reaction can be measured. At some substrate concentration, all the enzyme molecules are bound to substrate and are involved in some stage of the catalytic reaction. Under these conditions the enzyme is saturated with substrate and no increase in reaction velocity.

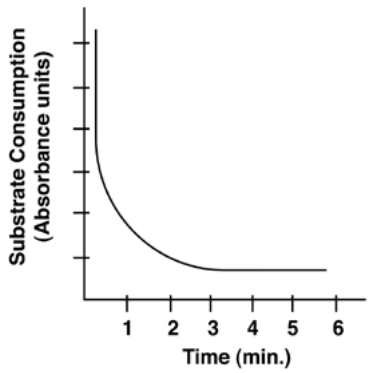


Figure 3

The initial reaction rate can also be expressed in terms of the appearance of product. To determine the rate of the reaction, pick any two points on the straight-line portion of the graph curve **(Figure 4)**. The amount of product formed between two points divided by the difference in time between the two points will be the rate of the reaction. It can be expressed as µmoles product/sec.

$$V = \frac{[P]_2 - [P]_1}{T_2 - T_1}$$

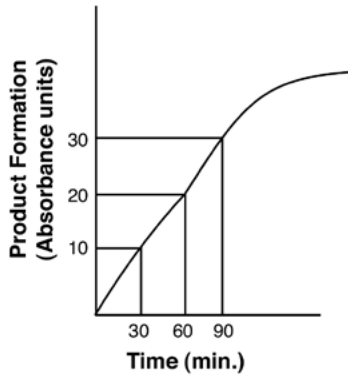


Figure 4

As the illustration of **Figure 4**:

$$\frac{\text{30 µmoles - 10 µmoles}}{\text{90 seconds - 30 seconds}} = \frac{20}{60} = \text{0.33 µmoles/sec}$$

There is no product formed at time 0; 10 µmoles have been formed after 30 seconds; 20 µmoles after 60 seconds; 30 µmoles after 90 seconds. For the initial period the rate of this reaction could be stated as 20 µmoles of product formed per minute. Typically, less additional µmoles of product are formed by the second, third and fourth minute **(Figure 4)**. For each successive minute after the initial 1.5 minutes, the amount of product formed is less than in the preceding minute.

MEASURING ENZYME ACTIVITY

In an enzyme catalyzed reaction, the **substrate (S)** is transformed to **product (P)**. Before the enzyme can transform the substrate it must first bind to it. Only a relatively small portion of the enzyme molecule is involved with substrate binding and catalysis. This region is called the **active site.** The active site contains the critical amino acid residues and, if applicable, **prosthetic groups** required for activity.

Initial binding is non-covalent and can be in rapid equilibrium. After productive binding has been achieved, the enzyme-substrate complex begins to generate product which is subsequently released. The **free enzyme (E)** can react with additional substrate and this reaction is repeated rapidly and effectively. The reaction is summarized using a single substrate and a single product in a non-reversible reaction:

$$\mathbf{E + S \rightarrow ES \rightarrow EP \rightarrow E + P}$$

Catalase is the enzyme used in this experiment and hydrogen peroxide is its substrate. Hydrogen peroxide is a toxic by-product of aerobic oxidation in intermediary metabolism. All aerobic life forms are capable of enzymatic peroxide detoxification. Catalase (H_2O_2:H_2O_2 oxidoreductase) catalyzes the rapid decomposition of hydrogen peroxide by the following reaction:

$$\mathbf{2\,H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2\ (gas)}$$

Catalase uses the hydrogen peroxide as a hydrogen acceptor and donor and the catalytic reaction is referred to as catalytic. Almost all the cell types in mammals contain catalase, with liver, kidney and erythrocytes being particularly rich sources.

Catalase has one of the highest catalytic turn over rates known. Over 3.6×10^7 molecules of hydrogen peroxide are converted to product by an enzyme molecule per minute.

In this experiment, a colorimetric assay will be used to measure the disappearance of substrate (hydrogen peroxide). It will measure the amount of hydrogen peroxide remaining after catalysis of H_2O_2 by catalase in a coupled secondary reaction with iodide (KI). As shown by the equation that follows:

$$\mathbf{2\,I^- + 2\,H^+ + H_2O_2 \rightarrow 2\,H_2O + I_2}$$

Catalase (enzyme) catalyzes the conversion of hydrogen peroxide to water and oxygen. To visualize the enzymatic time course reaction, equal volumes of the catalase incubation reaction mixture are transferred at various time points to an acidic solution of iodide (KI). In this acidic environment, catalase is denatured and the enzymatic reaction is terminated. In the second reaction, which is the chemical (non-enzymatic) reaction the remaining hydrogen peroxide converts Iodide (I^-) to iodine resulting in the generation of a brown-red color that is characteristic of iodine. Thus, the most hydrogen peroxide, the darker the brown color. During the enzymatic reaction, the remaining hydrogen peroxide (substrate) will decrease in the incubation reaction. As a consequence, the amount of brown color due to the generation of iodine (I_2) in the chemical reaction will also proportionally decrease.

Experiment Overview

EXPERIMENT OBJECTIVE

The objective is to understand enzyme catalysis. Students will perform an enzyme assay and determine the rate of a biochemical reaction.

WORKING HYPOTHESIS

If the disappearance of substrate can be measured as a function of time during a reaction, then a corresponding qualitative observation and/or quantitative determination of enzyme rate can be made.

IN THIS EXPERIMENT

- Catalase will be added to a buffered solution of hydrogen peroxide. A time course of the reaction will be obtained by removing aliquots from the reaction tube every 30 seconds.
- Aliquots removed during the time course reaction will be added to "assay solution for the remaining hydrogen peroxide" tubes that contain a catalase denaturing solution. To determine the amount of substrate remaining in the time course reaction the remaining hydrogen peroxide will be measured.
- Hydrogen peroxide that is not catalyzed by the enzyme catalase will oxidize iodide (I^-) in a coupled reaction to give the brown-red (Iodine) color. The color intensity increases with the amount of hydrogen peroxide remaining from the time course reaction. Therefore, in the enzymatic reaction as the incubation time (between substrate and enzyme) increases there will be a corresponding decrease in the amount of hydrogen peroxide and therefore a decrease in the intensity of the red-brown color.
- The color intensity will be quantified by the use of a spectrophotometer and the reaction rate will be determined.

LABORATORY SAFETY

Wear gloves and safety goggles

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Exercise caution when using any electrical equipment in the laboratory.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.

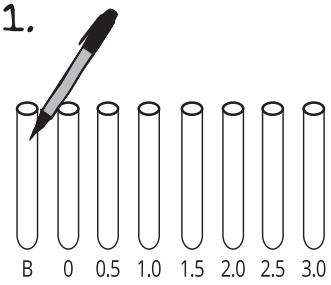
Experimental Procedures

The enzyme catalase catalyzes the decomposition of hydrogen peroxide (substrate) to water and oxygen gas (products). In this experiment:

- Catalase will be added to a buffered solution of hydrogen peroxide. A time course of the reaction will be obtained by removing aliquots from the reaction tube every 30 seconds.
- These aliquots will be added to separate tubes of assay solution. The assay solution contains KI which denatures the enzyme catalase, destroying its activity.
- The iodide (I^-) in the assay solution is oxidized by any remaining peroxide, producing a red-brown iodine (I_2) solution. The color intensity can be quantitated in the spectrophotometer and the rate of the reaction determined.
- The concentrations of peroxide and enzyme in the reaction are approximately 1.8 milliMolar and 5 nanoMolar respectively.

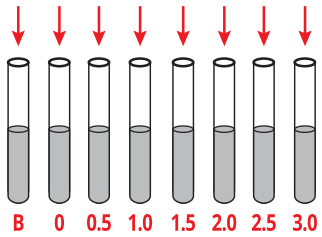
PREPARATION OF ASSAY TUBES

1.



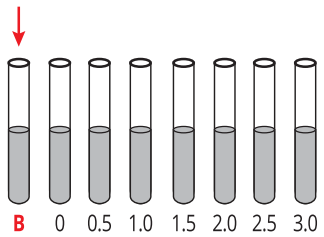
With a water resistant pen, **LABEL** 8 empty test tubes (at the top) to indicate blank and the various reaction times (0 to 3 minutes) as follows: B (blank), 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0.

2. 3 mL Assay Solution to each tube.



With a 5 mL pipet, **TRANSFER** 3 mL of assay solution to each of the tubes. **RINSE** pipet liberally with distilled water (about 200 mL). **SAVE** for Step 5.

3. 0.3 mL diluted buffer to "B".

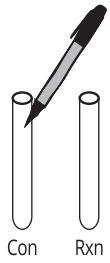


With a 1 mL pipet, **TRANSFER** 0.3 mL of diluted buffer to the blank. **MIX** by tapping or vortex. **SAVE** pipet and use in Step 6.

Experimental Procedures, continued

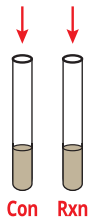
PREPARATION OF CONTROL AND REACTION TUBES

4.



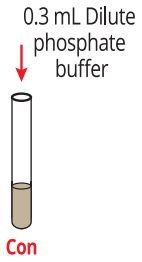
LABEL one of two remaining test tubes "Con" (for control) and the other "Rxn" (for reaction).

5. 1.8 mL Enzyme Rxn Cocktail



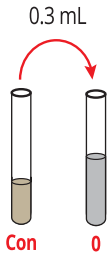
With the rinsed 5 mL pipet, **DISPENSE** 1.8 mL of Enzyme Reaction Cocktail to each of the two tubes.

6.



With the 1 mL pipet, **ADD** 0.3 mL of dilute phosphate buffer to the control tube. **SAVE** the pipet for Step 7.

7.

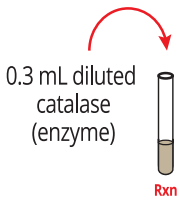


REMOVE 0.3 mL of liquid from the control tube and **ADD** to the assay tube labeled "0". Set the "Con" tube aside and continue with the "Rxn" tube. **DISCARD** pipet.

Next, the enzyme will be added. Hydrogen peroxide that is not catalyzed by the enzyme catalase will oxidize iodide to give a brown-red color. Therefore, the more peroxide in the reaction, the darker the color.

PREPARATION AND MONITORING OF TIMED REACTIONS

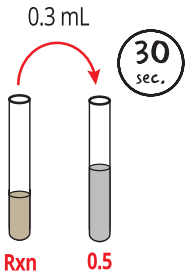
8.



With a **FRESH** 1 mL pipet, **ADD** 0.3 mL of diluted catalase (Enzyme) to the tube labeled "Rxn" and **MIX**. **START** timer, or note the second hand on the clock or watch. **RETAIN** pipet to take samples. **RINSE** pipet liberally with distilled water (about 200 mL) after adding catalase.

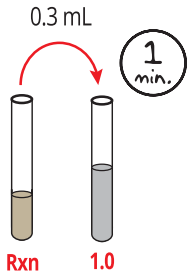
NOTE: Upon addition of enzyme catalase, the reaction will begin. Start timing the reaction immediately and aliquot 0.3 mL of this mixture to tubes labeled 0.5, 1.0, 1.5 and 2.0, 2.5, and 3.0 at 30 second intervals.

9.



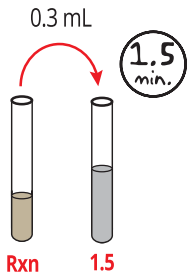
With the 1 mL pipet, **REMOVE** 0.3 mL from Rxn tube. At 0.5 minute (30 seconds), **ADD** it to tube 0.5. Mix.

10.



With the 1 mL pipet, **REMOVE** 0.3 mL from Rxn tube. At 1.0 minute, **ADD** it to tube 1. **MIX**.

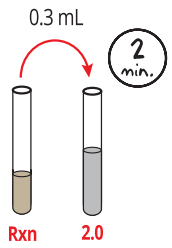
11.



With the 1 mL pipet, **REMOVE** 0.3 mL from Rxn tube. At 1.5 minutes, **ADD** it to tube 1.5. **MIX**.

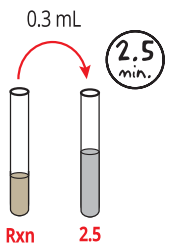
Experimental Procedures, continued

12.



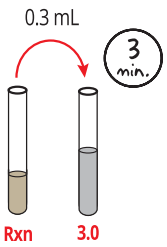
With the 1 mL pipet, **REMOVE** 0.3 mL from Rxn tube. At 2.0 minutes, **ADD** it to tube 2.0. **MIX**.

13.



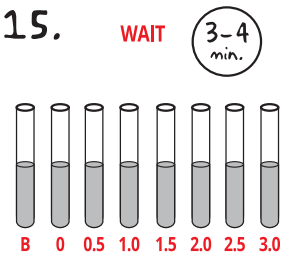
With the 1 mL pipet, **REMOVE** 0.3 mL from Rxn tube. At 2.5 minutes, **ADD** it to tube 2.5. **MIX**.

14.



With the 1 mL pipet, **REMOVE** 0.3 mL from Rxn tube. At 3.0 minutes, **ADD** it to tube 3.0. **MIX**.

15.



WAIT 3 - 4 minutes after your last time point to allow full color development.

Experimental Procedures, continued

DATA COLLECTION

16. Spectral readings can now be taken. Depending on the spectrophotometer, you may be able to **INSERT** your test tubes directly into the instrument. Otherwise **TRANSFER** the entire enzyme reactions in tubes provided by your instructor starting with 0 to 3.0.

17. **ZERO** the instrument with the tube "B" solution (Blank) according to your instructor's directions. Be sure the instrument is **SET at 500 nm wavelength**. The instrument should **READ 0 absorbance** with the blank solution (no color).

18. **REMOVE** the blank and record the absorbancy values for each solution in tubes 0 to 3.0. **RECORD** the results in Table 1, below. If you are using an instrument such as a Spec 20, it should take approximately two to three minutes to complete your readings.

TABLE 1

TIME *(min.)*	Assay Solution	Diluted Buffer	Volume Con	Volume Rxn	A_{500}
Blank	3 mL	0.3 mL	---	---	
0	3 mL	---	0.3 mL	---	
0.5	3 mL	---	---	0.3 mL	
1.0	3 mL	---	---	0.3 mL	
1.5	3 mL	---	---	0.3 mL	
2.0	3 mL	---	---	0.3 mL	
2.5	3 mL	---	---	0.3 mL	
3.0	3 mL	---	---	0.3 mL	

Enzyme Data Analysis

The reaction rate can be obtained by graphing the absorbancy data *versus* time. However, the rate can also be expressed in terms of substrate consumed.

1. To express your data in terms of molar concentration of peroxide:

$$\frac{\text{Absorbance}}{\varepsilon} \times 11 = \text{Molarity of hydrogen peroxide in Rxn tube.}$$

   ε, the extinction coefficient for this assay system, is 5.8×10^3. **The molar concentration of either a decrease in substrate or increase in the product can be obtained by dividing the absorbance at A_{500} by the molar extinction coefficient.** Multiplication by 11 (dilution factor) gives the peroxide concentration in the reaction tube. Scientific notation will make the calculations more convenient.

2. **GRAPH** the peroxide concentration on the y-axis *versus* time on the x-axis provided.

3. **DRAW** the best straight line through the data points. You may notice some curvature to the data points. This is normal, especially between 0 and the first time point, and between later time points. You are making a linear approximation.

4. **DETERMINE** the rate of change in the molarity of hydrogen peroxide with time. The rate is equivalent to the slope of the line. Pick a time, go vertically up to the line, then horizontally to the y-axis. **DETERMINE** the concentration in this way for the next time point.

$$\text{rate} = \frac{[\text{peroxide}_2 - \text{peroxide}_1]}{[\text{time}_2 - \text{time}_1]}$$

Express the rate as molarity change per minute.

Study Questions

1. Why did you observe bubbles in the experiment? Assume you had boiled the enzyme solution before adding it to the peroxide. Would you expect to see bubbles? What gas do the bubbles contain?

2. Why did the color intensity of your peroxide assays decrease with time?

3. What makes the rate of a reaction of an enzymatic reaction decrease?

4. An active preparation of catalase was exposed to the proteolytic enzyme, trypsin. The catalase preparation was found to be inactive when it was re-assayed. Why?

5. The velocity of a catalase reaction was found to increase with increasing hydrogen peroxide concentrations as expected. However, at high peroxide concentrations, the reaction rate decreased and eventually went to zero. What could explain this observation?

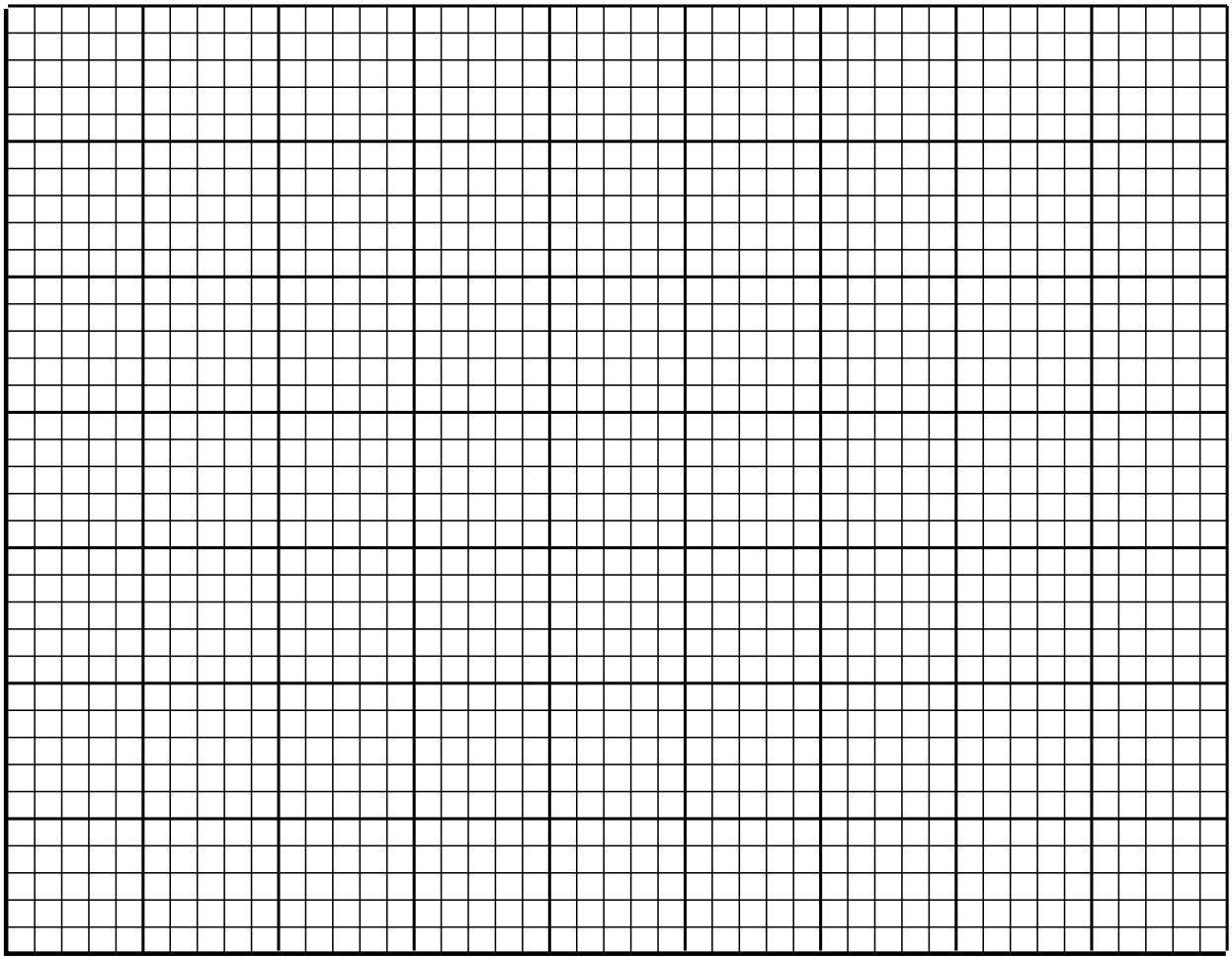
6. Graph the data obtained from the enzyme-catalase H_2O_2 decomposition by plotting the amount of H_2O_2 used as a function of time. See graph on page 15.

Study Questions, continued

a. Use the horizontal (x) axis for the independent variable. Label as: Time (min.)

b. Use the vertical (y) axis for the dependent variable. Label as: Peroxide concentration.

c. Graph : Title_____



Instructor's Guide

OVERVIEW OF LABORATORY INVESTIGATIONS

The "hands-on" laboratory experience is a very important component of the science courses. Laboratory experiment activities allow students to identify assumptions, use critical and logical thinking, and consider alternative explanations, as well as help apply themes and concepts to biological processes.

EDVOTEK experiments have been designed to provide students the opportunity to learn very important concepts and techniques used by scientists in laboratories conducting biotechnology research. Some of the experimental procedures may have been modified or adapted to minimize equipment requirements and to emphasize safety in the classroom, but do not compromise the educational experience for the student. The experiments have been tested repeatedly to maximize a successful transition from the laboratory to the classroom setting. Furthermore, the experiments allow teachers and students the flexibility to further modify and adapt procedures for laboratory extensions or alternative inquiry-based investigations.

ORGANIZING AND IMPLEMENTING THE EXPERIMENT

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances.

If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK web site: **www.edvotek.com**

In addition, Technical Service is available from 8:00 am to 5:00 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

Safety Data Sheets can be found on our website: **www.edvotek.com/safety-data-sheets**

Pre-Lab Preparations

STANDARDIZATION OF THE SPECTROPHOTOMETER

Differences in light paths, cuvette pathlengths, bulb intensities and other parameters can cause absorbancy values for the same solution to vary from one instrument to another. The spectrophotometer will require standardization and an evaluation to see if the assay color intensities fall in the useful reading range of your instrument (generally 0.1 to 1.0 absorbancy units). In order to obtain accurate results it is important that the instrument be maintained and be properly calibrated to the manufacturer's specifications. **Review the Operating Instructions**.

1. **ALLOW** the spectrophotometer to warm up for one half hour. **SET** the wavelength to 500 nanometers.

2. **LABEL** 6 clean 13 x 100 mm (10 mL) test tubes. Near the tops of the tubes, **LABEL** one of them 'Blank' and the others 1 through 5.

3. To a clean, dry 100 mL beaker or flask:
   - **ADD** 15 mL of distilled water .
   - Wearing gloves and using a FRESH pipet for each solution, **ADD** 5 mL each of potassium iodide (D), 0.1 M HCl (E), color enhancer (F) and color developer (G) to the water.
   - **MIX**.
   - **LABEL** the vessel "Assay Solution" and time made.
   - Keep cool and in the dark. **USE** within 1 hour.

4. To a clean, dry 50 mL beaker or flask:
   - **ADD** 19 mL of distilled water
   - **ADD** 1 mL of phosphate buffer, 20X (C).
   - **MIX**.
   - **LABEL** the beaker "Diluted Phosphate Buffer".

5. To a clean dry 15 mL tube:
   - **TRANSFER** 9 mL of diluted phosphate buffer
   - **ADD** 1 mL of 1.2% hydrogen peroxide (B) to the buffer.
   - **MIX**.
   - **LABEL** the beaker "0.12% peroxide". Set the pipet aside.

6. To a clean, dry 50 mL beaker or flask:
   - **ADD** 40 mL of distilled water.
   - With a FRESH 1 mL pipet, **ADD** 0.5 mL of the 0.12% peroxide solution.
   - **MIX**.
   - **LABEL** the beaker "600 mM Peroxide" and time made. **USE** within 1 hour.

TABLE 2

Tube Solution	Assay	Diluted Phosphate Buffer	600 mM Peroxide
Blank	2 mL	1 mL	0 mL
1	2 mL	0 mL	1.0 mL
2	2 mL	0.2 mL	0.8 mL
3	2 mL	0.5 mL	0.5 mL
4	2 mL	0.7 mL	0.3 mL
5	2 mL	0.8 mL	0.2 mL

7. Using a FRESH pipet, add prepared solutions (see **Table 2**).

8. **MIX** each tube and let the color develop for 5 minutes. The final volume in all tubes should be 3 mL. The color is stable for one hour. **PROCEED** with Step 9.

Pre-Lab Preparations

STANDARDIZATION OF THE SPECTROPHOTOMETER, CONTINUED

Several days before the lab, continued:

9. The Spectronic 20 is first adjusted to read 0% transmittance WITHOUT a tube in the sample holder, which should be closed.
   - **TURN** the left knob (power switch - zero control) until the instrument reads 0% transmittance.
   - **PLACE** the blank solution into the instrument.
   - **ADJUST** the instrument to read 0 absorbance by turning the right knob (transmittance/absorbance control).
10. **OBTAIN** absorbancy readings for the solutions in tubes 1-5.

    The absorbances should be **approximately** in the range of 1.0 to 0.1. **DISCARD** a point if it is 20% or greater outside this range.
    Generally, Tube 1 values are 1.1 to 0.95, depending on the instrument and allowing for pipetting error.
11. **REPEAT** the standardization procedure Steps 2, 7, 8, 9 and 10 one more time to obtain average absorbance values. If you wish to do the experiment using triplicate samples, there are enough reagents to do so and accommodate the students' laboratory. A third standardization will require more assay solution (Step 3).
12. **AVERAGE** your absorbance data for each solution.

Pre-Lab Preparations

Graphing

1. On linear graph paper, **PLOT** the average absorbance on the y-axis *versus* the peroxide concentration on the x-axis.

2. **DRAW** the best straight line through the data points. The slope of the line is the molar extinction coefficient for iodine in this assay system on your spectrophotometer. The coefficient is a proportionality constant for an absorbing species under specific conditions of pH, solvent, wave length, etc.

3. **DETERMINE** the slope of the line over a middle section, such as 5×10^{-4} to 1×10^{-4} M peroxide, by dividing the difference in absorbance values by the difference in concentrations.

   As an example, assume the standard graphed line gave absorbancy readings at 5×10^{-5} and 1×10^{-4} M peroxide of 0.28 and 0.57 respectively. The slope would be:

$$\varepsilon = \frac{0.57 - 0.28}{1 \times 10^{-4}\text{M} - 5 \times 10^{-5}\text{M}} = \frac{0.29}{5 \times 10^{-5}\text{M}} = 5800 = 5.8 \times 10^{3}$$

   The extinction coefficient (ε) is a constant for an absorbing species that is measured under specific reaction conditions such as buffer, pH, temperature. These values are obtained experimentally. The molar concentration of either a decrease in substrate or increase in the product can be obtained by dividing the absorbance at A500 by the molar extinction coefficient.

If your spectrophotometer is a Spectronic 20, or similar model, you may place the tubes directly into the instrument for reading. Transfer to special cuvettes for the Spec 20 is not required. Otherwise, you will have to transfer the appropriate volumes to cuvettes (assumed to have a 1 cm pathlength). The pathlength is the distance the light beam has to travel through the cuvette. Zero baseline adjustments for double beam spectrophotometers require the blank solution in the reference and sample cuvettes.

TABLE 3

TUBE	TRIAL 1	TRIAL 2	AVERAGE	[PEROXIDE]
1				1.95×10^{-4} M
2				1.56×10^{-4} M
3				9.8×10^{-5} M
4				5.9×10^{-5} M
5				3.9×10^{-5} M

Pre-Lab Preparations

ON THE DAY OF THE LAB, PREPARE THE FOLLOWING:

1. Dilute Phosphate Buffer pH 7.2 (1x)

a. **POUR** 190 mL of distilled water into a clean 250 mL beaker.
b. **TRANSFER** 10 mL of phosphate buffer concentrate (C) to the beaker containing the water.
c. **MIX**.
d. **LABEL** the vessel "Dilute Phosphate Buffer".
e. **STORE** on ice or in the refrigerator. Each group requires 1 mL.

2. Peroxide Substrate (0.12% Hydrogen Peroxide)

a. **TRANSFER** 27 mL diluted phosphate buffer to clean 50 mL beaker.
b. Using a 5 mL pipet, **ADD** exactly 3 ml of 1.2% peroxide (B) to the 27 mL of buffer.
c. **MIX**.
d. **LABEL** the vessel "0.12% Peroxide".
e. Let the diluted peroxide **COOL** on ice or in the refrigerator in the dark.

WITHIN 30 MINUTES OF THE LAB, PREPARE THE FOLLOWING:

3. Potassium Iodide Solution (Assay Solution)

a. **ADD** 150 mL of distilled water to a clean 300 mL beaker.
b. **ADD** 25 mL of potassium iodide solution (D).
c. **ADD** 25 mL of the 0.1 M HCl solution (E).
d. **ADD** 25 mL of the enhancer solution (F).
e. **ADD** 25 mL of the developer solution (G).
f. **MIX**.
g. **LABEL** the vessel "Assay Solution".
h. **STORE** in the dark, on ice or in the refrigerator. Each group requires 25 mL.

NOTE:
If the assay solution is prepared too far in advance it will begin to yellow due to oxidation of the iodide. The yellowing will create higher blank values for the spectrophotometer.

4. Enzyme Reaction Cocktail (Buffered Substrate)

a. **ADD** 60 mL of diluted 1x phosphate buffer to a 100 mL beaker (from Step 1).
b. Using a washed or a fresh 5 mL pipet, **ADD** 3 mL of 0.12% peroxide to the buffer (from Step 2).
c. **MIX**.
d. **LABEL** the vessel "Reaction Cocktail". Each group will need 6 mL.

Pre-Lab Preparations

WITHIN 20 MINUTES OF THE LAB, PREPARE THE FOLLOWING:

5. Catalase Enzyme

a. With a pipet or graduate cylinder, **TRANSFER** 13 mL of ice cold, diluted 1x phosphate buffer to the 15 mL plastic tube provided.
b. **LABEL** "Enzyme". **KEEP** the tube on ice.
c. Carefully **ADD** 0.2 mL of catalase stock (A) to the buffer. The enzyme solution is viscous. **RINSE** the pipet in the buffer to remove residual enzyme.
d. **CAP** the tube and gently **MIX** by inverting so that no enzyme solution remains on the bottom.
e. **USE** within 20 to 30 minutes. Each group requires 1 mL.

6. Spectrophotometer

A well maintained and calibrated spectrophotometer will give good results for your lab activities. If your spectrophotometer requires 13 x 100 mm test tubes and a 3 mL sample volume, the assay solutions generated by students will be ready to read directly. If your instrument requires cuvettes (assumed to have a 1 cm pathlength), you will have to instruct your students to transfer their developed assay solutions to cuvettes.

a. **ALLOW** the spectrophotometer to warm up one half hour before the lab.
b. **SET** the wavelength at 500 nm. Absorbance will be measured. Color is stable for approximately 1 hour.

EACH LAB GROUP REQUIRES:

6 mL	"Reaction cocktail"
25 mL	"Assay solution" (Do not to contaminate with vessels or pipets that contained peroxide while dispensing)
1 mL	"Enzyme" on ice (Diluted catalase)
1 mL	"Dilute phosphate buffer"
1	Permanent marker to label tubes
10	13 x 100 mm, clean test tubes
2	1 mL pipets (0.1 mL divisions)
1	5 mL pipets (0.1 mL divisions)
1	Half a liter of distilled water in several clean beakers for washing pipets.

Experiment Results and Analysis

Absorbancy reading should decrease with time. The pipetting accuracy and the spectrophotometer will affect observed reaction velocities. The results of representative experiment are presented below:

MINUTES	ABSORBANCE*	MOLARITY**
0	1.087	2.06×10^{-3}
0.5	0.915	1.74×10^{-3}
1.0	0.811	1.54×10^{-3}
1.5	0.693	1.31×10^{-3}
2.0	0.594	1.13×10^{-3}

* Determined at 500 nm with cuvettes of 1 cm pathlength.
** This represents the Absorbance divided by the molar extinction coefficient multiplied by 11.
Rate between 0.5 and 1 min. is 4×10^{-4} Molar/min.

INSTRUCTOR NOTES

Catalase, the enzyme catalyzes the conversion of hydrogen peroxide to water and oxygen. In this coupled reaction, the molar concentrations of peroxide (substrate) consumed and I_2, the product from the non enzymatic reaction are equimolar. To visualize the enzymatic reaction, equal samples of the catalase incubation reaction are transferred at various time points to an acidic solution of potassium iodide (KI). In the acidic environment catalase is denatured and the enzymatic reaction stops.

In the second reaction, which is a non-enzymatic chemical reaction, the remaining hydrogen peroxide is used at each of the time points to convert the Iodide (I^-) to iodine (I_2). Over the time course of the enzymatic reaction, the amount of the substrate (hydrogen peroxide) will decrease and consequently the brown-red color in the chemical reaction will also decrease corresponding to the amount of iodine (I_2) generated.

Please refer to the kit insert for the Answers to Study Questions