



G-Biosciences ♦ 1-800-628-7730 ♦ 1-314-991-6034 ♦ [technical@GBiosciences.com](mailto:technical@GBiosciences.com)

A Geno Technology, Inc. (USA) brand name

# Enzyme Analysis

## Teacher's Guidebook

(Cat. # BE-405)



think proteins! think G-Biosciences [www.GBiosciences.com](http://www.GBiosciences.com)

|   |    |
|---|----|
| MATERIALS INCLUDED .....                                  | 3  |
| SPECIAL HANDLING INSTRUCTIONS .....                       | 3  |
| ADDITIONAL EQUIPMENT REQUIRED .....                       | 3  |
| TIME REQUIRED .....                                       | 3  |
| OBJECTIVES .....  | 4  |
| BACKGROUND .....  | 4  |
| ENZYME ASSAY AND KINETICS .....                           | 4  |
| ENZYME INHIBITION .....                                   | 5  |
| ALKALINE PHOSPHATASE ASSAY AND KINETICS .....             | 5  |
| TEACHER'S PRE EXPERIMENT SET UP .....                     | 6  |
| PREPARE ENZYME SOLUTION .....                             | 6  |
| PREPARE SUBSTRATE SOLUTION .....                          | 6  |
| MISCELLANEOUS.....  | 6  |
| MATERIALS FOR EACH GROUP .....                            | 7  |
| PROCEDURE .....   | 8  |
| I. ALKALINE PHOSPHATASE ASSAY & KINETICS .....            | 8  |
| II. EFFECT OF PH ON ALKALINE PHOSPHATASE ACTIVITY. ....   | 9  |
| III. ALKALINE PHOSPHATASE AND COMPETITIVE INHIBITION..... | 10 |
| RESULTS, ANALYSIS & ASSESSMENT .....                      | 12 |
| I. ALKALINE PHOSPHATASE ASSAY & KINETICS .....            | 12 |
| II. EFFECT OF PH ON ALKALINE PHOSPHATASE ACTIVITY. ....   | 13 |
| III. ALKALINE PHOSPHATASE AND COMPETITIVE INHIBITION..... | 14 |

## **MATERIALS INCLUDED**

This kit has enough materials and reagents for 24 students (6 groups of 4 students each).

- 1 bottle Enzyme Assay Buffer (pH 8.0)
- 1 bottle Enzyme Assay Buffer (pH 9.0)
- 1 bottle Enzyme Assay Buffer (pH 7.2)
- 1 bottle Enzyme Assay Buffer (pH 5.0)
- 1 vial Enzyme Substrate (pNPP)
- 1 vial Enzyme (AP)
- 1 bottle Enzyme Stop Solution
- 1 vial Enzyme Inhibitor
- 330 1.5ml Tubes

## **SPECIAL HANDLING INSTRUCTIONS**

- Buffers and Enzyme Inhibitor can be stored at room temperature
- Enzyme should be stored at -20°C
- Enzyme Substrate should be stored at -20°C, prepare solutions 30 minutes before lab.
- Briefly centrifuge all small vials before opening to prevent waste of reagents.

## **ADDITIONAL EQUIPMENT REQUIRED**

- Marker pens
- Stopwatch or timer
- Pipettes and tips
- Cuvettes (1ml) and Spectrophotometer
- 15ml Tubes

## **TIME REQUIRED**

- 2-3 hours

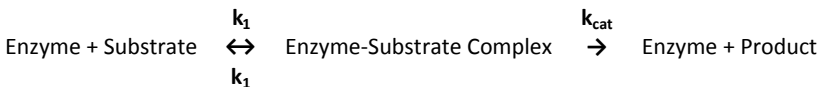
## OBJECTIVES

- To understand the principle of enzyme assays and kinetics.
- To investigate action of an enzyme.
- To see the effect of pH on enzyme activity.
- To see the effect of an enzyme inhibitor on enzyme activity.
- To establish enzyme importance in disease conditions.

## BACKGROUND

Enzymes are biocatalysts that increase the rate of otherwise slow reactions by decreasing the reactions activation energy, without undergoing any net change in their structures at the end of a reaction. They are mostly protein in nature and mediate all synthesis and degradation reactions carried out by living organisms. Ribozyme is a RNA molecule that has enzymatic properties. Enzymes catalyze almost all the biological reactions, converting a target molecule (the substrate) into product(s). Enzymes are considered as an integral part to biological systems because they allow the body to carry out reactions that normally would require too much energy to initiate.

An enzyme acts on a substrate to form an enzyme-substrate complex, which then forms a new product and the free enzyme. The active site of an enzyme, where the substrate binds, only recognizes the specific substrate and holds it in a set confirmation. The enzyme activity is a measure of the rate or velocity of the conversion of the substrate to the product per unit time by an enzyme. The enzymes generally work under mild conditions of temperature, pressure and pH that decrease the energy requirement.



The following terms are used to define enzyme more specifically:

- **Apoenzyme** - The protein portion of an enzyme is called the apoenzyme.
- **Cofactor** - It is the non-protein part of an enzyme. Cofactors can be loosely bound, **coenzymes**, or tightly bound, **prosthetic groups**.
- **Holoenzyme**- The complete enzyme (apoprotein + cofactor) is termed the Holoenzyme.

### *Enzyme Assay and Kinetics*

Enzyme kinetics, which refers to the rate of an enzyme-catalyzed reaction, can be affected by numerous factors, including enzyme, substrate concentration, pH and inhibitors. When an enzyme concentration is kept constant in a system, increasing the substrate concentration leads to an increase in reaction rate until a maximum velocity,  $V_{\text{max}}$ , is reached. The substrate concentration at which the velocity is  $\frac{1}{2} V_{\text{max}}$  is defined as the Michaelis constant,  $K_m$ . At low substrate concentrations, many of the enzyme active sites are empty and readily bind to substrate. The reaction rate stabilizes when no more active sites are open. Similarly, increasing the enzyme concentration, while the

substrate amount remains constant, causes an increase in the rate of the reaction until a maximum point is reached where all enzyme is bound to the substrate present. Precautions should be taken while handling enzymes, since parameters like pH, temperature, and presence of ions affect the enzyme activity.

**Enzyme Inhibition**

Enzyme inhibitors act to decrease the rate of an enzyme reaction. The following are examples of enzyme inhibition:

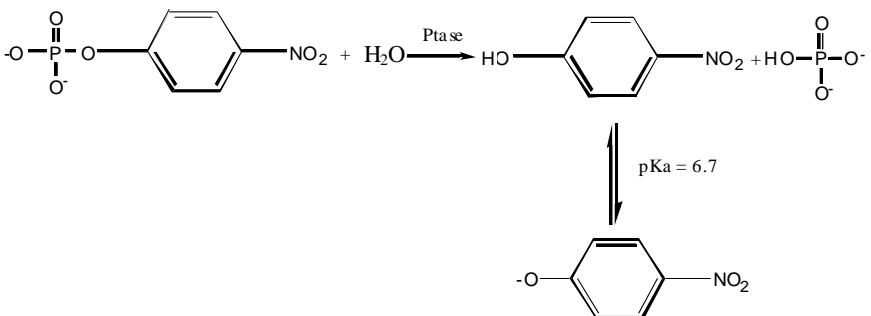
Competitive inhibition occurs when the inhibitor molecule binds to the active site of the enzyme. As a result, the substrate and inhibitor compete for the same spot. Competitive inhibition can be overcome by increasing the substrate concentration so that it is much greater than the inhibitor concentration.

Uncompetitive inhibition, however, occurs when the inhibitor molecule binds to a site on the enzyme other than the active site and results in a conformational change of the enzyme resulting in an inability to bind the substrate. Thus, inhibition is not removed by increasing substrate concentration because they are not competing for the same spot.

Noncompetitive inhibition occurs when the inhibitor binds both the active site of the enzyme and the enzyme-substrate complex. One way to measure the degree of inhibition is by calculating the inhibitor constant  $K_i$ , which is the concentration of inhibitor that causes a 2 fold increase in the slope of the  $1/(\text{substrate concentration})$  versus  $1/(\text{velocity})$  plot, also known as the Lineweaver-Burk plot.

**Alkaline phosphatase Assay and Kinetics**

Alkaline phosphatase is an enzyme that is found in the blood, intestine, liver, and bone cells of the human body. Like all enzymes, it is needed in small amounts to trigger specific chemical reactions. However, high levels of this enzyme in the body may be due to a variety of factors, which include bone fracture healing, liver diseases, bone diseases, hepatitis, or skeletal diseases; low levels may be indicative of poor nutrition, magnesium deficiency, or protein deficiency. Thus, alkaline phosphatase plays a crucial role in the body.



Alkaline phosphatase catalyzes the hydrolysis of phosphate monoesters to give inorganic phosphate and the corresponding alcohol, phenol, or sugar. As indicated by the name of this enzyme, catalytic activity is higher in the alkaline pH range. A convenient assay for the activity of alkaline phosphatase uses p-nitrophenylphosphate (pNPP), a colored chromogenic substrate. Hydrolysis of this phosphate monoester gives inorganic phosphate and p-nitrophenol (pNP). The colored end product of this reaction is measured with the help of a standard plot, the amount of product formed is calculated and from that measurement, the amount of alkaline phosphatase in the given sample is determined. This reaction is shown below:

## TEACHER'S PRE EXPERIMENT SET UP

### **Prepare Enzyme Solution**

1. Transfer 2ml of Assay Buffer (pH 8.0) to the vial of Enzyme (AP). Mix by vortexing or inverting the tube.
2. When completely dissolved aliquot 300 $\mu$ l to each group.

### **Prepare Substrate Solution**



*Prepare the enzyme substrate the morning of the experiment, no more than two hours before the start of the experiment.*

1. To prepare 5mM Enzyme Substrate (pNPP) solution: Add 10ml distilled water to a 15ml tube.
2. Remove 1ml water and add to the vial of Enzyme Substrate (pNPP). Mix until the substrate is completely dissolved. Transfer the 1ml of dissolved substrate back into the 15ml tube.
3. Mix by inverting 4-6 times and supply each group with 1.5ml. Label vials Substrate Solution.

### **Miscellaneous**

1. Aliquot 9ml of Enzyme Assay Buffer pH 8.0 and 1ml each of Enzyme Assay Buffers pH 9.0, 7.2, and 5.0 to each group.
2. Aliquot 200 $\mu$ l Enzyme Inhibitor (10mM) into six tubes and supply each group with one tube.
3. Aliquot 13ml Enzyme Stop Solution to each group.

## **MATERIALS FOR EACH GROUP**

Supply each group with the following components. Several components are to be shared by the whole class and should be kept on a communal table.

- 4 cuvettes
- 48 1.5ml Tubes
- Marker Pen
- 1 vial 300 $\mu$ l Enzyme (AP)
- 1 vial 1.5ml Substrate Solution
- 1 tube 9ml Enzyme Assay Buffer pH 8.0
- 1 vial 1ml Enzyme Assay Buffer pH 9.0
- 1 vial 1ml Enzyme Assay Buffer pH 7.2
- 1 vial 1ml Enzyme Assay Buffer pH 5.0
- 1 vial 200 $\mu$ l Enzyme Inhibitor (10mM)
- 1 tube 13ml Enzyme Stop Solution
- 100ml distilled water (or pure lab water)

## PROCEDURE



Do **NOT** add enzyme until ready to start your timer.

### I. Alkaline phosphatase Assay & Kinetics.

The aim is to follow the kinetics of alkaline phosphatase enzyme using three different substrate concentrations over a 20 minute period.

1. *Prepare Enzyme Stop Solution:* Label three sets of twelve 1.5ml centrifuge tubes for each assay of different substrate concentrations. Mark each set with a blank and time course intervals from 0-20 minutes with 2 minute intervals, as follows:

**Assay A:** Blank A, A-0m, A-2m, A-4m, A-6m, ..., A-20m

**Assay B:** Blank B, B-0m, B-2m, B-4m, B-6m, ..., B-20m

**Assay C:** Blank C, C-0m, C-2m, C-4m, C-6m, ..., C-20m

2. Add 0.1ml Enzyme Stop Solution and 0.8ml distilled water (or pure lab water) to all the Assay A, B and C tubes.
3. *Prepare enzyme reaction tubes:* Label three 1.5ml centrifuge tubes "Assay A", "Assay B", and "Assay C". Set up the following reactions as shown in the table and mix the contents by pipetting up and down several times.

| Assay # | Substrate Solution (5mM) |                          | Enzyme Assay Buffer (pH8.0) |
|---------|--------------------------|--------------------------|-----------------------------|
|         | Volume ( $\mu$ l)        | Final Concentration (mM) | Volume ( $\mu$ l)           |
| Assay A | 60                       | 0.25                     | 1160                        |
| Assay B | 120                      | 0.50                     | 1100                        |
| Assay C | 240                      | 1.00                     | 980                         |

4. *Prepare reaction blanks:* Transfer 0.1ml solution from the above enzyme reaction tubes to the appropriate tubes marked Blank and mix. They are your reaction controls or blanks.

0.1ml solution from tube **Assay A** to tube Blank A; 0.1ml solution from tube **Assay B** to tube Blank B; and 0.1ml solution from tube **Assay C** to tube Blank C.

5. *Initiate the enzyme reaction:* Add 25 $\mu$ l Enzyme (AP) to only the Assay A enzyme reaction tube and quickly mix by inverting the tube twice. **Immediately**, remove 0.1ml reaction solution and mix with Enzyme Stop Solution in tube A-0m, this is your zero time point. Start the timer or stop watch immediately.
6. Transfer 0.1ml reaction solution every 2 minutes from the assay tube to the corresponding Assay A Enzyme Stop Solution.



- On completion of Assay A, start Assay B by repeating steps 5 and 6 using the Assay B enzyme reaction tube. Once completed, carry out Assay C by repeating steps 5 and 6.



*Instruct each group how to use the spectrophotometer. Instruct students to reuse the assay tubes.*

- Set the spectrophotometer at 410-415nm. Blank the spectrophotometer with Blank A when measuring the absorbance of Assay A tubes. Followed by the Assay B and Assay C. Record the absorbances in the table in the results section.
- Save Blank B for experiments II and III. Save all remaining assay tubes and rinse them with water for the following experiments.

## **II. Effect of pH on alkaline phosphatase activity.**

This experiment uses one concentration (0.5mM) of Substrate Solution to test the effects of pH on enzyme activity.

- Prepare Enzyme Stop Solution:* Label 4 sets of eleven 1.5ml tubes: Mark each set with time course intervals from 0-20 minutes with 2 minute intervals, as follows:

**Assay A:** A-0m, A-2m, A-4m, A-6m, ..., A-20m

**Assay B:** B-0m, B-2m, B-4m, B-6m, ..., B-20m

**Assay C:** C-0m, C-2m, C-4m, C-6m, ..., C-20m

**Assay D:** D-0m, D-2m, D-4m, D-6m, ..., D-20m

- Add 0.1ml Enzyme Stop Solution and 0.8ml distilled water (or pure lab water) to all the Assay A, B, C and D tubes.
- Prepare enzyme reaction tubes:* Label four 1.5ml centrifuge tubes "Assay A", "Assay B", "Assay C" and "Assay D". Set up the following reactions as shown in the table and mix the contents by pipetting up and down several times:

| Assay#.        | Substrate Solution (5mM) | Enzyme Assay Buffer |                   |
|----------------|--------------------------|---------------------|-------------------|
|                |                          | (pH)                | Volume ( $\mu$ l) |
| <b>Assay A</b> | 110 $\mu$ l              | pH 5.0              | 1000              |
| <b>Assay B</b> | 110 $\mu$ l              | pH 7.2              | 1000              |
| <b>Assay C</b> | 110 $\mu$ l              | pH 8.0              | 1000              |
| <b>Assay D</b> | 110 $\mu$ l              | pH 9.0              | 1000              |

- Initiate the enzyme reaction:* Add 25 $\mu$ l Enzyme (AP) to only the Assay A enzyme reaction tube and quickly mix by inverting the tube twice. **Immediately**, remove 0.1ml reaction solution and mix with Enzyme Stop Solution in tube A-0m, this is your zero time point. Start the timer or stop watch immediately.

5. Transfer 0.1ml reaction solution every 2 minutes from the assay tube to the corresponding Assay A Enzyme Stop Solution.
6. On completion of Assay A, start Assay B by repeating steps 4 and 5 using the Assay B enzyme reaction tube. Once completed, carry out Assay C by repeating steps 5 and 6, then Assay D.



*Instruct each group how to use the spectrophotometer. Instruct students to reuse the assay tubes.*

7. Set the spectrophotometer at 410-415nm. Blank the spectrophotometer with Blank 2 saved from the first experiment. Measure the absorbance of all tubes and record the values in the table in the results section.
8. Save the blank 2 for following experiment III. Save all assay tubes and rinse them with water for following experiment III.

### **III. Alkaline phosphatase and competitive inhibition.**

This experiment tests the effects of different concentrations of an inhibitor on enzyme activity.

1. *Prepare 1 to 10 serial dilutions of the Enzyme Inhibitor:* The provided 10mM Enzyme Inhibitor needs to be diluted to two additional dilutions (1mM and 0.1mM), as follows:
  - a. Label two 1.5ml tubes with 1.0mM and 0.1mM.
  - b. To each tube, add 180 $\mu$ l Assay Buffer pH 8.0.
  - c. Transfer 20 $\mu$ l Enzyme Inhibitor (10mM) to the 1mM labeled tube and pipette up and down to mix.
  - d. Transfer 20 $\mu$ l from the 1mM Enzyme Inhibitor tube to the 0.1mM labeled tube and pipette up and down to mix.
2. *Prepare Enzyme Stop Solution:* Label 4 sets of eleven 1.5ml tubes: Mark each set with time course intervals from 0-20 minutes with 2 minute intervals, as follows:

**Assay A:** A-0m, A-2m, A-4m, A-6m, ..., A-20m  
**Assay B:** B-0m, B-2m, B-4m, B-6m, ..., B-20m  
**Assay C:** C-0m, C-2m, C-4m, C-6m, ..., C-20m  
**Assay D:** D-0m, D-2m, D-4m, D-6m, ..., D-20m
3. Add 0.1ml Enzyme Stop Solution and 0.8ml distilled water (or pure lab water) to all the Assay A, B, C and D tubes.

4. *Prepare enzyme reaction tubes:* Label four 1.5ml centrifuge tubes “Assay A”, “Assay B”, “Assay C” and “Assay D”. Set up the following reactions as shown in the table and mix the contents by pipetting up and down several times:

| Assay #. | Substrate Solution (5mM) | Enzyme Assay Buffer pH 8.0 | Enzyme Inhibitor   |        |
|----------|--------------------------|----------------------------|--------------------|--------|
|          |                          |                            | Concentration (mM) | Volume |
| Assay A  | 110µl                    | 1010µl                     | 0                  | 0µl    |
| Assay B  | 110µl                    | 900µl                      | 0.1                | 110µl  |
| Assay C  | 110µl                    | 900µl                      | 1.0                | 110µl  |
| Assay D  | 110µl                    | 900µl                      | 10                 | 110µl  |

5. *Initiate the enzyme reaction:* Add 25µl Enzyme (AP) to only the Assay A enzyme reaction tube and quickly mix by inverting the tube twice. **Immediately**, remove 0.1ml reaction solution and mix with Enzyme Stop Solution in tube A-0m, this is your zero time point. Start the timer or stop watch immediately.
6. Transfer 0.1ml reaction solution every 2 minutes from the assay tube to the corresponding Assay A Enzyme Stop Solution.
7. On completion of Assay A, start Assay B by repeating steps 4 and 5 using the Assay B enzyme reaction tube. Once completed, carry out Assay C by repeating steps 5 and 6, then Assay D.



*Instruct each group how to use the spectrophotometer.*

8. Set the spectrophotometer at 410-415nm. Blank the spectrophotometer with Blank 2 saved from the first experiment. Measure the absorbance of all tubes and record the values in the table in the results section.

## RESULTS, ANALYSIS & ASSESSMENT

### I. Alkaline phosphatase Assay & Kinetics

| Substrate Conc. | Absorbance |   |   |   |   |    |    |    |    |    |    |
|-----------------|------------|---|---|---|---|----|----|----|----|----|----|
|                 | 0          | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
| 0.25mM          |            |   |   |   |   |    |    |    |    |    |    |
| 0.5mM           |            |   |   |   |   |    |    |    |    |    |    |
| 1.0mM           |            |   |   |   |   |    |    |    |    |    |    |

Plot Absorbance vs. Time for each concentration on a graph. Include all concentrations on the same graph for easy comparison

Q. Describe which part of the graphs refer to the rate of the enzyme and the  $V_{max}$  of the reaction.

*The rate of the enzyme is the gradient of the slope of the enzyme. The  $V_{max}$  is the plateau of the curve, where it flattens out.*

Q. Describe the effects of different concentrations of substrate on enzyme activity, discuss the effect on the rate of the enzyme,  $V_{max}$  and the rate to reach  $V_{max}$ .

*With increasing substrate concentration there is an increase in the rate of the reaction due to more substrate being converted to product by the enzyme present. At low concentrations not all the enzyme is working as there is not enough substrate.*

*With increasing concentration of substrate the  $V_{max}$  of the reaction is reached quicker as the more substrate the faster the enzyme active sites are filled.*

**II. Effect of pH on alkaline phosphatase activity.**

| pH  | Absorbance |   |   |   |   |    |    |    |    |    |    |
|-----|------------|---|---|---|---|----|----|----|----|----|----|
|     | 0          | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
| 5.0 |            |   |   |   |   |    |    |    |    |    |    |
| 7.2 |            |   |   |   |   |    |    |    |    |    |    |
| 8.0 |            |   |   |   |   |    |    |    |    |    |    |
| 9.0 |            |   |   |   |   |    |    |    |    |    |    |

Plot Absorbance vs. Time for each concentration on a graph. Include all curves on the same graph for easy comparison

Q. Describe the effects of pH on the enzyme activity, what is the optimal pH for the enzyme?

*Enzymes are sensitive to pH and the graph shows that acidic and neutral pH decreases the activity of the enzyme. This is expected as the enzyme used in the assay is Alkaline Phosphatase, an enzyme with optimal activity in alkaline conditions. The optimal activity is pH8.0.*

Q. What other environmental factors can affect enzyme activity?

*Other factors include temperature and pressure. Chemicals can also affect activity due to inhibition of the enzyme.*

### III. Alkaline phosphatase and competitive inhibition.

| Inhibitor<br>Concentration (mM) | Absorbance |   |   |   |   |    |    |    |    |    |    |
|---------------------------------|------------|---|---|---|---|----|----|----|----|----|----|
|                                 | 0          | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
| 0                               |            |   |   |   |   |    |    |    |    |    |    |
| 0.1                             |            |   |   |   |   |    |    |    |    |    |    |
| 1.0                             |            |   |   |   |   |    |    |    |    |    |    |
| 10.0                            |            |   |   |   |   |    |    |    |    |    |    |

Make a graph of Absorbance vs. Time for each concentration of substrate with the different inhibitor concentrations.

Q. Describe the effects of the inhibitor on the enzyme?

Q. Describe an experiment to determine if the inhibitor used is a competitive or non-competitive inhibitor.

*Select a single concentration of inhibitor and incubate it with increasing amounts of substrate in the presence of the enzyme. If increasing concentrations of substrate prevent inhibition then the inhibitor is a competitive inhibitor.*

Last saved: 9/5/2012 CMH

*This page is intentionally left blank*



[www.GBiosciences.com](http://www.GBiosciences.com)





G-Biosciences ♦ 1-800-628-7730 ♦ 1-314-991-6034 ♦ [technical@GBiosciences.com](mailto:technical@GBiosciences.com)

A Geno Technology, Inc. (USA) brand name

# Enzyme Analysis

## Student's Handbook

(Cat. # BE-405)



think proteins! think G-Biosciences [www.GBiosciences.com](http://www.GBiosciences.com)

|   |    |
|---|----|
| OBJECTIVES.....   | 3  |
| BACKGROUND .....  | 3  |
| ENZYME ASSAY AND KINETICS.....                            | 3  |
| ENZYME INHIBITION .....                                   | 4  |
| ALKALINE PHOSPHATASE ASSAY AND KINETICS .....             | 4  |
| MATERIALS FOR EACH GROUP .....                            | 5  |
| PROCEDURE.....  | 6  |
| I. ALKALINE PHOSPHATASE ASSAY & KINETICS.....             | 6  |
| II. EFFECT OF PH ON ALKALINE PHOSPHATASE ACTIVITY.....    | 8  |
| III. ALKALINE PHOSPHATASE AND COMPETITIVE INHIBITION..... | 10 |
| RESULTS, ANALYSIS & ASSESSMENT .....                      | 12 |
| I. ALKALINE PHOSPHATASE ASSAY & KINETICS .....            | 12 |
| II. EFFECT OF PH ON ALKALINE PHOSPHATASE ACTIVITY.....    | 13 |
| III. ALKALINE PHOSPHATASE AND COMPETITIVE INHIBITION..... | 14 |

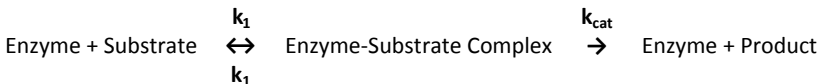
## OBJECTIVES

- To understand the principle of enzyme assays and kinetics.
- To investigate action of an enzyme.
- To see the effect of pH on enzyme activity.
- To see the effect of an enzyme inhibitor on enzyme activity.
- To establish enzyme importance in disease conditions.

## BACKGROUND

Enzymes are biocatalysts that increase the rate of otherwise slow reactions by decreasing the reactions activation energy, without undergoing any net change in their structures at the end of a reaction. They are mostly protein in nature and mediate all synthesis and degradation reactions carried out by living organisms. Ribozyme is a RNA molecule that has enzymatic properties. Enzymes catalyze almost all the biological reactions, converting a target molecule (the substrate) into product(s). Enzymes are considered as an integral part to biological systems because they allow the body to carry out reactions that normally would require too much energy to initiate.

An enzyme acts on a substrate to form an enzyme-substrate complex, which then forms a new product and the free enzyme. The active site of an enzyme, where the substrate binds, only recognizes the specific substrate and holds it in a set confirmation. The enzyme activity is a measure of the rate or velocity of the conversion of the substrate to the product per unit time by an enzyme. The enzymes generally work under mild conditions of temperature, pressure and pH that decrease the energy requirement.



The following terms are used to define enzyme more specifically:

- **Apoenzyme** - The protein portion of an enzyme is called the apoenzyme.
- **Cofactor** - It is the non-protein part of an enzyme. Cofactors can be loosely bound, **coenzymes**, or tightly bound, **prosthetic groups**.
- **Holoenzyme**- The complete enzyme (apoprotein + cofactor) is termed the Holoenzyme.

### *Enzyme Assay and Kinetics*

Enzyme kinetics, which refers to the rate of an enzyme-catalyzed reaction, can be affected by numerous factors, including enzyme, substrate concentration, pH and inhibitors. When an enzyme concentration is kept constant in a system, increasing the substrate concentration leads to an increase in reaction rate until a maximum velocity,  $V_{\text{max}}$ , is reached. The substrate concentration at which the velocity is  $\frac{1}{2} V_{\text{max}}$  is defined as the Michaelis constant,  $K_m$ . At low substrate concentrations, many of the enzyme active sites are empty and readily bind to substrate. The reaction rate stabilizes when

no more active sites are open. Similarly, increasing the enzyme concentration, while the substrate amount remains constant, causes an increase in the rate of the reaction until a maximum point is reached where all enzyme is bound to the substrate present. Precautions should be taken while handling enzymes, since parameters like pH, temperature, and presence of ions affect the enzyme activity.

### **Enzyme Inhibition**

Enzyme inhibitors act to decrease the rate of an enzyme reaction. The following are examples of enzyme inhibition:

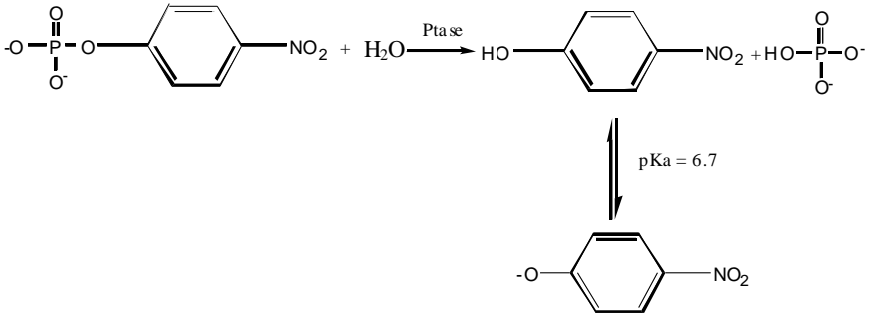
Competitive inhibition occurs when the inhibitor molecule binds to the active site of the enzyme. As a result, the substrate and inhibitor compete for the same spot.

Competitive inhibition can be overcome by increasing the substrate concentration so that it is much greater than the inhibitor concentration.

Uncompetitive inhibition, however, occurs when the inhibitor molecule binds to a site on the enzyme other than the active site and results in a conformational change of the enzyme resulting in an inability to bind the substrate. Thus, inhibition is not removed by increasing substrate concentration because they are not competing for the same spot.

Noncompetitive inhibition occurs when the inhibitor binds both the active site of the enzyme and the enzyme-substrate complex. One way to measure the degree of inhibition is by calculating the inhibitor constant  $K_i$ , which is the concentration of inhibitor that causes a 2 fold increase in the slope of the  $1/(\text{substrate concentration})$  versus  $1/(\text{velocity})$  plot, also known as the Lineweaver-Burk plot.

### **Alkaline phosphatase Assay and Kinetics**



Alkaline phosphatase is an enzyme that is found in the blood, intestine, liver, and bone cells of the human body. Like all enzymes, it is needed in small amounts to trigger specific chemical reactions. However, high levels of this enzyme in the body may be due to a variety of factors, which include bone fracture healing, liver diseases, bone diseases, hepatitis, or skeletal diseases; low levels may be indicative of poor nutrition, magnesium deficiency, or protein deficiency. Thus, alkaline phosphatase plays a crucial role in the body.

Alkaline phosphatase catalyzes the hydrolysis of phosphate monoesters to give inorganic phosphate and the corresponding alcohol, phenol, or sugar. As indicated by the name of this enzyme, catalytic activity is higher in the alkaline pH range. A convenient assay for the activity of alkaline phosphatase uses p-nitrophenylphosphate (pNPP), a colored chromogenic substrate. Hydrolysis of this phosphate monoester gives inorganic phosphate and p-nitrophenol (pNP). The colored end product of this reaction is measured with the help of a standard plot, the amount of product formed is calculated and from that measurement, the amount of alkaline phosphatase in the given sample is determined. This reaction is shown below:

### **MATERIALS FOR EACH GROUP**

Supply each group with the following components. Several components are to be shared by the whole class and should be kept on a communal table.

- 4 cuvettes
- 48 1.5ml Tubes
- Marker Pen
- 1 vial 300 $\mu$ l Enzyme (AP)
- 1 vial 1.5ml Substrate Solution
- 1 tube 9ml Enzyme Assay Buffer pH 8.0
- 1 vial 1ml Enzyme Assay Buffer pH 9.0
- 1 vial 1ml Enzyme Assay Buffer pH 7.2
- 1 vial 1ml Enzyme Assay Buffer pH 5.0
- 1 vial 200 $\mu$ l Enzyme Inhibitor (10mM)
- 1 tube 13ml Enzyme Stop Solution
- 100ml distilled water (or pure lab water)

## PROCEDURE



Do **NOT** add enzyme until ready to start your timer.

### I. Alkaline phosphatase Assay & Kinetics.

The aim is to follow the kinetics of alkaline phosphatase enzyme using three different substrate concentrations over a 20 minute period.

1. *Prepare Enzyme Stop Solution:* Label three sets of twelve 1.5ml centrifuge tubes for each assay of different substrate concentrations. Mark each set with a blank and time course intervals from 0-20 minutes with 2 minute intervals, as follows:

**Assay A:** Blank A, A-0m, A-2m, A-4m, A-6m, ..., A-20m

**Assay B:** Blank B, B-0m, B-2m, B-4m, B-6m, ..., B-20m

**Assay C:** Blank C, C-0m, C-2m, C-4m, C-6m, ..., C-20m

2. Add 0.1ml Enzyme Stop Solution and 0.8ml distilled water (or pure lab water) to all the Assay A, B and C tubes.
3. *Prepare enzyme reaction tubes:* Label three 1.5ml centrifuge tubes "Assay A", "Assay B", and "Assay C". Set up the following reactions as shown in the table and mix the contents by pipetting up and down several times.

| Assay # | Substrate Solution (5mM) |                          | Enzyme Assay Buffer (pH8.0) |
|---------|--------------------------|--------------------------|-----------------------------|
|         | Volume ( $\mu$ l)        | Final Concentration (mM) | Volume ( $\mu$ l)           |
| Assay A | 60                       | 0.25                     | 1160                        |
| Assay B | 120                      | 0.50                     | 1100                        |
| Assay C | 240                      | 1.00                     | 980                         |

4. *Prepare reaction blanks:* Transfer 0.1ml solution from the above enzyme reaction tubes to the appropriate tubes marked Blank and mix. They are your reaction controls or blanks.

0.1ml solution from tube **Assay A** to tube Blank A; 0.1ml solution from tube **Assay B** to tube Blank B; and 0.1ml solution from tube **Assay C** to tube Blank C.

5. *Initiate the enzyme reaction:* Add 25 $\mu$ l Enzyme (AP) to only the Assay A enzyme reaction tube and quickly mix by inverting the tube twice. **Immediately**, remove 0.1ml reaction solution and mix with Enzyme Stop Solution in tube A-0m, this is your zero time point. Start the timer or stop watch immediately.
6. Transfer 0.1ml reaction solution every 2 minutes from the assay tube to the corresponding Assay A Enzyme Stop Solution.

7. On completion of Assay A, start Assay B by repeating steps 5 and 6 using the Assay B enzyme reaction tube. Once completed, carry out Assay C by repeating steps 5 and 6.



*Instruct each group how to use the spectrophotometer. Instruct students to reuse the assay tubes.*

8. Set the spectrophotometer at 410-415nm. Blank the spectrophotometer with Blank A when measuring the absorbance of Assay A tubes. Followed by the Assay B and Assay C. Record the absorbances in the table in the results section.
9. Save Blank B for experiments II and III. Save all remaining assay tubes and rinse them with water for the following experiments.

## II. Effect of pH on alkaline phosphatase activity.

This experiment uses one concentration (0.5mM) of Substrate Solution to test the effects of pH on enzyme activity.

1. *Prepare Enzyme Stop Solution:* Label 4 sets of eleven 1.5ml tubes: Mark each set with time course intervals from 0-20 minutes with 2 minute intervals, as follows:

**Assay A:** A-0m, A-2m, A-4m, A-6m, ..., A-20m

**Assay B:** B-0m, B-2m, B-4m, B-6m, ..., B-20m

**Assay C:** C-0m, C-2m, C-4m, C-6m, ..., C-20m

**Assay D:** D-0m, D-2m, D-4m, D-6m, ..., D-20m

2. Add 0.1ml Enzyme Stop Solution and 0.8ml distilled water (or pure lab water) to all the Assay A, B, C and D tubes.
3. *Prepare enzyme reaction tubes:* Label four 1.5ml centrifuge tubes "Assay A", "Assay B", "Assay C" and "Assay D". Set up the following reactions as shown in the table and mix the contents by pipetting up and down several times:

| Assay#. | Substrate Solution (5mM) | Enzyme Assay Buffer |                   |
|---------|--------------------------|---------------------|-------------------|
|         |                          | (pH)                | Volume ( $\mu$ l) |
| Assay A | 110 $\mu$ l              | pH 5.0              | 1000              |
| Assay B | 110 $\mu$ l              | pH 7.2              | 1000              |
| Assay C | 110 $\mu$ l              | pH 8.0              | 1000              |
| Assay D | 110 $\mu$ l              | pH 9.0              | 1000              |

4. *Initiate the enzyme reaction:* Add 25 $\mu$ l Enzyme (AP) to only the Assay A enzyme reaction tube and quickly mix by inverting the tube twice. **Immediately**, remove 0.1ml reaction solution and mix with Enzyme Stop Solution in tube A-0m, this is your zero time point. Start the timer or stop watch immediately.
5. Transfer 0.1ml reaction solution every 2 minutes from the assay tube to the corresponding Assay A Enzyme Stop Solution.
6. On completion of Assay A, start Assay B by repeating steps 4 and 5 using the Assay B enzyme reaction tube. Once completed, carry out Assay C by repeating steps 5 and 6, then Assay D.



*Instruct each group how to use the spectrophotometer. Instruct students to reuse the assay tubes.*

7. Set the spectrophotometer at 410-415nm. Blank the spectrophotometer with Blank 2 saved from the first experiment. Measure the absorbance of all tubes and record the values in the table in the results section.



8. Save the blank 2 for following experiment III. Save all assay tubes and rinse them with water for following experiment III.

### III. Alkaline phosphatase and competitive inhibition.

This experiment tests the effects of different concentrations of an inhibitor on enzyme activity.

1. *Prepare 1 to 10 serial dilutions of the Enzyme Inhibitor:* The provided 10mM Enzyme Inhibitor needs to be diluted to two additional dilutions (1mM and 0.1mM), as follows:
  - a. Label two 1.5ml tubes with 1.0mM and 0.1mM.
  - b. To each tube, add 180 $\mu$ l Assay Buffer pH 8.0.
  - c. Transfer 20 $\mu$ l Enzyme Inhibitor (10mM) to the 1mM labeled tube and pipette up and down to mix.
  - d. Transfer 20 $\mu$ l from the 1mM Enzyme Inhibitor tube to the 0.1mM labeled tube and pipette up and down to mix.
2. *Prepare Enzyme Stop Solution:* Label 4 sets of eleven 1.5ml tubes: Mark each set with time course intervals from 0-20 minutes with 2 minute intervals, as follows:

**Assay A:** A-0m, A-2m, A-4m, A-6m, ..., A-20m

**Assay B:** B-0m, B-2m, B-4m, B-6m, ..., B-20m

**Assay C:** C-0m, C-2m, C-4m, C-6m, ..., C-20m

**Assay D:** D-0m, D-2m, D-4m, D-6m, ..., D-20m

3. Add 0.1ml Enzyme Stop Solution and 0.8ml distilled water (or pure lab water) to all the Assay A, B, C and D tubes.
4. *Prepare enzyme reaction tubes:* Label four 1.5ml centrifuge tubes "Assay A", "Assay B", "Assay C" and "Assay D". Set up the following reactions as shown in the table and mix the contents by pipetting up and down several times:

| Assay #. | Substrate Solution (5mM) | Enzyme Assay Buffer pH 8.0 | Enzyme Inhibitor   |             |
|----------|--------------------------|----------------------------|--------------------|-------------|
|          |                          |                            | Concentration (mM) | Volume      |
| Assay A  | 110 $\mu$ l              | 1010 $\mu$ l               | 0                  | 0 $\mu$ l   |
| Assay B  | 110 $\mu$ l              | 900 $\mu$ l                | 0.1                | 110 $\mu$ l |
| Assay C  | 110 $\mu$ l              | 900 $\mu$ l                | 1.0                | 110 $\mu$ l |
| Assay D  | 110 $\mu$ l              | 900 $\mu$ l                | 10                 | 110 $\mu$ l |

5. *Initiate the enzyme reaction:* Add 25 $\mu$ l Enzyme (AP) to only the Assay A enzyme reaction tube and quickly mix by inverting the tube twice. **Immediately**, remove 0.1ml reaction solution and mix with Enzyme Stop Solution in tube A-0m, this is your zero time point. Start the timer or stop watch immediately.

6. Transfer 0.1ml reaction solution every 2 minutes from the assay tube to the corresponding Assay A Enzyme Stop Solution.
7. On completion of Assay A, start Assay B by repeating steps 4 and 5 using the Assay B enzyme reaction tube. Once completed, carry out Assay C by repeating steps 5 and 6, then Assay D.



*Instruct each group how to use the spectrophotometer.*

8. Set the spectrophotometer at 410-415nm. Blank the spectrophotometer with Blank 2 saved from the first experiment. Measure the absorbance of all tubes and record the values in the table in the results section.

## RESULTS, ANALYSIS & ASSESSMENT

### I. Alkaline phosphatase Assay & Kinetics

| Substrate Conc. | Absorbance |   |   |   |   |    |    |    |    |    |    |
|-----------------|------------|---|---|---|---|----|----|----|----|----|----|
|                 | 0          | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
| 0.25mM          |            |   |   |   |   |    |    |    |    |    |    |
| 0.5mM           |            |   |   |   |   |    |    |    |    |    |    |
| 1.0mM           |            |   |   |   |   |    |    |    |    |    |    |

Plot Absorbance vs. Time for each concentration on a graph. Include all concentrations on the same graph for easy comparison

Q. Describe which part of the graphs refer to the rate of the enzyme and the  $V_{max}$  of the reaction.

---

---

---

---

Q. Describe the effects of different concentrations of substrate on enzyme activity, discuss the effect on the rate of the enzyme,  $V_{max}$  and the rate to reach  $V_{max}$ .

---

---

---

---

---

---

---

---

**II. Effect of pH on alkaline phosphatase activity.**

| pH  | Absorbance |   |   |   |   |    |    |    |    |    |    |
|-----|------------|---|---|---|---|----|----|----|----|----|----|
|     | 0          | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
| 5.0 |            |   |   |   |   |    |    |    |    |    |    |
| 7.2 |            |   |   |   |   |    |    |    |    |    |    |
| 8.0 |            |   |   |   |   |    |    |    |    |    |    |
| 9.0 |            |   |   |   |   |    |    |    |    |    |    |

Plot Absorbance vs. Time for each concentration on a graph. Include all curves on the same graph for easy comparison

Q. Describe the effects of pH on the enzyme activity, what is the optimal pH for the enzyme?

---

---

---

---

---

---

---

---

---

---

Q. What other environmental factors can affect enzyme activity?

---

---

---

---

**III. Alkaline phosphatase and competitive inhibition.**

| Inhibitor<br>Concentration (mM) | Absorbance |   |   |   |   |    |    |    |    |    |    |
|---------------------------------|------------|---|---|---|---|----|----|----|----|----|----|
|                                 | 0          | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
| 0                               |            |   |   |   |   |    |    |    |    |    |    |
| 0.1                             |            |   |   |   |   |    |    |    |    |    |    |
| 1.0                             |            |   |   |   |   |    |    |    |    |    |    |
| 10.0                            |            |   |   |   |   |    |    |    |    |    |    |

Make a graph of Absorbance vs. Time for each concentration of substrate with the different inhibitor concentrations.

Q. Describe the effects of the inhibitor on the enzyme?

---

---

---

---

Q. Describe an experiment to determine if the inhibitor used is a competitive or non-competitive inhibitor.

---

---

---

---

---

---

---

---

---

*This page is intentionally left blank*



[www.GBiosciences.com](http://www.GBiosciences.com)