Properties of an Enzyme: Wheat Germ Acid Phosphatase

Experiment #10

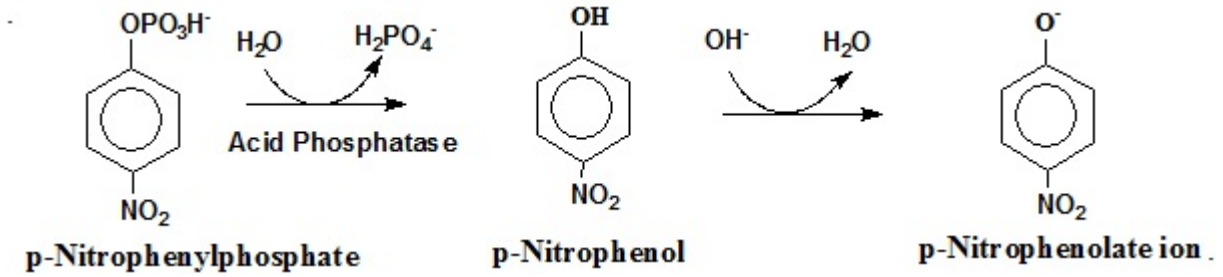
Objective

To show the catalysis of a chemical reaction by an active enzyme and to observe the effects of temperature, killing the enzyme with heat, concentration of substrate, and the presence of an inhibitor of the enzyme.

Introduction

Phosphatases catalyze the hydrolysis of phosphate monoesters with consequent release of inorganic phosphate and the corresponding alcohol (or phenol in this case). These enzymes are widely distributed in nature. Some essential points of enzyme studies are (1) to show the assay response is due to enzyme action, and (2) to show the response is dependent on conditions of the assay, such as pH and temperature.

In this experiment, p-nitrophenyl phosphate (pNPP) will be used as the substrate. The degree of hydrolysis of the substrate is determined by photometric measurement of the p-nitro-phenol liberated in the reaction. In alkaline solution, the p-nitrophenolate ion is bright yellow.



You will measure the relative amount of product formed by comparing the intensity of the yellow color in each tube following the reaction. The reaction will be stopped by transferring the enzyme reaction mixture to a solution of sodium hydroxide (strong base). This not only stops the enzyme reaction, but also converts the p-nitrophenol product to its anion form that gives the intense yellow color.

You will study the effects of heat and of pH on enzyme activity. You will also study the inhibition of this enzyme by sodium fluoride (or the fluoride ion).

Materials

A 37°C water bath will be available in the lab, 0.10 M sodium hydroxide and 0.10 M sodium fluoride solutions; 0.05 M citrate buffer solutions at pH 3.0, pH 5.0 and pH 8.0; 0.5 mM p-nitrophenyl-phosphate substrate solution; wheat germ acid phosphatase enzyme solution (keep on ice). A Spectronic 20 spectrometer for measuring absorbance at 410 nm.

Procedure

Add about 100 mL of water to a 400 mL beaker and heat to boiling. You will also need a beaker or styrofoam cup for an ice bath. There will be a water bath adjusted to 37°C set up in the lab for you to use at this temperature, with several test tube racks, keep track of your test tubes to

avoid confusing them with others. A scheme for each part of the experiment is outlined in tables. **Notice:** Each group should transfer some acid phosphatase enzyme solution to a small test tube to about half full or more and transfer some p-nitrophenylphosphate substrate solution to another clean small test tube. This should be enough for the entire experiment.

A. Demonstration of Enzyme Action

Schematic for Test Tubes, Part A

Tube # ➜	1	2	3	4	5
Buffer	1.0 mL of Citrate Buffer, pH 5.0 in each tube				
Enzyme	5 Drops of stock enzyme solution in each tube				
Conditions	Ice Bath	Room Temp	37°C Bath	37°C Bath	Boiling Water then 37°C Bath
Substrate Soln (after temp equil)	5 drops	5 drops	5 drops	No substr	5 drops
Incubation	Incubate 10 min each at their respective temperatures				
Stop Reaction	Transfer the contents of each tube to another large test tube containing 3.0 mL of 0.10 M NaOH solution to stop reaction and develop yellow color.				

1. Add 1.0 mL of citrate buffer, pH 5.0 and 5 drops of acid phosphatase enzyme solution (using a plastic dropper) into each of 5 clean test tubes. Make sure each tube has the same volume and label them 1 thru 5 (you may also want to label each with your initials to avoid mixing them up with other peoples tubes in the water bath).

2. Place tube #1 in an ice bath. Leave tube #2 on the bench top (room temp). Place tubes #3 and #4 in the water bath at 37°C. Place tube #5 in a boiling water bath for about 5 min.

3. Remove tube #5 from the boiling water bath after 5 min and place it in the 37°C water bath along with tubes #3 and #4 for 2 min before adding substrate solution.

4. Add exactly 5 drops (using a plastic dropper) of substrate solution (p-nitrophenylphospate) to tubes #1, #2, #3 and #5 containing buffer and enzyme. Do **NOT** add any substrate to tube #4. This will be used to show that substrate is necessary for the reaction. Mix the solutions well and allow them to stand for exactly 10 min (time these as accurately as possible) at their respective temperatures. [Note: Tube 1 is still in ice, tube 2 is at room temperature, tubes 3, 4 and 5 are in the 37°C water bath].

5. While the enzyme reaction is proceeding for 10 minutes, set up 5 clean large test tubes and label them 1B through 5B (B for Base). Add 3.0 mL of 0.1 M sodium hydroxide (NaOH) solution to each of the 5 large test tubes.

6. At the end of 10 min incubation, pour each of the enzyme solutions into their respective

tubes containing the 0.1 M sodium hydroxide solution (i.e., tube #1 containing enzyme and buffer is poured into tube #1B containing the 0.1 M sodium hydroxide solution). This will stop the reaction and convert any p-nitrophenol that was formed into p-nitrophenolate ion.

7. Transfer the solutions after mixing them to a small test tube, measure the absorbance of each solution at 410 nm using the spectrometer and record the absorbance value on the Report Sheet. The instructor will zero the spectrometer using a water blank before measuring the absorbance. Do not make any adjustments to the spectrometer.

8. After measuring the absorbance you can discard these solutions in the hazardous waste bottle in the hood.

B. Dependence of the Reaction on Enzyme Concentration and Enzyme Inhibition by Fluoride

Schematic for Test Tubes, Part B

Tube # ➜	0	5	10	F
Buffer	1.0 mL of Citrate Buffer, pH 5.0 in each tube			
Enzyme	0 drops	5 drops	10 drops	5 drops
Sodium Fluoride Soln	0 drops	0 drops	0 drops	5 drops
Deionized Water	10 drops	5 drops	0 drops	0 drops
Substrate Soln (after temp equilibration)	5 drops	5 drops	5 drops	5 drops
Incubation	Incubate 10 min each at 37°C in water bath			
Stop Reaction	Transfer contents of each tube to another test tube containing 3.0 mL of 0.10 M NaOH solution to stop reaction and develop yellow color.			

1. Add 1.0 mL of citrate buffer, pH 5.0 to each of four test tubes as you did in part A, and label them 0, 5, 10 and F (the numbers represent the number of drops of enzyme added to each, F represents the presence of fluoride inhibitor). Do not add any enzyme to the tube labeled “0", add 5 drops (using a plastic dropper) of enzyme solution to the tube labeled “5" and the tube labeled “F”, add 10 drops of enzyme solution to the tube labeled “10". Add 5 drops of sodium fluoride solution to the tube labeled "F". Add 10 drops of deionized water to tube “0" and 5 drops of water to tube “5" to give each tube the same volume of liquid. **No** water is added in tubes "10" and "F".

2. Place each tube in the 37°C water bath for 5 min before adding substrate. Be careful not to mix them up with other sets of tubes.

3. After the temperature has equilibrated, add 5 drops of p-nitrophenylphosphate substrate solution (using a plastic dropper) to each test tube and allow them to incubate in the 37°C

water bath for 10 min.

4. While they are incubating, prepare 4 test tubes with 3.0 mL of 0.1 M NaOH solution and label them 0B, 5B, 10B and FB. Alternatively, you can use the tubes labeled 1B thru 4B from part A, but be sure to keep track of which solution is added to each of these tubes.

5. After each buffer/enzyme solution has been in the 37°C water bath for exactly 10 min, pour the contents of the tube into the respective tubes containing 0.1 M NaOH solution.

6. Measure the absorbance of the solution in each tube and report it in the second table on the Report Sheet. These solutions can be discarded in the hazardous waste bottle.

C. pH Dependence of the Enzyme Catalyzed Reaction

Schematic for Test Tubes, Part C

Tube # ➜	3	5	8
Buffer, 1.0 mL in each tube	pH 3	pH 5	pH 8
Enzyme	5 Drops of stock enzyme solution in each tube		
Substrate Soln (after temp equil)	5 drops	5 drops	5 drops
Incubation	Incubate 10 min each at 37°C		
Stop Reaction	Transfer the contents of each tube to another test tube containing 3.0 mL of 0.10 M NaOH solution to stop reaction and develop yellow color.		

1. Add 1.0 mL of citrate buffer, pH 3 to one test tube, 1.0 mL of citrate buffer, pH 5 to another test tube and 1.0 mL of citrate buffer, pH 8 to a third test tube. Label these tubes. Add 5 drops of enzyme solution to each tube and mix well. Place each tube in the 37°C water bath for 5 min for temperature equilibration.

2. After the temperature has equilibrated, add 5 drops of p-nitrophenylphosphate substrate solution (using a plastic dropper) to each test tube and allow them to incubate in the 37°C water bath for 10 min.

3. While they are incubating, prepare 3 test tubes with 3.0 mL of 0.1 M sodium hydroxide solution and label these “3B”, “5B” and “8B”, or use the labeled tubes from part A.

4. After each buffer solution has been in the 37°C water bath for exactly 10 min, pour the contents of the tube into the respective tubes containing 0.1 M sodium hydroxide solution.

5. Measure the absorbance of each solution and report it in the table on the Report Sheet. You can discard these solutions in the hazardous waste bottle in the hood.

Name _____ Section _____

Properties of an Enzyme: Wheat Germ Acid Phosphatase

Experiment #10 Pre-Lab Exercise

1. What is an enzyme? Give a description of what an enzyme is in terms of its chemical composition (what kind of biomolecule) and in terms of what it does (its function).

2. A chemical reaction catalyzed by the enzyme acid phosphatase is shown in the introduction. How would you classify this enzyme in terms of the enzyme classification scheme described in the text book, *i.e.*, oxidoreductase, transferase, hydrolase, lyase, isomerase, or ligase?

3. List at least four factors that can influence enzyme activity and indicate what is meant by the term "enzyme activity" in this context (see the text book).

4. Considering the biochemical (protein) nature of enzymes, what are some factors or chemical and physical agents that can destroy or denature enzymes? Consider the earlier experiment regarding properties of proteins. You should list at least three.

Name _____ Section _____

5. Would you say that boiling water would be an effective way to destroy the enzymes essential for microbes to live? Explain.

6. Fluoride ion is considered to be toxic to most organisms at relatively high concentrations. There is currently a controversy over the use of fluoride in municipal drinking water. What is the basis for putting fluoride in drinking water? What would be the reason for not adding it? You may want to check the internet for discussion of this issue.

Name _____ Section _____

Properties of Enzymes: Acid Phosphatase

Experiment #10 Data & Report Sheet

Part A. Demonstration of Enzyme Action

Record absorbance at 410 nm of each solution after adding to 3.0 mL of 0.10 M sodium hydroxide.

Tube	Conditions	Absorbance @ 410 nm
1	On ice, 0°C	
2	Room Temperature, 22°C	
3	Body Temperature, 37°C, with Substrate	
4	Body Temperature, 37°C, No Substrate	
5	Boiled Enzyme, Incubated at 37°C, with Substrate	

A-1. Comparing tubes 3 and 4, what conclusion can you make about the need for substrate in order to demonstrate enzyme activity?

A-2. Explain why tube 5 would have lower activity than tube 3.

A-3. Explain the relative absorbance for tubes 1, 2 and 3. What accounts for the differences?

Part B. Dependence of the Reaction on Enzyme Concentration and Fluoride Inhibition

Tube	Conditions	Absorbance @ 410 nm
0	No Enzyme Added	
5	5 Drops of Enzyme Solution Added	
10	10 Drops of Enzyme Solution Added	
F	5 Drops of Enzyme Solution Added, with Fluoride Inhibitor	

B-1. Would you expect any p-nitrophenol product to be formed when there is no enzyme present in the solution? Explain.

B-2. Would you expect fluoride to occur naturally in drinking water? Explain.

Part C. pH Dependence of the Enzyme Catalyzed Reaction

pH	Absorbance @ 410 nm
3	
5	
8	

C-1. Discuss the observed effect of pH on the activity of acid phosphatase. Why is it called "acid" phosphatase?

C-2. Do most enzymes have the same optimum pH?