EDVOTEK®

AP BIOLOGY BIG IDEA 4 INVESTIGATION 13

EDVO-Kit: AP13

Enzyme Activity

See Page 3 for storage instructions.

EXPERIMENT OBJECTIVE:

The objective of this experiment is to introduce students to the concept of enzyme catalysis. Students will study the effect of various factors on the rate of reaction between an enzyme and its substrate. Students also learn to determine the optimal conditions under which the enzyme activity is maximized.

Experiment Components

A Hydrogen peroxide solution
B Guaiacol solution
C Phosphate buffer pH 3
D Phosphate buffer pH 7
E Phosphate buffer pH 10
F Phosphate buffer pH 14

Store entire experiment in the refrigerator.

This experiment is designed for 10 lab groups.

Requirements

- Turnip root
- Distilled or deionized water
- Serological pipets (2 ml and 5 ml)
- Pipet pumps or bulbs
- Erlenmeyer flask, 500 ml
- Spectrophotometer
- Water baths (for 4°, 37°, 60°, and 100° C)
- Filter paper and funnel
- Test tube racks
- Test tubes
- Thermometer
- Cheesecloth
- Parafilm
- Hot plate
- Timer or clock with second hand
- Lab permanent markers
- Ice
- Razor
- Goggles
- Blender

Background Information

Enzymes as Biological Catalysts

A biological **catalyst** is used in trace amounts and accelerates the rate of a biochemical reaction without being consumed or transformed during the reaction. The equilibrium constant of reactions are not altered by catalysts. Only the rate of approach to equilibrium is changed.

Reactions in cells are catalyzed by biological catalysts known as **enzymes,** which can accelerate reactions by as much as 10^{14} to 10^{20} times. Enzymes function best under mild physiological conditions of neutral pH, and temperatures of 37° C. Enzymes are generally very specific for the reactions they catalyze. Certain enzymes are regulated by intracellular concentrations of key metabolites that are not directly involved with the reaction they catalyze. This regulation (increase or decrease for an enzyme activity) is often regulated by a cell's physiological requirements at a given time. Enzymes that are regulated in this way are termed **allosteric**.

In 1897, Eduord Buchner demonstrated yeast cell free extracts to catalyze the fermentation of sugar to produce alcohol. In 1926, J.B. Sumner demonstrated that an enzyme was a protein. 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 enzyme activities are known to have diverse and complex structures. There are however certain common structural features that are shared by all proteins.

Conformation of Proteins

Proteins consist of specific sequences of amino acid residues linked to each other by peptide bonds. The sequence of residues in a polypeptide chain is called **primary structure**. The unique amino acid sequences is the most important feature of primary structures. 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 variety 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

Background Information

which enable the protein to bind and react with 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.

Biological Activity of Enzymes

Certain proteins contain, as integral parts 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 metal ions. Such moieties are called **prosthetic groups**. A well known prosthetic group is **heme,** which consists of an iron atom coordinated with nitrogen moieties that are part of complex organic ring compounds called **porphyrin.**

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 dodecylsulfate, 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.

Measuring Enzyme Activity

The reactant molecule in an enzyme catalyzed reaction is called the **substrate**. 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, the **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, single product in a non-reversible reaction:

$$E + S \rightarrow ES \rightarrow EP \rightarrow E + P$$

Background Information

Peroxidase is the enzyme used in this experiment.

Enzyme + Substrate → Enzyme - Substrate Complex → Enzyme + Products + ΔG

For this investigation the specific reaction is as follows:

Peroxidase + Hydrogen Peroxide → Complex → Peroxidase + Water + Oxygen

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

The appearance of product (P) or the disappearance of substrate (S) can be measured as a function of time during a reaction. One can measure the amount of product formed or the decrease in substrate at regular intervals (in this experiment at 30-sec intervals). This quantity can be plotted as a graph. Typical results are shown in Figure 1 which demonstrate the **rate of an enzymatic reaction.**

An enzymatic reaction measurement is referred to as an **assay**. At a fixed concentration and reaction conditions, an enzyme reaction rate can increase by higher substrate concentrations. The probability of forming ES complex increases with more substrate molecules present. Generally, the substrate concentration is thousands of times greater than the enzyme concentration for *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.

$$[S]_1 - [S]_2$$

$$T_1 - T_2$$

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 1)**. Within short 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.

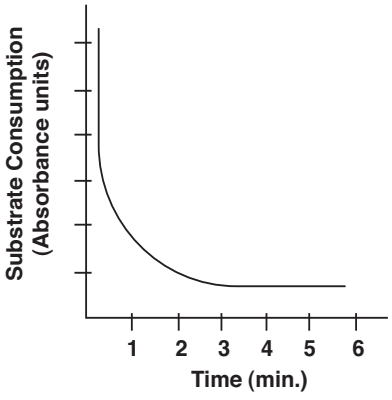


Figure 1

Experiment Procedure

Background Information

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 2)**. 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.

$$[P]_2 - [P]_1$$

$$T_2 - T_1$$

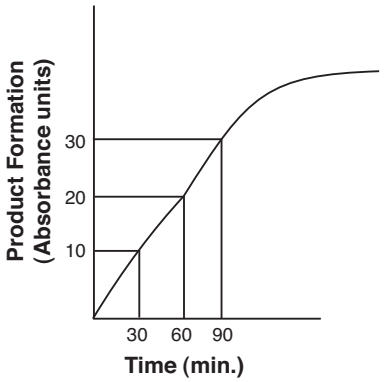


Figure 2

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 2)**. For each successive minute after the initial 1.5 minutes, the amount of product formed is less than in the preceding minute.

As the illustration of Figure 2:

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

In this experiment, an indicator for oxygen will be used. The compound Guaiacol has a high affinity for oxygen. When in solution, Guaiacol binds instantly with oxygen to form tetraguaiacol, turning the product into a brownish solution. The greater the amount of oxygen produced, the darker brown the product solution will become.

Baseline is a line that is used as a point of reference. In this investigation the term "baseline" is used to establish a standard for a reaction. Thus when manipulating components of a reaction (enzyme concentration, pH, temperature), students will use the baseline as their reference to help understand what occurred in the reaction.

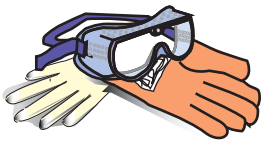
Experiment Overview and General Instructions

EXPERIMENT OBJECTIVE

The objective of this experiment is to introduce students to the concept of enzyme catalysis. Students will study the effect of various factors on the rate of reaction between an enzyme and its substrate. Students also learn to determine the optimal ranges under which the enzyme activity is maximized.

EXPERIMENT OVERVIEW

In this experiment, students will first develop a method for measuring peroxidase in turnips and determine a baseline. Then they will study the effect of various factors such as enzyme concentration, pH, and temperature on the activity of the turnip peroxidase enzyme.



LABORATORY SAFETY GUIDELINES

1. Always wear gloves and goggles when working in the laboratory.
2. Exercise caution when working in the laboratory as you will be using equipment that can be dangerous if used incorrectly.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Always wash hands thoroughly with soap and water after working in the laboratory.
5. If you are unsure of something, ASK YOUR INSTRUCTOR!

LABORATORY NOTEBOOKS

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you will be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

- Record your observations.

After the Experiment:

- Interpret the results - does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

Experimental Procedures

A few notes before the start of the experiment:

- In this experiment, students will be graphing various charts to demonstrate the effect of different abiotic and biotic factors on enzyme activity of Peroxidase. You may do this by hand, using the graph paper provided, or on a spreadsheet program such as Excel.
- In Investigation I, students will construct a baseline activity of Turnip Peroxidase, and then compare this baseline activity with enzyme activity under different conditions in Investigations II and III.

Investigation I: Determining a Baseline by Measuring Peroxidase in Turnips

A. SETTING UP THE REACTIONS

1. Turn on the spectrophotometer and adjust the Absorbance to 500 nm. Allow the spectrophotometer to warm up for approximately 15 minutes.
2. Using a permanent marker, label one test tube "S" (Substrate) and label the other test tube "E" (Enzyme). Also label one additional test tube as "B" (Blank).
3. Add the following components to the tubes as summarized in Chart 1. Use a FRESH micropipet tip for each transfer of the chemicals. Cover the test tubes and gently mix well.

Chart 1 – Summary of Substrate and Enzyme Tubes

Tube	Hydrogen Peroxide solution	Guaiacol solution	Turnip Peroxidase	Distilled or deionized water	Final Volume
S (Substrate)	0.15 ml	0.1 ml	------	3.5 ml	3.75 ml
E (Enzyme)	-----	-----	0.75 ml	3.0 ml	3.75 ml
Blank	-----	------	------	7.5 ml	7.5 ml

Investigation I: Determining a Baseline by Measuring Peroxidase in Turnips

B. DATA COLLECTION

1. Be sure the spectrophotometer is set at 500 nm. 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 appropriate sized tubes provided by your instructor.

2. Zero the instrument with the tube B solution (Blank) according to your instructor's directions. The instrument should read 0 absorbance with the blank solution (no color).

3. Remove the blank.

4. When ready to begin a reaction, carefully combine the contents of tube S and tube E. Cover the test tube with a piece of Parafilm. Quickly invert to mix well.

5. Place the test tube in the spectrophotometer cuvette holder, and immediately start timing.

6. Measure and record the absorbance every 30 seconds for the next 5 minutes in Table 1 below.

   - If you feel comfortable removing the tube from the spectrophotometer cell holder and putting it back before the 30 second interval elapses, do so and observe the color change within the 5 minute period.
   - Rotate the tube before each reading. Record the observed color at the end of every 30 second interval.
   - A cell phone and/or camera are excellent ways to record color change.

7. Repeat the above steps to determine the absorbance of different enzyme concentrations (1/2 X and/or 2X) on peroxidase activity.

8. Record the results in the table.

Time (sec)	Absorbance ½ X Enzyme	Absorbance 1X Enzyme	Absorbance 2X Enzyme
0			
30			
60			
90			
120			
150			
180			
210			
240			
270			
300			

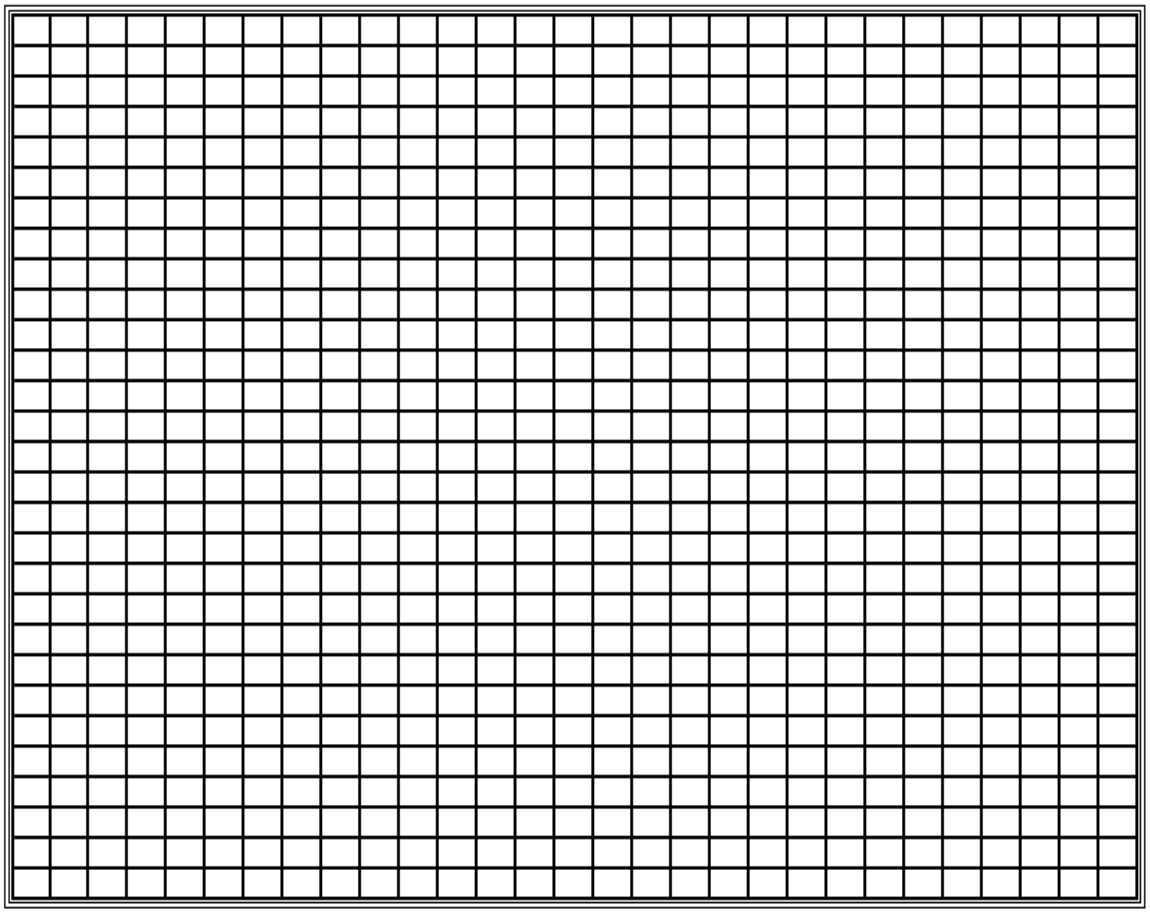
Table 1. Absorbance from turnip peroxidase baseline.

Investigation I: Determining a Baseline by Measuring Peroxidase in Turnips

C. GRAPHING AND CALCULATING THE REACTION RATE

1. You should have three sets of "absorbance vs. time" data from Investigation I, one from each enzyme concentration.

2. Plot the data from three sets on the same graph using the graph paper provided below. Extrapolate a straight line through the linear portion of the reaction curve.



3. Next, calculate the slopes of each line to determine the enzyme activity. The slope of the linear portions of the curves is a measure of enzymatic activity.

Question 1: Based on the data obtained from the graph, determine which concentration of enzyme is most suitable for the analysis. Explain.

Enzyme Activity—Opportunities for Inquiry

- Investigations II and III are suggestions for the student-directed lab activities. Students investigate the effects of pH and temperature on the rate of the peroxidase-catalyzed reaction.

- Students are encouraged to conduct several trials to determine the enzymatic activity of Peroxidase in response to such environmental conditions. Analyze and graph the result for each trial.

Investigation II: Determining the Effect of pH on Enzymatic Activity

A. SETTING UP THE REACTIONS:

1. Using a permanent marker, label four pairs of tubes with each pair containing the different pH buffers, as follows:

   Set 1: pH3 "S" and pH3 "E"
   Set 2: pH7 "S" and pH7 "E"
   Set 3: pH10 "S" and pH10 "E"
   Set 4: pH14 "S" and pH14 "E"

2. Add the following components to the tubes as summarized in Chart 2. Use a FRESH micropipet tip for each transfer of the chemicals. Cover the test tubes and gently mix well.

   Note: Add the different pH buffer to the four "E" tubes. Do not add the different pH buffer to the four "S" (substrate) tubes!

Chart 2 – Summary of Substrate and Enzyme Tubes with Different pH Buffers

Tube	Hydrogen Peroxide solution	Guaiacol solution	Turnip Peroxidase	pH Buffer (various pH)	Distilled or deionized water	Final Volume
pH 3 "S"	0.15 ml	0.1 ml	------	------	3.5 ml	3.75 ml
pH 3 "E"	-----	-----	0.75 ml	3.0 ml (pH 3)	----	3.75 ml
pH 7 "S"	0.15 ml	0.1 ml	------	------	3.5 ml	3.75 ml
pH 7 "E"	-----	-----	0.75 ml	3.0 ml (pH 7)	----	3.75 ml
pH 10 "S"	0.15 ml	0.1 ml	------	------	3.5 ml	3.75 ml
pH 10 "E"	-----	-----	0.75 ml	3.0 ml (pH 10)	----	3.75 ml
pH 14 "S"	0.15 ml	0.1 ml	------	------	3.5 ml	3.75 ml
pH 14 "E"	-----	-----	0.75 ml	3.0 ml (pH 14)	----	3.75 ml

Investigation II: Determining the Effect of pH on Enzymatic Activity

B. DATA COLLECTION

1. Remember to blank the spectrophotometer with the blank solution at 500 nm. The instrument should read 0 absorbance. No color change should occur. Remove the blank.

2. When ready to begin the reaction, carefully combine the contents of tube S and tube E containing pH 3 buffer. Cover the test tube with a piece of Parafilm. Quickly invert to mix well.

3. Place the test tube in the spectrophotometer cuvette holder, and immediately start timing.

4. Measure and record the absorbance every 30 seconds for the next 5 minutes in Table 2 below.

   Note:
   - *If you feel comfortable removing the tube from the spectrophotometer cell holder and putting it back before the 30 second interval elapses, do so and observe the color change within the 5-minute period.*
   - *Rotate the tube before each reading. Record the observed color at the end of every 30-second intervals.*
   - *A cell phone and/or camera are excellent ways to record color change.*

5. Repeat the above steps for the remaining 3 pairs of tubes containing buffers at pH 7, pH 10, and pH 14.

6. Record the results in the table.

Time (sec)	Absorbance at **pH 3**	Absorbance at **pH 7**	Absorbance at **pH 10**	Absorbance at **pH 14**
0				
30				
60				
90				
120				
150				
180				
210				
240				
270				
300				

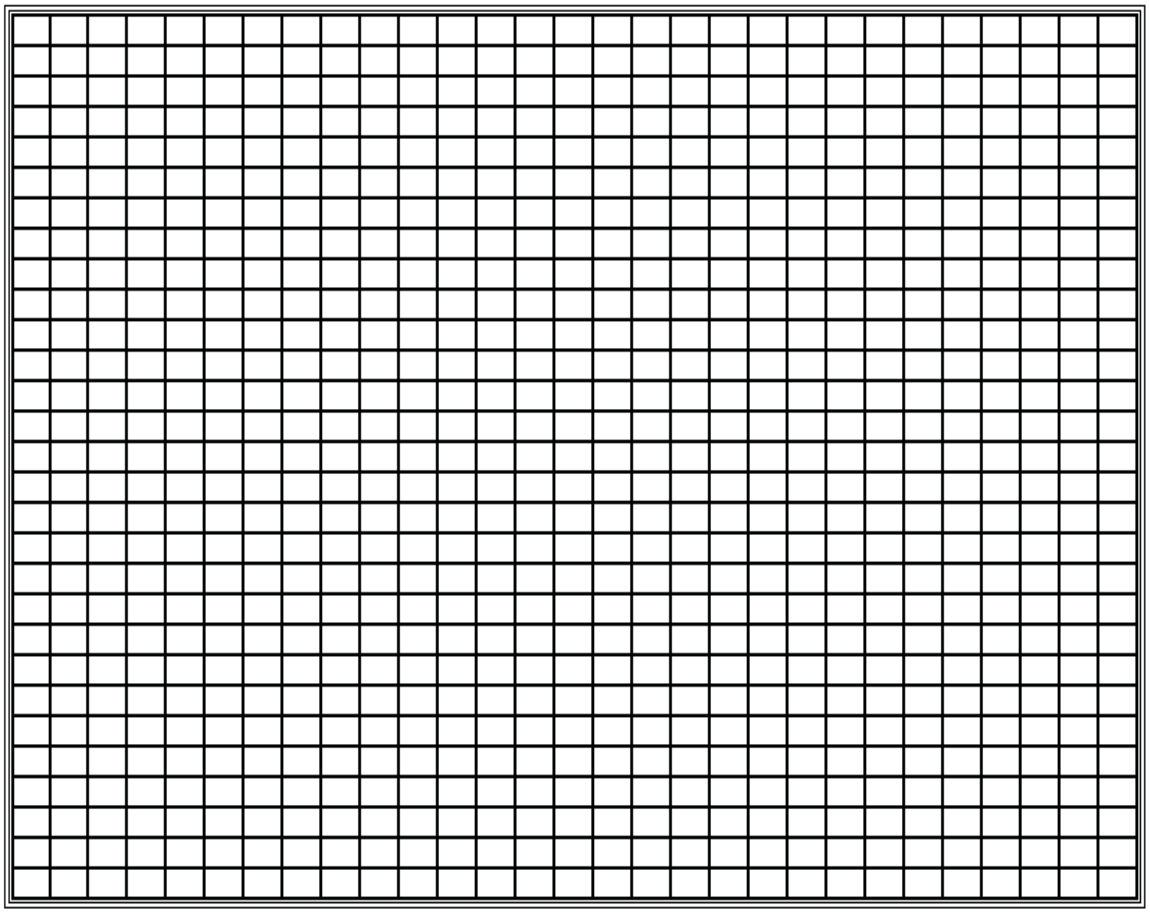
Table 2. Absorbance at different pH.

Investigation II: Determining the Effect of pH on Enzymatic Activity

C. GRAPHING THE ABSORBANCE AT DIFFERENT pH

1. You should have four sets of "absorbance vs. time" data from Investigation II, one from each pH.

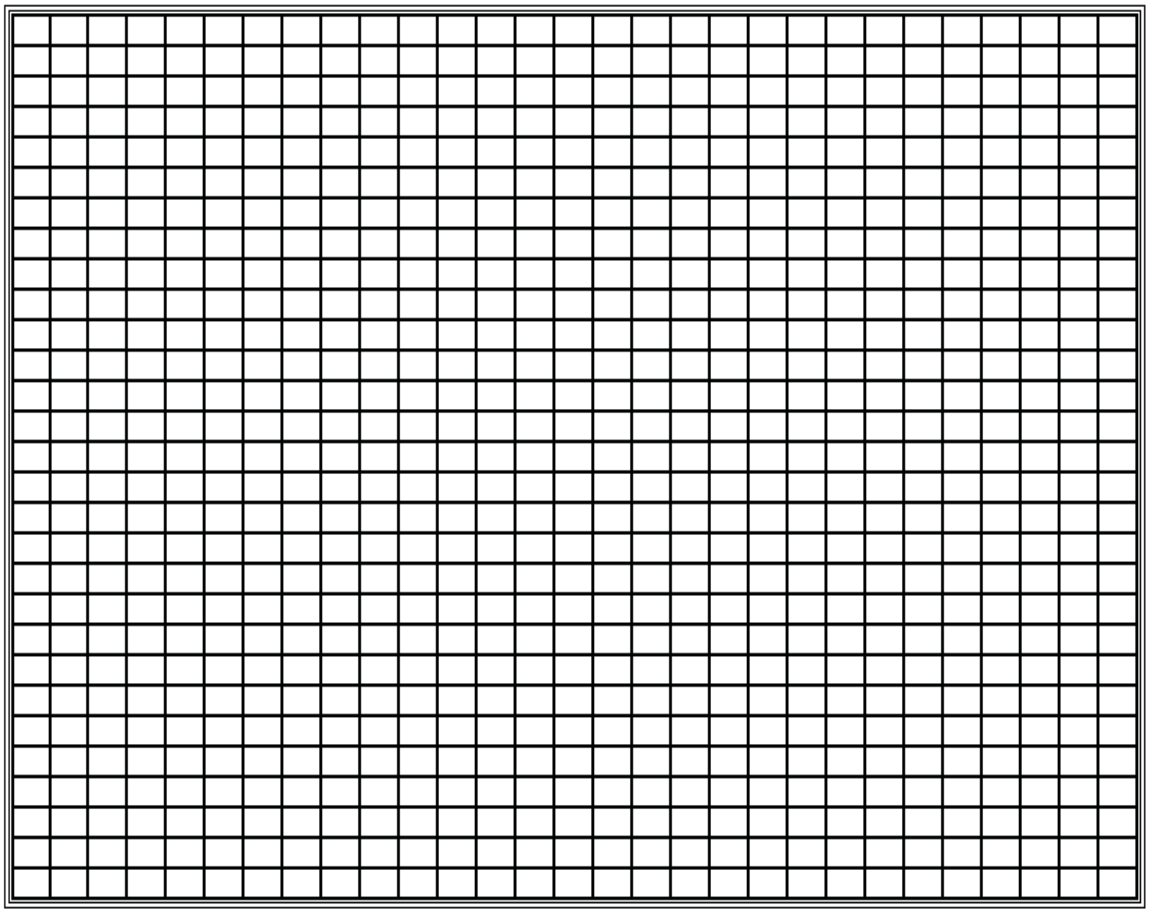
2. Plot the data from four sets on the same graph using the graph paper provided below.



3. Next, calculate the slopes of each line to determine the enzyme activity.

Investigation II: Determining the Effect of pH on Enzymatic Activity

4. Plot another graph using the graph paper below to show correlation between enzyme activity and pH. (You should have four points for this chart, try to connect the points to form a smooth curve for these sets of data.)



Question 2: At what pH is the turnip peroxidase enzyme most active at?

Investigation III: Determining the Effect of Temperature on Enzymatic Activity

A. SETTING UP THE REACTIONS

1. Using a permanent marker, label four sets of pairs of tubes, as follows

   Set 1: 4 °C "S" and 4 °C "E"
   Set 2: 37 °C "S" and 37 °C "E"
   Set 3: 60 °C "S" and 60 °C "E"
   Set 4: 100 °C "S" and 100 °C "E"

2. Add the following components to the tubes as summarized in Chart 1. Use a FRESH micropipet tip for each transfer of the chemicals. Cover the test tubes and gently mix well.

   Note: *Each of the four sets will consist of a Substrate and an Enzyme tube for a total of 8 tubes or 4 pairs. Each set will be treated at different temperature settings to observe the effect of temperature on the enzymatic activity of Peroxidase.*

Chart 3 – Summary of Substrate and Enzyme Tubes

Tube (4 of each)	Hydrogen Peroxide solution	Guaiacol solution	Turnip Peroxidase	Distilled or deionized water	Final Volume
S (Substrate)	0.15 ml	0.1 ml	------	3.5 ml	3.75 ml
E (Enzyme)	-----	-----	0.75 ml	3.0 ml	3.75 ml

B. DATA COLLECTION

1. Remember to blank the spectrophotometer with the blank solution at 500 nm. Your reading should read 0, and no color changes should occur. Remove the blank.

2. Incubate the 1st set of Substrate and Enzyme tubes labeled 4° C "S" and 4° C "E" in a 4° C ice water bath for 10 minutes.

3. After the incubation time is up, carefully combine the contents of tube S and tube E. Cover the test tube with a piece of Parafilm. Quickly invert to mix well.

4. Place the test tube in the spectrophotometer cuvette holder, and immediately start timing.

Investigation III: Determining the Effect of Temperature on Enzymatic Activity

5. Measure and record the absorbance every 30 seconds for the next 5 minutes in Table 3 below.

   Note:
   - *If you feel comfortable removing the tube from the spectrophotometer cell holder and putting it back before the 30 second interval elapses, do so and observe the color change within the 5-minute period.*
   - *Rotate the tube before each reading. Record the observed color at the end of every 30-second interval.*
   - *A cell phone and/or camera are excellent ways to record color change.*

6. Repeat the above steps for the remaining 3 pairs of tubes at 37 °C, 60 °C, and 100 °C.

7. Record the results in the table.

Time (sec)	Absorbance at **4° C**	Absorbance at **37° C**	Absorbance at **60° C**	Absorbance at **100° C**
0				
30				
60				
90				
120				
150				
180				
210				
240				
270				
300				

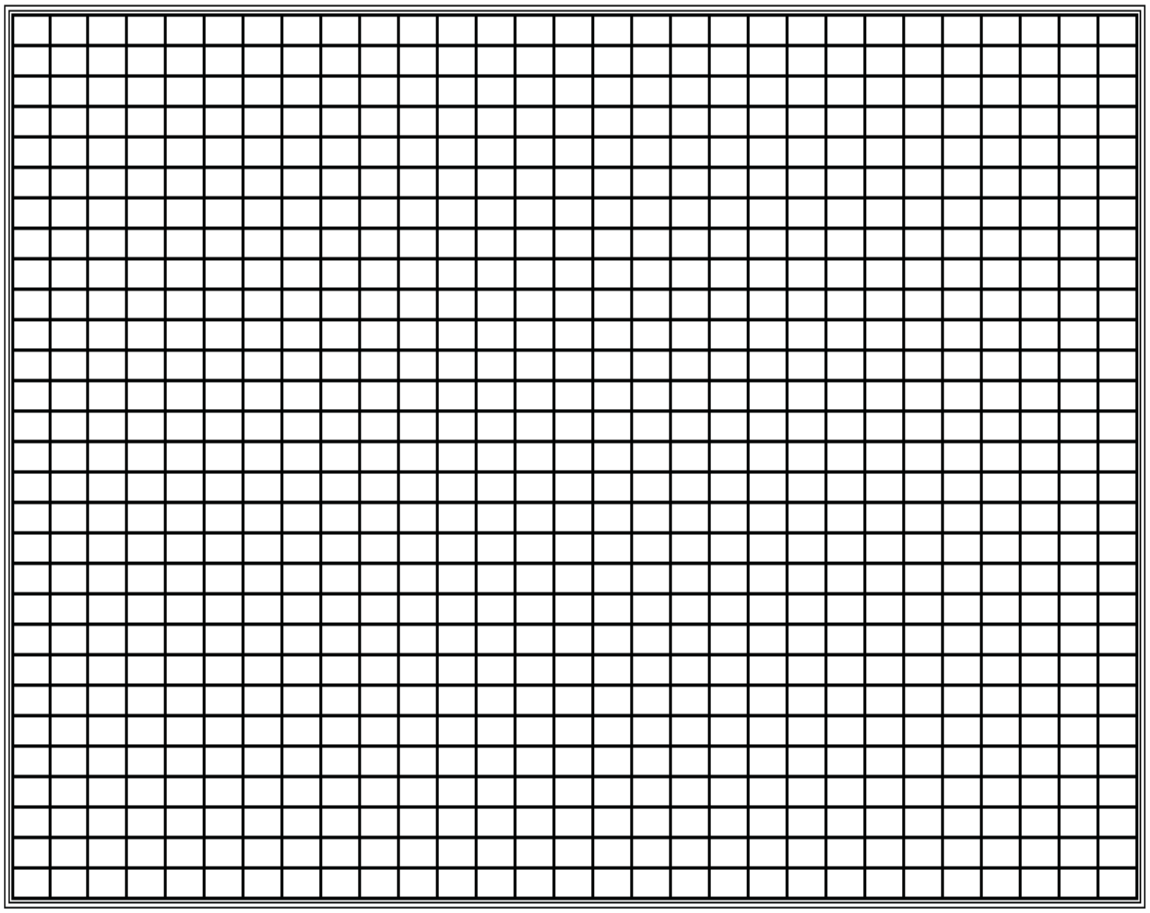
Table 3. Absorbance at different temperatures.

Investigation III: Determining the Effect of Temperature on Enzymatic Activity

C. GRAPHING THE ABSORBANCE AT DIFFERENT TEMPERATURE

1. You should have four sets of "absorbance vs. time" data from Investigation III, one from each temperature.

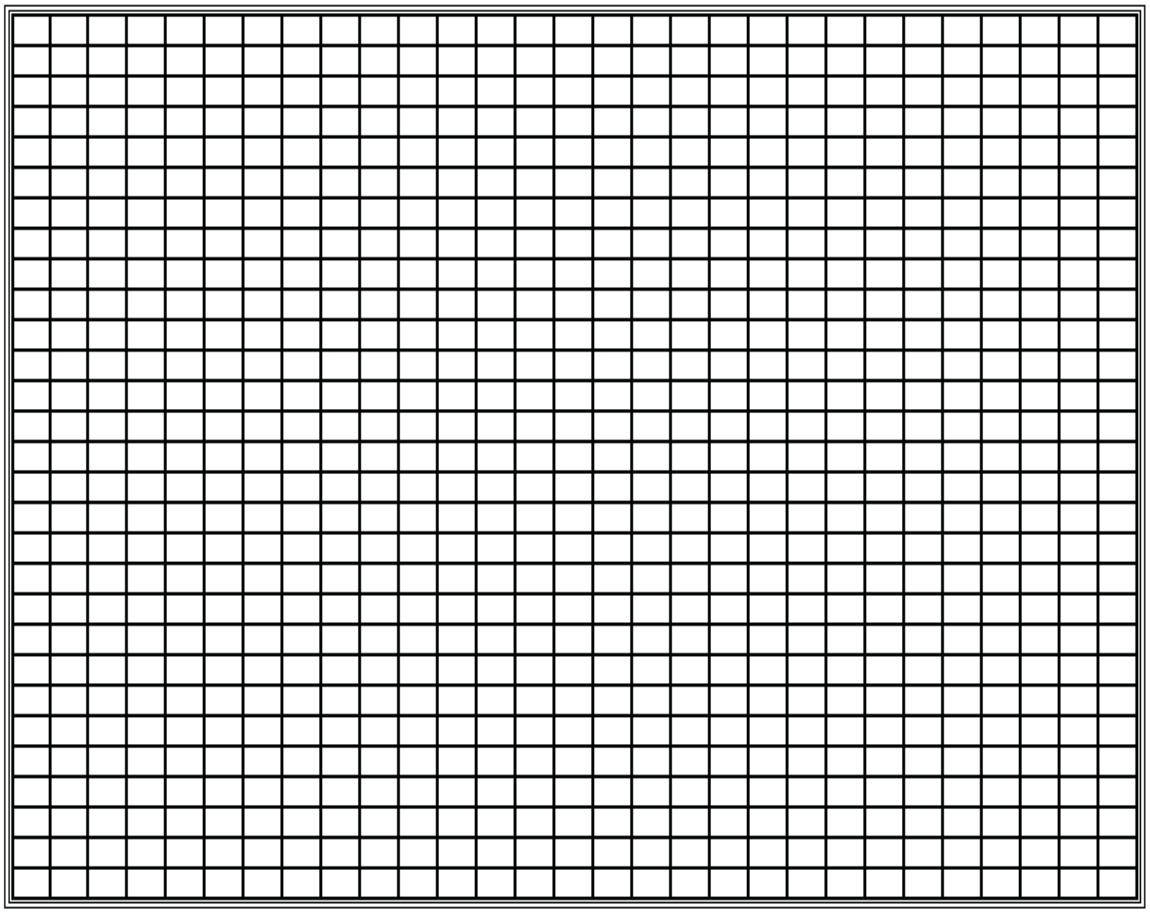
2. Plot the data from four sets on the same graph using the graph paper provided below.



3. Next, calculate the slopes of each line to determine the enzyme activity.

Investigation III: Determining the Effect of Temperature on Enzymatic Activity

4. Plot another graph using the graph paper below to show correlation between enzyme activity and temperatures. (You should have four points for this chart, try to connect the points to form a smooth curve for these sets of data.)



Question 3: At what temperature is the turnip peroxidase enzyme most active at?

Experiment Results and Analysis

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

- Record your observations.

After the Experiment:

- Interpret the results - does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

Study Questions

1. Why was the standardization of enzyme concentration performed?
2. What is the enzyme in this reaction?
3. What is the substrate in this reaction?
4. What are the products in this reaction?
5. What is the function of the Guaiacol?
6. Explain why the color intensity of the peroxide assays increased with time?
7. What makes the rate of a reaction of an enzymatic reaction decrease?
8. Assuming optimal reaction conditions (pH, temperature, etc.), how could you increase the rate of the reaction other than increasing the substrate concentration?