Enzymes and Enzymatic Reactions

Adapted with permission from the original author, Charles Hoyt

Introduction

All living things use energy, give off waste, reproduce and interact with the environment. To carry out these and other life processes, biochemicals must be made, transformed and eventually broken down. These biochemical reactions are rapid, specific and occur at temperatures compatible with life. What makes these reactions possible are a class of globular proteins called enzymes. Without these enzymes, life processes would not be possible.

Learning Objectives

Upon completion of this lab you should be able to:

1. Define

rate of reaction	enzyme	catalyst	substrate
competitive inhibitors	product	active site	activation energy
non-competitive inhibitors	blank	reaction rate	control

2. Describe the physical characteristics of an enzyme.
3. Describe several environmental factors that affect enzyme activity. Use your pipeting and measuring skills to set up an experiment to test enzyme activity.
5. Collect absorbance data using a Spectrophotometer.
6. Construct a best-fit curve of the data.
7. Understand why is blanks are important in experiments determining enzyme activity.

Introduction to Enzymes

Enzymes have several important characteristics.

1. Enzymes are able to take a specific biomolecule called a **substrate** and change it into another specific biomolecule called **product** (or products).

2. Enzymes have an **active site,** which is a pocket or cleft in the protein that is shaped so that it specifically fits a certain substrate. It is here that the substrate is converted into a product.

3. Enzymes are biological **catalysts**. They participate in a biological reaction and guide the reaction, but they **are not changed** by the reaction. Since they are not changed by the reaction they are used over and over to carry out the same reaction.

4. Enzymes speed up reaction rate by decreasing the **activation energy** required to start the reaction. Activation energy is the energy required to break certain bonds in the substrate so that other bonds can form. The formation of these new bonds results in the formation of the product.

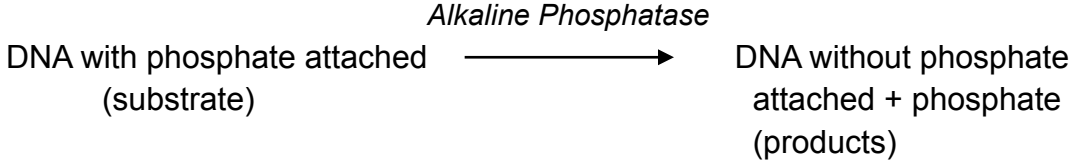
5. Enzymes, like all proteins, are affected by the environment. Changes in temperature, pH and the ionic strength of the surrounding solution all affect enzyme activity.

6. Certain chemicals, as well as environmental factors can also inhibit enzyme activity. Some chemicals inhibit enzymes by attaching permanently to the enzyme so that the enzyme is unable to function. These inhibitors are known as **non-competitive inhibitors** because they are able to inhibit an enzyme without competing with the normal substrate for the active site.

Other chemicals are so similar to the enzyme substrate that they can bind to the active site of the enzyme. Since the chemical is not the normal substrate, it can not be converted into the product. These particular chemicals do not permanently bind to the enzyme and therefore they inhibit the enzyme by competing with the normal substrate for the active site. You can probably guess that these inhibitors are called **competitive inhibitors**.

<u>Alkaline Phosphatase</u>

There are 100,000 or more available from the human body. Today we will investigate the properties of alkaline phosphatase, which is found in the liver. It's natural function is to remove a phosphate group from DNA molecules:



You will not use real DNA as a substrate in this experiment because it is difficult to measure the removal of the phosphate group. We will use another compound called

paranitrophenyl phosphate as the substrate:

Alkaline Phosphatase

Paranitrophenyl phosphate → Nitrophenol + Phosphate

This reaction can be easily measured because the paranitrophenyl phosphate is colorless and one of the products, nitrophenol, is a bright yellow color. As more nitrophenol is produced, the solution becomes a darker yellow and the absorbance increases.

SAFETY NOTE: Nitrophenol is a hazardous material and contact with skin should be avoided. Wash thoroughly if contact does occur.

The Experiments

Exercise A: You are going to set up reactions in test cuvettes which can measure the formation of product. You will use 5 different concentrations of the enzyme to determine the optimal concentration of the enzyme to use in part B.

Exercise B: You are going to change some of the environmental parameters and see how that affects enzyme activity.

A Quick Note about Blanks:

There are several other reagents in the tubes that are necessary for the reaction to occur (like the buffer, the substrate, and the enzyme itself) that are not the chemical that we are trying to measure. We are only interested in changes in the absorbance due to the substrate being changed into product. Therefore, if we can exclude these other chemicals from our spectrophotometer reading we get clearer results. The blank is a way of doing exactly that. The blanks in this experiment include all of the reagents except the enzyme. Additionally, there may be a color change in the experimental tubes due to oxidation of the substrate or other non-enzymatic factors. Since the blanks also contain the substrate, any oxidation will occur in the blanks as well. Therefore if you "zero" the spectrophotometer with the blank before measuring the absorbance of your experimental tube, any absorption due to oxidation will not show up in the spectrophotometer reading of the experimental tube. See Table 1 for the make up of the blanks.

Experimental Procedures (all experiments are run at room temperature except where noted)

1. Turn on the Spectrophotometer and set the wavelength to 410nm.

2. **Set up the blank cuvettes as shown in Table 1**. Remember to treat blanks the same way you treat experimental tubes! Get a set of reagents, a test tube rack and 10 cuvettes. Label 5 as: 1B, 2B, 3B, 4B, 5B. Use one pipet for each reagent. It is best to label a pipet for use with one reagent. If you don't contaminate the pipet, you can keep using it for that reagent.

Exercise A, Blanks

	1B	2B	3B	4B	5B
Buffer	1.65 ml	1.60 ml	1.50 ml	1.30 ml	0.90 ml
DI water	0.05 ml	0.10 ml	0.20 ml	0.40 ml	0.80 ml
Substrate	1.3 ml	1.3 ml	1.3 ml	1.3 ml	1.3 ml
Total	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml

3. **Set up your experimental tubes as shown in Table 2**. Label 5 new tubes: 1E, 2E, 3E, 4E, 5E. **Remember that when the enzyme and substrate come together, the reaction will begin**, so set up the tubes leaving out either the substrate or the enzyme until you are ready to take your readings.

Exercise A, Experimental Cuvettes

	1E	2E	3E	4E	5E
Buffer	1.65 ml	1.60 ml	1.50 ml	1.30 ml	0.90 ml
Enzyme	0.05 ml	0.10 ml	0.20 ml	0.40 ml	0.80 ml
Substrate	1.3 ml	1.3 ml	1.3 ml	1.3 ml	1.3 ml
Total	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml

4. Begin taking readings. Begin with your first blank, cuvette 1B. Invert the cuvette with Parafilm to mix and immediately place it in the spectrophotometer. Press the "0 ABS" key and the window will read "SETTING BLANK." When the absorbance reads 0.0000, remove the cuvette. Quickly add the enzyme to cuvette 1E, mix by inverting and immediately place the tube in the spectrophotometer. Take the reading

immediately and every 30 seconds for 3.5 minutes. Record your data in your lab notebook.

5. When you have taken all of your readings, **remember to dispose of all solutions in the hazardous waste container. Do not dump the solutions down the drain. Clean up.**

6. Graph your results in your lab notebook. Use ABS on the y axis and time in seconds on the X axis. Make your graphs one page in size, with complete title, labeled axes with units, etc.

7. This experiment determines how much enzyme to use in Exercise B. The amount to be used in the following experiments is that amount that showed a linear absorbance change for 3 min. on your graph. Show your graph to the instructor.

Exercise B: How do Environmental Factors Affect the Activity of Alkaline Phosphatase?

After you complete part A, your instructor will assign your group ONE of the following THREE exercises: temperature, substrate concentration or pH. The goal is to determine how these environmental changes will affect the enzyme's ability to catalyze the reaction. Choose the enzyme concentration based on part A with your instructor's assistance.

NOTES FOR ALL GROUPS

- Make sure that all conditions for the experiment are the same except for the environmental factor to be studied.
- Use the correct blank(s)
- Use the same basic <u>set up</u> and <u>procedures</u> followed in Exercise A

<u>Group #1. Variation in Temperature</u>

All the experimental tubes will have the <u>same</u> composition. Use 5 experimental temperatures:

- ice bath (0° C)
- room temperature (usually about 24° C)
- 37° C
- 50° C
- 70° C

Make your tubes and blanks with all reagents, **making sure not to mix the substrate and enzyme until you are ready to take your readings, as before**. Incubate each tube in the appropriate temperature for 5 minutes. Using the same procedure as in part A, combine substrate + enzyme and take your readings every 30 seconds for 3.5 minutes. Record your data, graph the results and safely dispose of waste.

Group #2. Variation in pH

All of your tubes will use the same components as the tube in Exercise A which gave you the most linear result EXCEPT you will experiment with different pH buffers.

Make your tubes and blanks with all reagents EXCEPT the enzyme or substrate, as before. Using the same procedure as in part A, add the enzyme and take your readings every 30 seconds for 3.5 minutes. Record your data, graph the results and safely dispose of waste.

Group #3. Variable Substrate Concentration

In varying the substrate concentration **(initial concentration of substrate is 800µg/ml)**, the volume of the substrate will change. Make the volume of substrate in the experimental cuvettes between 0.1ml and 1.0 ml to avoid making the buffer too dilute. The volume of the substrate changes but the total volume in each cuvette must stay the same. Make your tubes and blanks with all reagents EXCEPT the enzyme or substrate, as before. Using the same procedure as in part A, add the enzyme and take your readings every 30 seconds for 3.5 minutes. Record your data, graph the results and safely dispose of waste.

Exercise B, Blanks for Group 3

	1B	2B	3B	4B	5B
Buffer					
Water					
Substrate					
Total	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml

Exercise B, Experimental Cuvettes for Group 3

	1E	2E	3E	4E	5E
Buffer					
Enzyme					
Substrate					
Total	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml

Summary

Make sure to summarize your findings. How did the various environmental factors affect enzyme activity? Share your results with your classmates and be sure to get a general idea of what the other 2 groups discovered.

Post-Lab Questions:

1. Why did we not use the native reaction for alkaline phosphatase in this lab?

2. Define: substrate, active site, enzyme optima, reactants, products.

3. What physical feature of the enzyme is being affected when we alter the environmental conditions of the reaction?

4. Where do the enzymes in your body come from? (Are they dietary?)