

Properties of an Enzyme: Wheat Germ Acid Phosphatase

Experiment #10

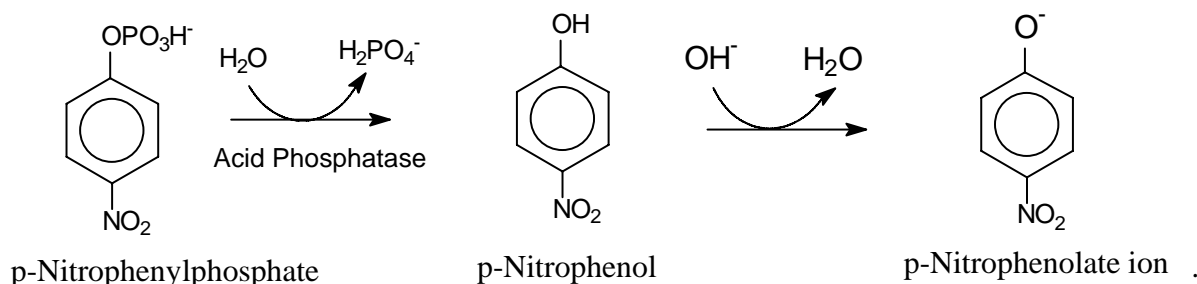
Objective

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-nitrophenol 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 adding NaOH solution to the enzyme reaction mixture. 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 NaOH and 0.10 M sodium fluoride solutions; 0.05 M citrate buffer solutions at pH 3.0, pH 5.0 and pH 7.0; 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.

A. Demonstration of Enzyme Action

Schematic for Test Tubes, Part A

Tube # →	1	2	3	4	5
Buffer	10 Drops 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 test tube containing 4.0 mL of 0.10 M NaOH solution to stop reaction and develop yellow color.				

1. Add 10 drops of citrate buffer, pH 5.0 and 5 drops of stock enzyme solution in each of 5 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. Place tube #2 at room temperature on the bench top. 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 for 5 min before adding substrate solution.
4. While the temperature of these solutions are equilibrating, add 4.0 mL of 0.1 M NaOH solution to each of 5 clean test tubes and label them 1B through 5B (B for Base).
5. After preparing the tubes with 0.1 M NaOH solution and allowing the enzyme solutions to equilibrate at their respective temperatures, add exactly 5 drops of substrate solution (p-nitrophenylphosphate) 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 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]. You may wish to start part B while you are waiting.

- At the end of 10 min, pour each solution into their respective tubes containing the 0.1 M NaOH solution (i.e., tube #1 containing enzyme and buffer is poured into tube #1B containing the 0.1 M NaOH solution). This will stop the reaction and convert any p-nitrophenol that was formed into p-nitrophenolate ion.
- Measure the absorbance of the solution in each test tube at 410 nm using the Spectronic 20 spectrometer and record the absorbance value on the Report Sheet. The instructor will show you how to zero the spectrometer before measuring the absorbance.

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	10 Drops 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 4.0 mL of 0.10 M NaOH solution to stop reaction and develop yellow color.			

- Add 10 drops 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 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 deionized water to tubes "0" and "5" to give each tube the same volume of liquid, that will be 10 drops of water in tube "0", 5 drops of water in tube "5", and no water in tubes "10" and "F".
- Place each tube in the 37°C water bath for at least 5 min before adding substrate. Be careful not to mix them up with the other set of tubes.
- After the temperature has equilibrated, add 5 drops of substrate solution to each test tube and allow them to incubate in the 37°C water bath for 10 min.

- While they are incubating, prepare 4 test tubes with 4 mL of 0.1 M NaOH solution and label them 0B, 5B, 10B and FB. Alternatively, you can use the tubes labeled 1B thru 5B from part A, but be sure to keep track of which solution is added to each of these tubes.
- After each buffer solution has been in the 37°C water bath for 10 min, pour the contents of the tube into the respective tubes containing 0.1 M NaOH solution.
- Measure the absorbance of the solution in each tube and report it in the second table on the Report Sheet.

C. pH Dependence of the Enzyme Catalyzed Reaction

Schematic for Test Tubes, Part C

Tube # →	3	5	7
Buffer, 10 drops in each tube	pH 3	pH 5	pH 7
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 2.0 mL of 0.10 M NaOH solution to stop reaction and develop yellow color.		

- Add 10 drops of citrate buffer, pH 3 to one test tube, 10 drops of citrate buffer, pH 5 to another test tube and 10 drops of citrate buffer, pH 7 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 at least 5 min for temperature equilibration.
- After the temperature has equilibrated, add 10 drops of substrate solution to each test tube and allow them to incubate in the 37°C water bath for 10 min.
- While they are incubating, prepare 3 test tubes with 4.0 mL of 0.1 M NaOH solution and label these “3B”, “5B” and “7B”, or use the tubes already labeled.
- After each buffer solution has been in the 37°C water bath for 10 min, pour the contents of the tube into the respective tubes containing 0.1 M NaOH solution.
- Measure the absorbance of each solution and report it in the table on the Report Sheet.

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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 previous experiment regarding properties of proteins. You should list at least three.

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Data & Report Sheet

Part A. Demonstration of Enzyme Action

Record the absorbance at 410 nm of each solution after adding to 4.0 mL of 0.10 M NaOH.

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	
15	15 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. Fluoride ion is considered to be toxic to most organisms at relatively high concentrations. There is currently a big 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.

B-3. 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	
7	

C-1. Discuss the observed effect of pH on the activity of acid phosphatase. Why is it called “acid” phosphatase? Is this a general characteristic of most enzymes?