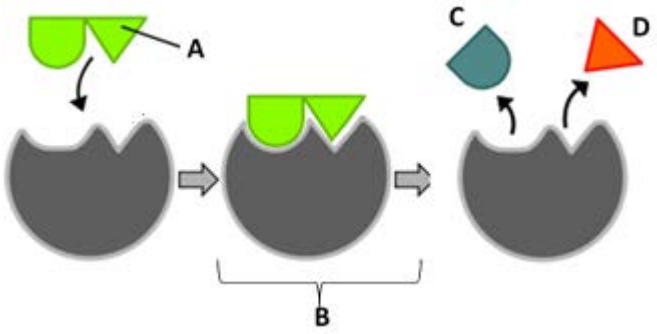
NAME: _____
(first) (last)

Instructor: _____

Generalized enzyme catalysis process:



1. Provide the name of the items labeled above:

A: _____

B: _____

C and D: _____

2. When an enzyme becomes denatured, it
A) increases its activities
B) is able to form the enzyme-substrate complex (ES)
C) loses its shape and can no longer catalyze the reaction
D) loses its primary structure and can now react with different substrates

3. Which of these substance has a brown color and is detected by the spectrophotometer in our reaction?
A) Guaiacol
B) Peroxidase
C) buffer
D) Tetraguaiacol
E) Hydrogen peroxide

4. What is the source of the peroxidase enzyme that we are assaying today?
A) Potatoes
B) Horseradish
C) Turnips
D) Onions

5. The amount of peroxidase will be changed by
A) diluting the turnip extract several times.
B) changing the amount of turnip extract used.
C) adding different amounts of water to the mixture.
D) using different types of turnip extracts prepared for us.

PRELAB ENZYMES

Proper lab attire includes no shorts, eye glasses and closed-toe shoes

6. Which of these will NOT be studied in the lab today?
A) The effect on an inhibitor on peroxidase.
B) The effect of enzyme concentration on the reaction rate of peroxidase.
C) The effect of substrate concentration on the reaction rate of peroxidase.
D) The effect of temperature on the reaction rate of peroxidase.
E) The effect of pH on the reaction rate of peroxidase.

7. Which temperatures will be used in this lab?
A) 0°C, room temperature, 40°C, 50°C and 100°C
B) 0°C, 25°C, 38°C, 40°C, 100°C
C) each group will use a set of temperature of their choice
D) a low temperature, medium-hot temperature and a very hot temperature

8. Which pHs will be tested in the enzyme runs? *(Select all that apply)*
A) pH 7.5
B) pH 3
C) pH 5
D) pH 8
E) pH 7
F) pH 9

9. What will we use to calibrate the spectrophotometer?
A) Water
B) Guaiacol
C) Buffer
D) A "blank" solution as described in each mixing table)

10. The icon  should be clicked 20 seconds after mixing substrates and enzyme (e.g. tubes 2 & 3)

_____True ____False

Laboratory 6 Properties of Enzymes: Turnip Peroxidase, A Case Study

Objectives

- Name the class of macromolecules to which peroxidase belongs and the monomers that make it up.
- Name the substrates and products of the peroxidase catalyzed reaction.
- Explain the role of guaiacol in this experiment.
- Define enzyme, activation energy, active site, pH, and denaturation.
- Distinguish between oxidation/reduction, activation energy/catalysis, substrate/product, and hydrogen peroxide/peroxidase.
- Describe how temperature, pH, enzyme concentration, and substrate concentration affect the reaction rate.
- Explain why peroxidase is a necessary enzyme for all aerobic or oxygen-using cells.

Background Information

The thousands of chemical reactions that occur in a cell at any given time do not occur randomly, but are highly under the control of biological catalysts called **enzymes**. A catalyst is a substance that speeds up a chemical reaction without being consumed by the reaction. Enzymes speed up reactions by lowering the **activation energy of a reaction**, which is that initial amount of energy necessary to bring reactants together with the proper amount of energy and in the proper orientation so that the products can be formed.

Most enzymes are proteins with particular primary structures dictated by genes. As proteins, upon their synthesis, enzymes assume particular shapes. This shape, especially in its "**active site"**, determines its catalytic effects. The active site of each enzyme binds to specific molecules – for example, the enzyme sucrase binds to sucrose but not to lactose, even though both are disaccharides. The reactant molecule that binds with to the active site of an enzyme and undergoes chemical modification is called the **substrate** of that enzyme. Some enzymes bind to two substrates to form products. Certain enzymes have metallic ions (such as Cu^{2+}, Fe^{2+}, Mn^{2+}) as part of their active site These metal ions are called **cofactors**. Sometimes a cofactor is organic in nature and not an ion (e.g. some vitamins); in that case the cofactor is properly called a **coenzyme**.

The binding between enzyme and substrate(s) consists of weak, non-covalent chemical bonds, forming and enzyme-substrate complex that exists for only a few milliseconds. During this instant, the covalent bonds of the substrate(s) either come under stress or are oriented in such a way that facilitates the formation of a different molecule or molecules. This results in the formation of a **product**. The product leaves the enzyme's active site and is used by the cell. The enzyme is unchanged by the reaction and may enter the catalytic cycle again if more substrate molecules are available.

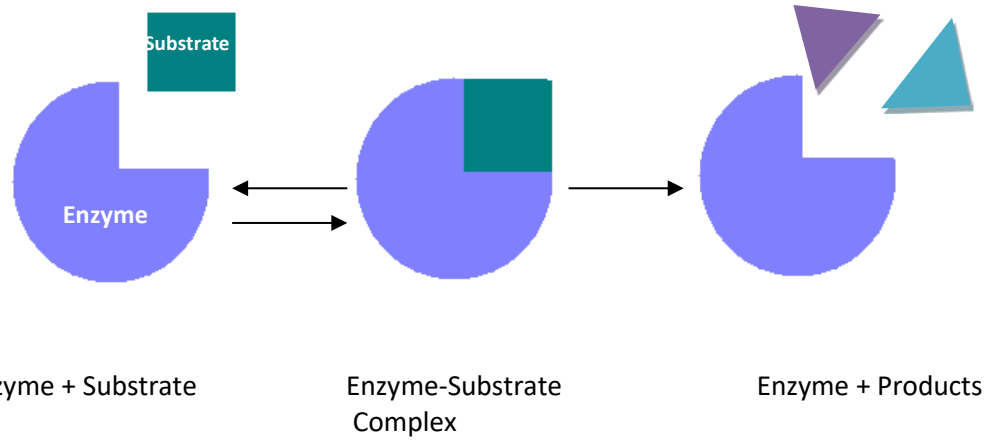


Fig. 6-1 A pictorial representation of an enzyme-catalyzed reaction.

An individual enzyme may convert about a thousand substrate molecules in one second.
Their fast rates combined with the fact that they are not used up during the reaction means that enzymes are needed in small amounts. Eventually enzymes wear out, break apart and lose their catalytic capacity. Cellular enzymes called "proteases" degrade inactive enzymes to amino acids, which are used by the cell to make other enzymes or other proteins.

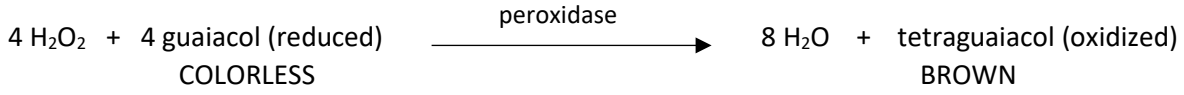
The amount of a particular enzyme found in a cell is determined by the balance between the processes that degrade the enzyme and those that synthesize it. When no enzyme is present, the chemical reaction catalyzed by the enzyme does not occur at an appreciable rate. Generally, if the **concentration of the enzyme** increases, the rate of the reaction will also increase. This is true up to a certain point. At some point **the amount of substrate** also plays a role in the rate of the reaction: adding more enzyme will not speed of a reaction if there is not sufficient substrate.

The **pH** (which is a measure of the concentration of H^+ in an aqueous solution) and high salt concentrations affect the shape of enzyme molecules by interfering with ionic bonds that are necessary to maintain their tertiary structure. This, in turn, affects the substrate-binding efficiency of the enzyme. **Temperature**, within the physiological limits of 0 to 40°C, affects the frequency with which the enzyme and its substrates collide and, hence, also affects binding. Extremely high temperatures typically cause enzyme denaturation, rendering the enzyme completely inactive. All factors that influence binding obviously affect the rate of enzyme-catalyzed reactions. Some of these factors will be investigated during this laboratory.

During this experiment you will study a type of enzyme called **peroxidase**, which is a large protein containing a heme cofactor in its active site. Peroxidase is a good experimental enzyme because it is easily prepared and assayed. Turnips, horseradish roots, and potatoes contain a large amount of this enzyme. For this experiment, we extracted the peroxidase from turnips.

The normal function of peroxidases is to rid the cell of toxic hydrogen peroxide (H_2O_2), which is produced as by-product in certain metabolic reactions. It is critical to remove this H_2O_2 before the cells become damaged by it.

We will carry out the peroxidase reaction "*in vitro*", and will monitor its activity by observing the production of an oxidized product. The dye **guaiacol** (extracted from the guaiacum tree of Central, South America and the Caribbean) becomes oxidized as the hydrogen peroxide is reduced to water. The complete reaction is as follows:



To quantitatively measure the amount of brown color in the final product, the enzyme and substrates can be mixed in a tube and immediately placed in a spectrophotometer. As color accumulates, the absorbance at 501.5 nm will increase. [The procedure for using a spectrophotometer was explained in the previous lab and you should take a few minutes to review those instructions before proceeding.]

For each particular run, plots of absorbance versus time can be constructed. The data points of each run are connected by drawing a best-fit line that goes through (0,0). The slope of each plot is directly related to the enzyme reaction rate for that run.

Factors Affecting Enzyme Activity

Your laboratory instructor may decide to divide your class up into teams; each team will tackle one or more of the following experimental variables: enzyme concentration, pH, temperature and the presence of an inhibitor. The results will then be shared at the end of the lab period and should be included in your lab report. Please see note at the end about how to share data with your classmates.

To perform each of the tests on the experimental variables, you will be following the same basic procedure. Please read over the instructions for all of the variables being studied in the lab, whether or not this is the variable to which your group has been assigned, and make sure you understand the instructions before you continue on with the experiment. This is the same basic procedure that you used in the Enzyme Lab Practice (Lab. 5)

For each of the following experimental variables, use the Mixing Table for each variable to set up the tubes. After you have all of the tubes set up, with the appropriate volumes of solutions, you are ready to begin the experiment. In each test, tube 1 is always the control and is used to blank the spectrophotometer. You will always be mixing tubes 2 & 3 together, tubes 4 &5 together, tubes 6 & 7 together, and tubes 8 & 9 together (when applicable). Remember to mix the solutions in the test tubes and then pour the contents of the tube into the cuvette.

The peroxidase extract has been prepared for you. The extract contains hundreds of different types of enzymes, including peroxidase.

Activity 6-1 The Effect of Enzyme Concentration:

Changing the amount of turnip extract changes the amount of peroxidase!

1. Obtain a beaker or cup of each of the following solutions: Turnip Extract, pH 5 buffer, 10mM H_2O_2, and 25 mM guaiacol. **You ONLY need to pour <u>about 20-mL</u> of each solution**. Make sure the beakers (or cups) are properly labeled.

2. Obtain two 1-mL pipets, one 5-mL and one 10-mL pipet. Use of the wrong pipet will cross contaminate your reagents and introduce errors into your subsequent experiments in this exercise. **<u>DO NOT CROSS CONTAMINATE!</u>**

3. Using the china marker on your trays, label seven test tubes from 1 to 7. Prepare these tubes according to Table 6-1. Amounts given are in mL. Tubes 3, 5 and 7 differ in the amount of turnip extract, providing different concentrations of the peroxidase enzyme to test.

Table 6-1 Mixing table for effect of enzyme concentration test. (**All volumes in milliters)**

Tube	Buffer (pH5)	H_2O_2	Extract	Guaiacol	Total Volume
1(Blank)	6.0	0	1.0	1.0	8
2	0	2.0	0	1.0	3
3	4.5	0	0.5	0	5
4	0	2.0	0	1.0	3
5	4.0	0	1.0	0	5
6	0	2.0	0	1.0	3
7	3.0	0	2.0	0	5

4. Pour the contents of tube 1 into a cuvette. This tube is used to "blank" the spectrophotometer, so that any color caused by contaminants in the reagents will not influence subsequent measurements.

5. Calibrate the spectrophotometer:
   - There is no on/off switch for the spectrophotometer; just make sure that the USB cable is connected to the computer. Double click on the LoggerPro 3.8.2 icon on the computer desktop.
   - Insert the blank cuvette in the sample chamber, making sure that the clear side of the cuvette is in the path of light.
   - On the top menu, click on Experiment. Scroll down to "Calibrate" and select Spectrometer:1" .
   - Wait 60 seconds for the lamp to warm up.
   - Click on "Finish Calibration" and on "OK".

6. Set-up the machine for data capture:
   - Configure  the machine to plot Absorbance vs. Time
   - Click on "Clear Selection" and select the optimum wavelength found in the previous lab period. If you don't have that information available, use 475 nm.
   - On the next screen, go to Experiment, Data Collection and set the experiment length to 120 seconds.

In your lab groups, one person should be the Timer, another the Spectrophotometer Reader, and another the Data Recorder.

7. Note the time to the nearest second and mix the contents of tubes 2 and 3 by pouring the contents of one tube into the other two or three times. **Mixing should be completed within ten seconds**.

8. Immediately after mixing, pour the reaction mixture into a cuvette, wipe the outside of the cuvette with a Kimwipe, and place the cuvette in the spectrophotometer.

   - Twenty seconds after the initial mixing of tubes 2&3 click on "Collect". 

   - After two minutes, remove the cuvette from the spectrophotometer, discard the solution and wash the cuvette.
   - Go to "File", "Save As". Give your file a name like "yourname enz concent". (You can save it on the desktop).

9. Repeat steps 7 and 8 with tubes 4 & 5 and 6 & 7. Click on "Store Latest Run" when asked if you want to erase the previous run. Save your data when the computer stops collecting data.

10. Click on "Linear Fit" 

on the top bar menu. Select Run 1. A box will appear with information about the plot. Recall that the value "m" represents the slope of the line, which in this case represents the "enzyme reaction rate". The units for this rate are s^{-1} or 1/s.

11. Click on "Linear Fit" for the other runs. You can click and drag these boxes so that they are not on top of each other. SAVE YOUR DATA on the desktop. Please see note at the end (page 6-13) about how to share data with your classmates.

Activity 6-2 Temperature Effects on Peroxidase Activity:

To determine the optimum temperature for peroxidase, perform the following experiment.

1. Set up four water baths: In an ice bath at approximately **0°C, at 40°C, at 50°C and at 100°C** (boiling water)
2. Obtain two 1-mL pipets, one 5-mL and one 10-mL pipet. Use of the wrong pipet will cross contaminate your reagents and introduce errors into your subsequent experiments in this exercise. **DO NOT CROSS CONTAMINATE!**
3. Number eleven test tubes 1 through 11. Refer to Table 6-3 for the volumes of reagents to be added to each tube.
4. Pre-incubate the test tubes at 0°C, 40°C and 50°C for at least 15 minutes before running the enzyme reaction.

5.**For the 100°C sample, take 2 mL of the turnip extract and place it in a boiling water bath. After five minutes, remove the tube, let it cool and add 1 mL of this boiled extract in tube 11.

Table 6-3 Mixing table for temperature experiment (**all values in milliliters**)

Temperature	**Tube**	**Buffer (pH5)**	**H_2O_2**	**Extract**	**Guaiacol**	**Total Volume**
	1(blank)	6.0	0	1.0	1.0	8
0°C	2	0	2.0	0	1.0	3
0°C	3	4.0	0	1.0	0	5
23°C	4	0	2.0	0	1.0	3
23°C	5	4.0	0	1.0	0	5
40°C	6	0	2.0	0	1.0	3
40°C	7	4.0	0	1.0	0	5
50°C	8	0	2.0	0	1.0	3
50°C	9	4.0	0	1.0	0	5
100°C	10	0	2.0	0	1.0	3
100°C	11	4.0	0	1.0**	0	5

6. Calibrate the spectrophotometer:
   - There is no on/off switch for the spectrophotometer; just make sure that the USB cable is connected to the computer. Double click on the LoggerPro 3.8.2 icon on the computer desktop.
   - Insert the blank cuvette in the sample chamber, making sure that the clear side of the cuvette is in the path of light.
   - On the top menu, click on Experiment. Scroll down to "Calibrate" and select "Spectrometer:1" .
   - Wait 60 seconds for the lamp to warm up.
   - Click on "Finish Calibration" and on "OK".

7. Set-up the machine for data capture:
   - Configure  the machine to plot Absorbance vs. Time
   - Click on "Clear Selection" and select the optimum wavelength found in the previous lab period. If you don't have that information available, use 475 nm.
   - On the next screen, go to Experiment, Data Collection and set the experiment length to 120 seconds.

In your lab groups, one person should be the Timer, another the Spectrophotometer Reader, and another the Data Recorder.

8. Note the time to the nearest second and mix the contents of tubes 4 and 5 by pouring the contents of one tube into the other. Repeat two or three times. **Mixing should be completed within ten seconds**.
9. Immediately after mixing, pour the reaction mixture into a cuvette, wipe the outside of the cuvette with a KimWipe, and place the cuvette in the spectrophotometer.
   - Twenty seconds after the initial mixing of tubes 4&5 click on "Collect". 
   - After two minutes, remove the cuvette from the spectrophotometer, discard the solution and wash the cuvette.
   - Go to "File", "Save As". Give your file a name like "yourname temperature exp". You can save it on the desktop.
10. Repeat steps 8 and 9 with tubes 2 & 3, 6 &7, 8 & 9 and 10 & 11. Click on "Store Latest Run"

when asked if you want to erase the previous run. Always save your data when the computer stops collecting data

11. Click on "Linear Fit"  on the top bar menu. Select Run 1. A box will appear with information about the plot. Recall that the value "m" represents the slope of the line, which in this case represents the "enzyme reaction rate". The units for this rate are s^{-1} or 1/s.

12. Click on "Linear Fit" for each of the other runs. You can click and drag these boxes so that they are not on top of each other. SAVE YOUR DATA on the desktop. Please see note at the end (page 6-13) about how to share data with your classmates.

Activity 6-3 pH Effects on Peroxidase Activity

To determine the optimum pH for peroxidase, perform the following experiment.

1. Obtain two 1-mL pipets, one 5-mL and one 10-mL pipet. Use of the wrong pipet will cross contaminate your reagents and introduce errors into your subsequent experiments in this exercise. **DO NOT CROSS CONTAMINATE!**

2. Number nine test tubes 1 through 9. Set up the test tubes by adding the reagents to these tubes as described in Table 6-5. Amounts given are in mL. The pH buffers have been provided for you in the laboratory.

Table 6-5 Mixing table for pH experiment (**all values in milliliters**).

Buffer	Tube	Amount of Buffer	H_2O_2	Extract	Guaiacol	Total Volume
pH 5	1 (blank)	6.0	0	1.0	1.0	8
pH 3	2	0	2.0	0	1.0	3
pH 3	3	4.0	0	1.0	0	5
pH 5	4	0	2.0	0	1.0	3
pH 5	5	4.0	0	1.0	0	5
pH 7	6	0	2.0	0	1.0	3
pH 7	7	4.0	0	1.0	0	5
pH 9	8	0	2.0	0	1.0	3
pH 9	9	4.0	0	1.0	0	5

3. Pour the contents of Tube 1 into a cuvette. This tube is used to "blank" the spectrophotometer, so that any color caused by contaminants in the reagents will not influence subsequent measurements.

4. Calibrate the spectrophotometer:
   - There is no on/off switch for the spectrophotometer; just make sure that the USB cable is connected to the computer. Double click on the LoggerPro 3.8.2 icon on the computer desktop.
   - Insert the blank cuvette in the sample chamber, making sure that the clear side of the cuvette is in the path of light.
   - On the top menu, click on Experiment. Scroll down to "Calibrate" and select "Spectrometer:1" .
   - Wait 60 seconds for the lamp to warm up. Click on "Finish Calibration" and on "OK".
5. Set-up the machine for data capture:

- Configure  the machine to plot Absorbance vs. Time
- Click on "Clear Selection" and select the optimum wavelength found in the previous lab period. If you don't have that information available, use 475 nm.
- On the next screen, go to Experiment, Data Collection and set the experiment length to 120 seconds.

In your lab groups, one person should be the Timer, another the Spectrophotometer Reader, and another the Data Recorder.

6. Note the time to the nearest second and mix the contents of tubes 2 and 3 by pouring the contents of tube 3 into tube 2 two or three times. **Mixing should be completed within ten seconds**.

7. Immediately after mixing, pour the reaction mixture into a cuvette, wipe the outside of the cuvette with a KimWipe, and place the cuvette in the spectrophotometer.

   - Twenty seconds after the initial mixing of tubes 2&3 click on "Collect". 
   - After two minutes, remove the cuvette from the spectrophotometer, discard the solution and wash the cuvette.
   - Go to "File", "Save As". Give your file a name like "yourname pH exp". You can save it on the desktop.

8. Repeat steps 7 and 8 with tubes 4 & 5, 6 & 7 and 8 & 9. Click on "Store Latest Run" when asked if you want to erase the previous run. Save your data when the computer stops collecting data.

9. Click on "Linear Fit"  on the top bar menu. Select Run 1. A box will appear with information about the plot. Recall that the value "m" represents the slope of the line, which in this case represents the "enzyme reaction rate". The units for this rate are s^{-1} or 1/s.
10. Click on "Linear Fit" for the other runs. You can click and drag these boxes so that they are not on top of each other. SAVE YOUR DATA on the desktop. Please see note at the end (page 6-13)about how to share data with your classmates.

Activity 6-4 The Effect of an Inhibitor on Peroxidase

Hydroxylamine ($HONH_2$) has a structure similar to hydrogen peroxide (HOOH). This molecule binds with the iron atom at the active site of peroxidase and prevents hydrogen peroxide from entering the site. What will be the effect on enzyme activity?

To test this hypothesis, follow this procedure:

1. Obtain two 1-mL pipets, one 5-mL and one 10-mL pipet. Use of the wrong pipet will cross contaminate your reagents and introduce errors into your subsequent experiments in this exercise. **DO NOT CROSS CONTAMINATE!**

2. Mix five drops of 1% hydroxylamine and 2 ml of enzyme extract in a test tube, letting the mixture stand for at least five minutes.

3. Prepare 5 test tubes as indicated in Table 6-7. Amounts given are in mL.

Table 6-7 Mixing table for inhibitor experiment (all volumes in milliliters).

Tube	Buffer (pH5)	H_2O_2	Extract	Guaiacol	Inhibitor and Extract	Total Volume
1 (Blank)	6.0	0	1.0	1.0	0	8
2	0	2.0	0	1.0	0	3
3	4.0	0	1.0	0	0	5
4	0	2.0	0	1.0	0	3
5	4.0	0	0	0	1.0	5

4. Pour the contents of Tube 1 into a cuvette. This tube is used to "blank" the spectrophotometer, so that any color caused by contaminants in the reagents will not influence subsequent measurements.

7. Calibrate the spectrophotometer:
   - There is no on/off switch for the spectrophotometer; just make sure that the USB cable is connected to the computer. Double click on the LoggerPro 3.8.2 icon on the computer desktop.
   - Insert the blank cuvette in the sample chamber, making sure that the clear side of the cuvette is in the path of light.
   - On the top menu, click on Experiment. Scroll down to "Calibrate" and select "Spectrometer:1" .
   - Wait 60 seconds for the lamp to warm up.
   - Click on "Finish Calibration" and on "OK".

6. Set-up the machine for data capture:

   - Configure  the machine to plot Absorbance vs. Time
   - Click on "Clear Selection" and select the optimum wavelength found in the previous lab period. If you don't have that information available, use 475 nm.
   - On the next screen, go to Experiment, Data Collection and set the experiment length to 120 seconds.

In your lab groups, one person should be the Timer, another the Spectrophotometer Reader, and another the Data Recorder.

7. Note the time to the nearest second and mix the contents of tubes 2 and 3 by pouring the contents of one tube into the other two or three times. **Mixing should be completed within ten seconds**.

8. Immediately after mixing, pour the reaction mixture into a cuvette, wipe the outside of the cuvette with a Kimwipe, and place the cuvette in the spectrophotometer.

   - Twenty seconds after the initial mixing of tubes 2&3 click on "Collect". 

   - After two minutes, remove the cuvette from the spectrophotometer, discard the solution and wash the cuvette.

- Go to “File”, “Save As”. Give your file a name like “yourname inhibitor”. You can save it on the desktop.

9. Repeat steps 7 and 8 with tubes 4 & 5. Click on “Store Latest Run” when asked if you want to erase the previous run. your data when the computer stops collecting data.
10. Click on “Linear Fit” on the top bar menu. Select Run 1. A box will appear with information about the plot. Recall that the value “m” represents the slope of the line, which in this case represents the “enzyme reaction rate”. The units for this rate are s^{-1} or 1/s.

11. Click on “Linear Fit” for the other run. You can click and drag these boxes so that they are not on top of each other. SAVE YOUR DATA on the desktop. Please see note at the end (page 6-13) about how to share data with your classmates.

Data Analysis

*NOTE: **Appendix 1** has instructions for plotting graphs in the computer using Microsoft Excel.*

QUESTIONS

1. The data of absorbance and time obtained for each experiment should be graphed as part of the lab report. The x-axis should be the independent variable (time in seconds) and the y-axis should be the dependent variable (absorbance units). Explain why absorbance is considered the dependent variable.

2. The slopes of the linear portions of the curves are a measure of enzyme reaction rate. What are the units?

3. Explain why the slopes are different.

4. Does the activity of peroxidase vary with temperature? What is the optimum temperature? How do you know this?

5. Does the activity of peroxidase vary with pH? What is the optimum pH?

6. How did boiling affect the activity of peroxidase?

7. Was hydroxylamine a strong, moderate or weak inhibitor? Give the basis for your answer.

SHARING DATA WITH YOUR CLASSMATES:

Since none of you have LoggerPro at home, you cannot just e-mail your classmates the file generated in the lab. To e-mail your linear plot, first right-click on top of the graph and select "Copy". Now open MSWord. Right-click and select "Paste". If you want to easily move the graph around, right-click on the graph, select "Format picture" and select "Square". You may also want to make the graph larger to facilitate the reading of the slope value. You can do this by dragging the squares on the corners of the picture.

To e-mail the plot to the whole class, log-on to e-campus. Click on the "Tools" tab on the left menu. Scroll down to "Send e-mail". Select "All users" (these are the users in your class). Click on the "Attach a file", located at the bottom of the page. Browse to find your file in the computer. Click on "Open" and on "Submit". Verify that your classmates received the file before leaving the lab.

References:

1. Dolphin, W.D., Exercise 4 (Properties of Enzymes) in: *Biology Laboratory Manual*, 4th edition, 1997, WCB Publishers
2. Eberhard,C., *General Biology Lab Manual*, 1996, Harcourt
3. MadScientist network: www.madsci.org
4. Vernier Biology Laboratories