Labs 9 and 10
Environmental Factors Affecting Phosphatase Activity

I Enzymes

As you may know, enzymes are catalysts that control the rates of chemical reactions in cells. Enzymes cause reactions to occur at rates thousands to millions of times faster than without the enzyme. Catalyzed reaction rates in cells range from the tortoise to the hare in speed. Carbonic anhydrase is an enzyme that converts **600,000** molecules of carbon dioxide to 600,000 molecules of bicarbonate in ONE second, in red blood cells. By comparison, Rubisco (ribulose 1,5 bisphosphate carboxylase/oxygenase), fixes (adds) **3** molecules of carbon dioxide to 3 molecules of ribulose 1,5 bisphosphate in ONE second, in green plant leaves. The rate at which different enzymes convert substrate(s) to product(s) is highly variable and subject to change due to a change in environmental conditions.

Enzyme assays measure how fast an enzyme works. These activity assays rely on using the spectrophotometer to measure either the decrease in substrate or the appearance of product. In continuous enzyme assays, the reaction uses either colored substrates or colored products. Cuvettes with an enzyme assay reaction mixture are placed in the spec and a change in absorbance of a substrate or product is measured over time. By plotting initial velocities of the reaction at various substrate concentrations, one can determine the Michaelis-Menton constant and affinity for the enzyme and reaction.

In the discontinuous enzyme assay that you will perform today and next week, the reaction is stopped after a set period of time and the absorbance is measured. Stopping the reaction requires a denaturing agent, such as heat, or severe pH change. The intensity of color in a given length of time is an indicator of the speed with which the enzyme has made product from substrate. This **activity** of the enzyme is usually expressed in the units of □*moles/minute* of product produced or substrate used.

In this phosphatase enzyme lab you will monitor the appearance of yellow color, indicating the product, p-nitrophenol is being produced. To quantify the amount of product, you will first construct a Standard Curve that plots absorbance at 6 p-nitrophenol concentrations. From this you can calculate the activity of phosphatase.

In the second week of this lab, you will hypothesize about the effect of environmental conditions (temperature, pH, enzyme concentration, substrate concentration, inhibitors) on the activity of phosphatase. You will then perform experiments of your own design to test your hypotheses.

II Phosphatase Assay

Phospahtases are enzymes that hydrolyze phosphate groups from a wide variety of organic substrates (phosphate esters), producing an alcohol and phosphoric acid. They are found in all cells and usually are classified as either acid phosphatases or alkaline phosphatases. Phosphatases can be studied in crude cellular extracts or in pure form. In this experiment an artificial substrate, p-nitrophenyl phosphate, will be used to study the activity of a phosphatase enzyme. One of the products of the reaction, p-nitrophenol, turns yellow under basic conditions and can be detected using a spectrophotometer.

p-Nitrophenyl Phosphate + H_2O + (Phosphatase) ----------> p-Nitrophenol + H_3PO_4

Draw a picture of the substrate and product structures (not the enzyme) in the reaction above.

III Challenge

The challenge is to characterize the activity of phosphatase and to determine how differing environmental factors affect this activity. *What are the optimal environmental conditions for this enzyme?*

IV Methodology

Week #1

Procedure for Standard Curve Determination

1. Using *serial dilution* techniques, prepare 5 mL each of six p-nitrophenol concentrations, ranging from 1-50 □M. Use the stock **p-Nitrophenol Solution** and the **0.05 M Standard Buffer** to make the dilutions in marked test tubes.

2. Take 1 mL of each dilution and place into 9 mL of 0.02 M NaOH. *(This is done because the structure of p- nitrophenol is such that it only appears yellow in the presence of a base.)*

3. Transfer 3 mL of each dilution (as prepared in #2) to a cuvette and measure the absorbance of each at 410 nm. Be sure to zero the absorbance first with a blank of pure standard buffer.

4. Graph your results with and draw a straight line of best fit through the points.

Standard Phosphatase Reaction Assay

1. The reaction mixture is simple, made of three parts:
    a. 3 mL of 0.05 M Standard (sodium citrate) Buffer, pH 4.8
    b. 3 mL of 1mM p-nitrophenyl phosphate (substrate)
    c. 3 mL of 1 mg/mL phosphatase

2. To perform an experiment, the buffer and substrate are added to a clean test tube. The enzyme is then added to initiate the reaction and the tube is quickly and briefly stirred.

3. The reaction is then left to incubate for 10 minutes.

4. At successive 2 minute intervals during the 10 minute incubation, 1 mL of the mixture is removed from the tube and placed into a tube containing 9 mL of 0.02 M NaOH. This halts the reaction. After 3 minutes, 3 mL of this second mixture is placed in a cuvette and the absorbance measured in a spectrophotometer at 410 nm. Make sure to blank first.

5. You should have a total of 5 absorbances: 2,4,6,8,and 10 minutes.

6. Using the Standard Curve that you generated, calculate the concentration of p-Nitrophenol at each time period.

7. Graph the results with time on the x-axis and [p-nitrophenol] on the y-axis.

8. Is the relationship linear? What does the shape of the line say about the reaction?

9. *You will use the product concentration at 10 minutes to determine the average phosphatase activity.*

10. Your TF will lead a discussion of how to determine *enzyme activity*, which should be expressed as *moles of product produced per minute*.

WEEK #2

Your goal this week is to hypothesize about changes in phosphatase activity due to a change in environmental conditions of the reaction and to determine the optimal conditions for the phosphatase activity. You and your group will determine which conditions to test. Use the same methodology as last week, but make changes as you see necessary. The following are in the lab for you to use:

1. Ice
2. Heated water baths
3. Standard Buffer, pH 6.8
4. Standard Buffer, ph 8.8
5. 10 mM p-Nitrophenyl Phosphate
6. 10 mg/mL Phosphatase
7. 1 mM Tartaric acid (a possible inhibitor)

You will then write a Lab Report that describes your experiments, your results and your inferences about the optimal conditions for phosphatase activity.