5 PROTEINS: THE KINETIC PROPERTIES OF WHEAT GERM ACID PHOSPHATASE

Enzymes are a specific class of proteins that catalyze the myriad biochemical reactions of the living cell. The precise number of enzymes in any given cell is not known but must be well into the thousands considering the vast number of reactions in the metabolic pathways. Although the details are not all worked out, it appears that an enzyme functions by forming a complex with the substrate molecule, thereby lowering the activation energy necessary to convert substrate to product. The sequence of events for a simple enzymatic reaction involving a single substrate and a single product is illustrated in Figure 5-1. Since it acts as a catalyst, the enzyme is unchanged by the reaction, its role being to speed up a process that is thermodynamically possible.

The speed or rate of the enzyme–catalyzed reaction can be determined by measuring the amount of substrate used up or product generated per unit time. This rate is called reaction velocity (v) and is most commonly expressed in terms of micromoles of product made per minute (μmoles P/minute). Since the rate of enzymatic reactions is of importance to the overall economy of the cell, it is of real significance that the biochemist learn as much as possible about the factors that affect the velocity of these reactions.

Enzyme Concentration.

When all other factors (temperature, substrate concentration, etc.) are held constant and an enzymatic reaction is performed at progressively higher concentrations of enzyme, a characteristic pattern emerges (Figure 5-2). At low enzyme concentrations, the relationship is a linear one such that each higher level of enzyme yields a correspondingly higher rate of reaction. This relationship does not hold at the highest enzyme concentrations, however, because the enzyme molecules overwhelm the substrate molecules and no further velocity increases are observed. Thus, the linear portion of the graph illustrated (from 0 to 4 mg/ml) delimits the range of enzyme concentrations that yield valid results at the given substrate concentration.

Substrate Concentration

As might reasonably be expected, there is also a direct correlation between substrate concentration and reaction velocity. As the substrate level is increased, the velocity increases in a hyperbolic fashion (Figure 5-3). The cause for this was first elaborated by Michaelis and Menten. The shape of this curve is best explained on the basis of the degree to which substrate molecules engage the active site(s) of the enzyme. At low substrate concentration, there are many more active sites than substrate molecules; consequently they are processed with little delay, and with each increase in substrate level there is a dramatic increase in velocity. There is eventually a point, however, at which the number of substrate molecules exceeds the number of active sites; at that point the enzyme is saturated and added substrate has little effect on the reaction velocity. The mathematical expression for this curve is called
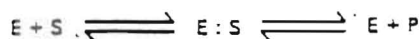
$$E + S \rightleftharpoons E:S \rightleftharpoons E + P$$

Fig. 5-1. A simple enzymatic reaction, wherein E represents enzyme, S the substrate, P the product, and E:S the one-substrate complex.
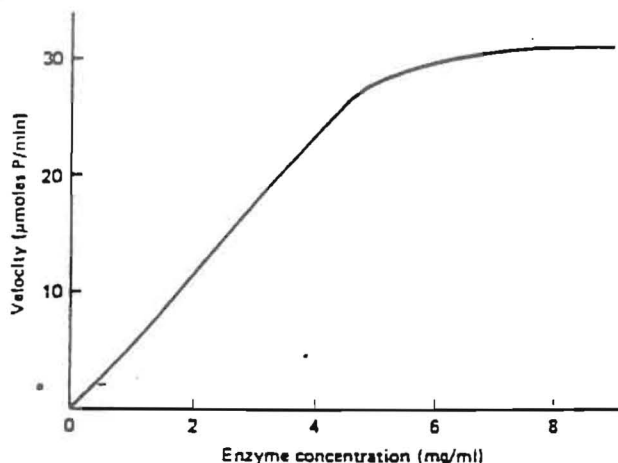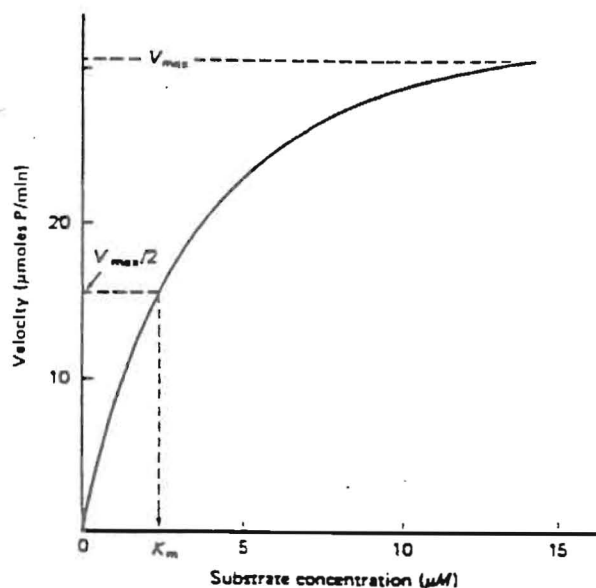

Fig. 5-2. The relationship between enzyme concentration and reaction velocity.

Fig. 5-3. The effect of increasing substrate concentration on reaction velocity. This is an example of classic Michaelis-Menten enzyme kinetics.

the Michaelis-Menten equation, wherein v equals the initial velocity, V_{max} equals the maximum initial velocity, [S] is the molar concentration of the substrate, and K_m is the Michaelis constant:

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

Approximate values for V_{max} and K_m can be determined graphically from the Michaelis-Menten curve (Figure 5-3). V_{max} is the maximum velocity attained under the prevailing conditions (32 μmoles P/minute for the case illustrated in Figure 5-3), and K_m is the substrate concentration at one half V_{max} ($V_{max}/2$)—in this case, about 2.3 μM. The K_m is useful because it indicates the affinity of the enzyme for the substrate; a low K_m indicating a high attraction between reaction components, and a higher K_m is evidence of a low affinity.

More precise values for K_m and V_{max} may be obtained if the same experimental data are displayed graphically in the double-reciprocal plot of Lineweaver and Burke (Figure 5-4). The reciprocal of the velocity ($1/v$) plotted against the reciprocal of the substrate concentration ($1/[S]$) yields a straight line with slope K_m/V_{max}.

V_{max} is equal to the reciprocal of the y intercept (35.7 μmoles P/minute in this case), and Km is equal to the negative reciprocal of the x intercept (3.13 μM substrate). Since these results for K_m and V_{max} are determined from the x and y intercepts of a straight line, they are considered to be more reliable than those obtained by approximation from the Michaelis-Menten curve (Figure 5-3). The equation for the Lineweaver-Burke plot is obtained by taking the reciprocal of both sides of the Michaelis-Menten equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$$

The information accumulated from enzyme and substrate concentration trials is extremely useful in designing subsequent experiments. One can now set up assay conditions with a level of substrate that is not limiting and a level of enzyme that yields a linear response at that substrate concentration. For the hypothetical enzymatic reaction described thus far, ideal assay conditions might include a substrate concentration of 15.0 μM and an enzyme concentration of 4.0 mg/ml.
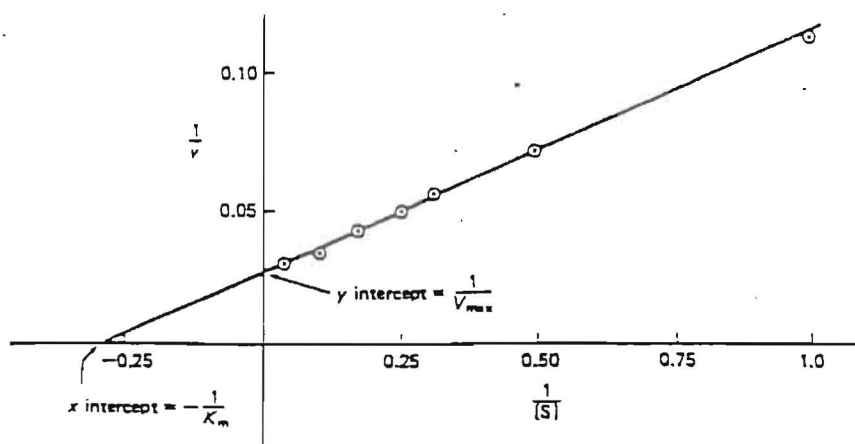
Fig. 5-4. The data presented in Figure 5-3 plotted in the double-reciprocal fashion of Lineweaver and Burke.

Enzyme Inhibitors

There exist a number of molecular species which, in the presence of an enzyme and its substrate, have the effect of binding to the enzyme (or to the enzyme-substrate complex) and totally or partially inhibiting the reaction. In those cases where the binding is irreversible, the reaction is inalterably inhibited and not subject to kinetic analysis. If the binding is reversible, however, the specific type of inhibition can be determined by kinetic analysis.

The three types of inhibition that can be clearly distinguished in this manner are competitive, noncompetitive, and uncompetitive. Experimentally, these are distinguished by performing the enzymatic reaction in the presence of a constant amount of the inhibitor at ever-increasing concentrations of the substrate. When the inhibited reaction is compared with the normal reaction using the graphic analyses of Michaelis and Menten or Lineweaver and Burke, the type of inhibition is clearly indicated. In the case of competitive inhibition, high substrate concentrations wipe out the inhibitory effect and the V_{max} for the inhibited reaction is identical to that for the uninhibited reaction (Figure 5-5).

The K_m of the inhibited reaction (K_i) is significantly higher than that of the reaction run in the absence of inhibitor, which indicates an apparent decrease in the affinity of the enzyme for its substrate. Noncompetitive inhibition yields the curve indicated in Figure 5-6, with a lower V_{max} and a K_m (K_i) identical to that of the reaction in the absence of inhibitor.

Uncompetitive inhibition is characterized by a lower V_{max}, a higher K_m, and a Michaelis-Menten curve quite similar to that of noncompetitive inhibition. The best

Fig. 5-5. A Michaelis-Menten plot for an enzymatic reaction performed in the presence (– · – · –) and absence (——) of a competitive inhibitor.
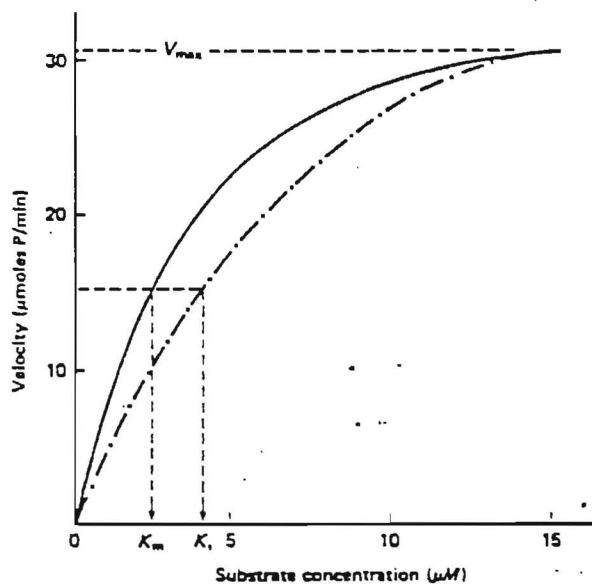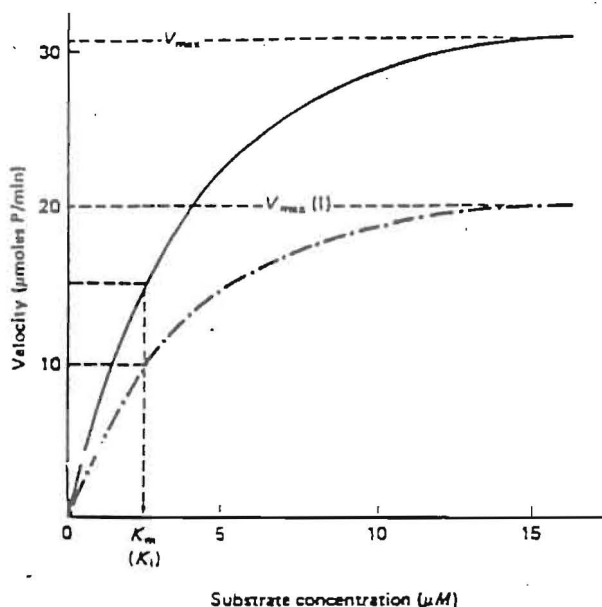
Fig. 5-6. A Michaelis-Menten plot for an enzymatic reaction performed in the presence (−·−·−) and absence (——) of a noncompetitive inhibitor.

way to distinguish the three types of inhibition graphically is to use the Lineweaver-Burke plot (Figure 5-7). Notice that in the case of uncompetitive inhibition the slope of the inhibited curve (K_m/V_{max}) is the same as that of the noninhibited curve, whereas in the other two types of inhibition, the slope of the inhibited plot is greater.

In summary, it is relatively simple to distinguish the three types of reversible inhibition by comparing the Michaelis-Menten and Lineweaver-Burke kinetics in the presence and absence of the inhibitor.

Hydrogen Ion Concentration

As mentioned earlier, enzymes are extremely sensitive to variations in pH. A change in the hydrogen ion concentration alters the charge on the amino acids of the protein, which alters the attractive forces governing the three-dimensional shape of the enzyme. Such a conformational change in the enzyme is usually reflected in the ability of the enzyme to catalyze its reaction. A typical pH curve (Figure 5-8) exhibits a peak of enzymatic activity, termed the pH optimum, and a rather precipi-

Fig. 5-7. A Lineweaver-Burke plot of an enzymatic reaction performed in the absence of inhibitor (——) and in the presence of competitive (− − − −), noncompetitive (−·−·−), and uncompetitive (····) inhibitors.
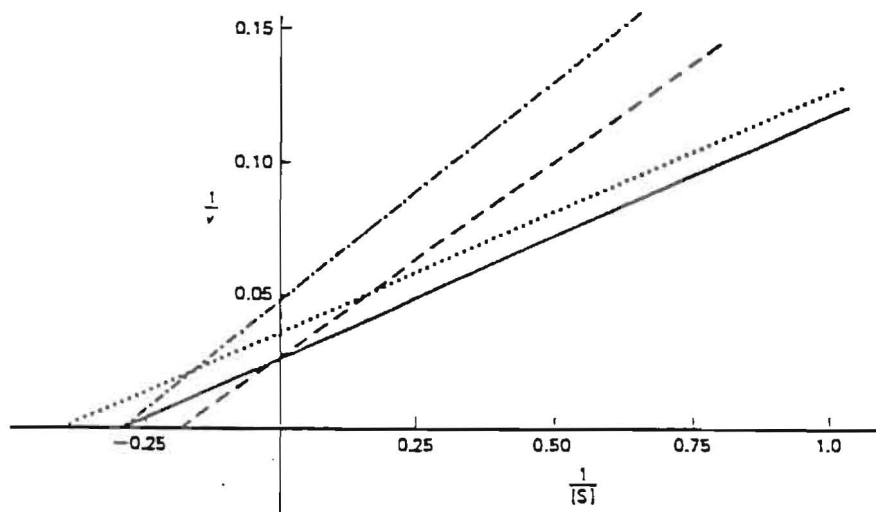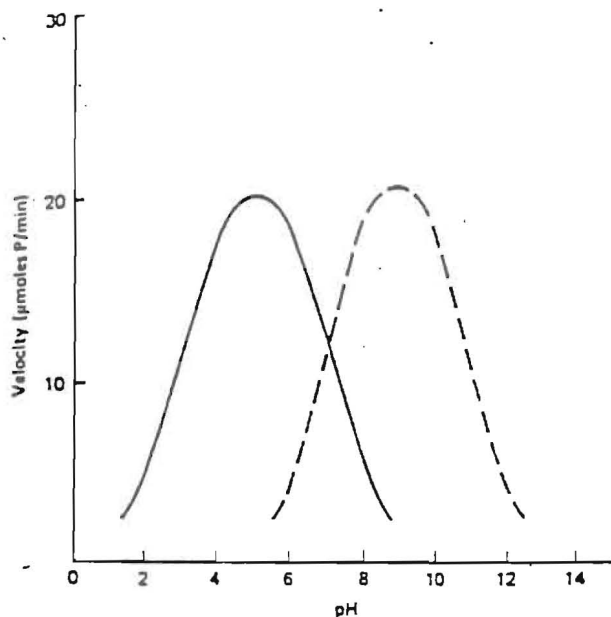
Fig. 5-8. The effect of different pH values on the reaction velocity of two different enzymes. One of the enzymes (———) has an acid pH optimum (5.0) and the other a basic pH optimum (9.0).

tous decline in activity on either side of the peak. The two curves depicted in Figure 5-8 are typical of enzymes such as acid phosphatase and alkaline phosphatase, which both catalyze the same reaction but differ in pH optimum. The sharp decline in enzymatic activity on either side of the optimum underscores the importance of maintaining an appropriately buffered solution when working with living systems *in vitro*.

Temperature

Temperature also has a profound effect on enzymatic reaction rate. At low temperatures, the enzyme is relatively inactive, which explains why ice-bath temperatures are often used in the isolation of proteins and other macromolecules sensitive to hydrolytic enzymes. At higher temperatures, there is a concomitant increase in reaction rate resulting from increased molecular motion, which continues until the optimum temperature is attained. For the majority of enzymes, activity rapidly

declines beyond that point as a result of heat denaturation of the protein (Figure 5-9).

Acid Phosphatase

The acid phosphatase you previously isolated from wheat germ will now be the object of detailed kinetic analysis. The series of experiments is designed to determine the effect of various factors (temperature, substrate concentration, etc.) on the velocity of the acid phosphatase reaction. There are five separate experiments, and you are expected to complete all of them during the next two laboratory periods. It is suggested that the experiments be performed in the following sequence:

Day 1. Time course
       Substrate concentration
       Phosphate inhibition
Day 2. Enzyme concentration
       Temperature effects

For all of these experiments, use the acid phosphatase assay described on pp. 30 to 31. Under acid con-

Fig. 5-9. The effect of different temperatures on enzymatic reaction velocity. The optimum temperature for this reaction is 30°C.
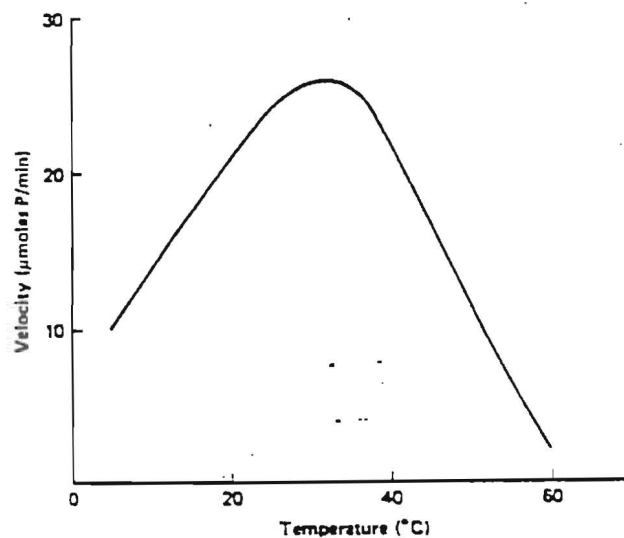
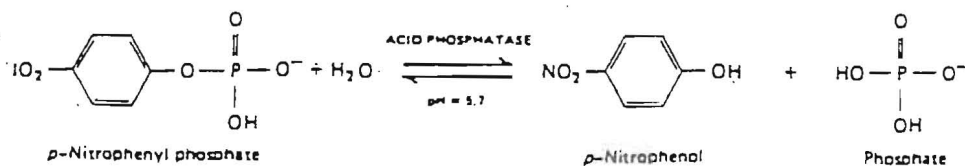Fig. 5-10. The hydrolysis of p-nitrophenyl phosphate by acid phosphatase.

ditions, the enzyme catalyzes the hydrolysis of p-nitrophenyl phosphate to inorganic phosphate and p-nitrophenol (Figure 5-10). If base is added to the mixture after the completion of the reaction, the p-nitrophenol is converted to a colored form which absorbs light at 405 nm. Assuming a path length of 1.0 cm and an extinction coefficient of 18.8×10^3 liter $mole^{-1}cm^{-1}$, you can use the absorbance at 405 nm to calculate the number of micromoles of p-nitrophenol released. Since this is a fixed-time assay that is stopped after 5 minutes, the velocity of the reaction (μmoles of p-nitrophenol/minute) can be easily computed.

Each of the following experiments is done in fundamentally the same fashion, except that one factor, such as temperature or enzyme concentration, is altered to determine the effect of that parameter. Each experiment must be performed a total of three times to provide enough data for valid conclusions.

The basic assay is performed in the following manner:

1. Prepare and label the required number of reaction tubes, and to each of them add 0.5 ml of 1.0 M sodium acetate buffer (pH 5.7), 0.5 ml of 0.1 M MgCl$_2$, 0.5 ml of 0.05 M p-nitrophenyl phosphate, and 3.3 ml of distilled water.
2. Add 0.2 ml of the appropriately diluted* enzyme solution and start a stopwatch.
3. Incubate the reaction tubes at 37°C for 5 minutes.
4. Stop the reaction by adding 2.5 ml of 0.5 M KOH.
5. If a cloudy precipitate forms, it should be removed by a brief centrifugation at top speed in a desktop clinical centrifuge.
6. Set the colorimeter at 405 nm, adjust to zero ab-

*An appropriate dilution of acid phosphatase yields an A_{405} of about 0.3 in 5 minutes under these assay conditions.

sorbance with a blank tube, and determine the A_{405} of all assay tubes. The blank tube will differ from experiment to experiment, depending on conditions. In some cases, it will be the reaction tube lacking enzyme; in others, it will be the reaction stopped immediately after the addition of enzyme.

Kinetic Properties I.
Time Course of the Reaction

Introduction
The assay you are using is called a fixed-time assay because the reaction is stopped after 5 minutes and the velocity is calculated assuming that the relationship between product yield and time has been linear throughout. The object of this exercise is to demonstrate the validity of that assumption. Set up a series of identical enzyme reaction tubes, each of which is allowed to incubate for a different period of time (zero through 30 minutes). The results should indicate how long the reaction is linear under the given conditions of substrate and enzyme concentration.

Procedure
1. Prepare a series of seven reaction tubes labeled 0 through 30 minutes at 5-minute intervals (0, 5, 10, . . . minutes).
2. To each of these tubes add 0.5 ml of 1.0 M sodium acetate buffer (pH 5.7), 0.5 ml of 0.1 M MgCl$_2$, 0.5 ml of 0.05 M p-nitrophenyl phosphate, and 3.3 ml of distilled water.
3. Place all the tubes in a test tube rack situated in a water bath maintained at 37°C and let the temperature equilibrate for 5 minutes.
4. Add 0.2 ml of the enzyme (appropriately diluted acid phosphatase) to the tube marked 0 minutes

and immediately stop the reaction by adding 2.5 ml of 0.5 M KOH. This zero-time tube will serve as the blank against which all the others will be compared.

5. Add 0.2 ml of the enzyme to the tube marked 5 minutes, mix, start the stopwatch, and let the reaction proceed for 5 minutes before adding the KOH to terminate the reaction.

6. Run all of the other reaction tubes in exactly the same fashion with the exception that each successive tube will be incubated for 5 minutes longer than the previous one (total reaction times to equal 0, 5, 10, . . . 30 minutes). An efficient way to do this is to start each reaction at 2-minute intervals, keeping an eye on the stopwatch and stopping each of the reactions at the appropriate time. It is helpful to prepare a schedule of events (Table 5-1) before you begin.

7. After all the reactions have been terminated, determine the absorbance at 405 nm for each sample. The zero-time sample should be used as the blank.

8. Repeat this experiment two more times.

Calculations and Questions

1. Use the extinction coefficient (18.8×10^3 liter mole^{-1} cm^{-1}) for p-nitrophenol to calculate the micromoles of product released at each time point.

2. Average the data for all three trials and prepare a graph, plotting μmoles of p-nitrophenol released against time.

3. Is the time course linear throughout? If the time course is not linear, what are some factors that might contribute to the changed velocity at longer time periods?

Table 5-1.
Schedule for time course experiment

| Total incubation time (min) | Clock time (min) | |
	Start reaction (add enzyme)	Stop reaction (add KOH)
0	0	0
5	0	5
10	2	12
15	4	19
20	6	26
25	8	33
30	10	40

4. Is the 5-minute fixed-time assay valid for acid phosphatase? If not, how should it be changed?

5. Determine the initial velocity (v) for the acid phosphatase reaction from the slope of the linear part of the graph.

Kinetic Properties II.
The Effect of Different Substrate Concentrations on Reaction Velocity

Introduction

The object of this exercise is to demonstrate the effect of performing the standard 5-minute assay in the presence of substrate concentrations ranging from 0 to 5.0 mM p-nitrophenyl phosphate. The results should provide classic Michaelis-Menten data from which approximations of V_{max} and K_m can be made. Double-reciprocal plots of the same data should be done to arrive at even more exact values for K_m and V_{max}.

Procedure

1. Prepare a series of substrate dilutions according to the protocol outlined in Table 5-2. The amount of each sample is sufficient to do both this experiment and the next (Kinetic Properties III), which requires the same range of substrate concentrations. Do not discard these samples until both experiments have been completed.

2. Set up eight assay tubes labeled according to the various substrate concentrations. To each of these tubes add 0.5 ml of 1.0 M sodium acetate buffer

Table 5-2.
Protocol for the dilution of substrate (P-nitrophenyl phosphate)

Tube	0.05 M PNPP* (ml)	Distilled water (ml)	Concentration of dilute PNPP (M)
A	0	5.0	0
B	0.05	4.95	0.0005
C	0.10	4.90	0.0010
D	0.25	4.75	0.0025
E	0.50	4.50	0.005
F	1.00	4.0	0.010
G	2.5	2.5	0.025
H	5.0	0	0.050

*PNPP = p-nitrophenyl phosphate

(pH 5.7), 0.5 ml of 0.1 M MgCl$_2$, and 3.3 ml of distilled water. To each tube add 0.5 ml of the correspondingly diluted substrate (p-nitrophenyl phosphate).

3. Place the tubes in a test tube rack situated in a 37°C water bath and let stand for 5 minutes.

4. Initiate each assay at 2-minute intervals by adding 0.2 ml of the enzyme, run each reaction for 5 minutes, and stop it by adding 2.5 ml of 0.5 M KOH. Note that the final concentration of substrate in each reaction tube during the assay is 0, 0.05, 0.10, 0.25, 0.50, 1.0, 2.5, and 5.0 mM p-nitrophenyl phosphate.

5. Using a colorimeter adjusted to 405 nm, determine the absorbance for each reaction mixture. The tube containing no substrate should be used as the blank.

6. Repeat this experiment two more times.

Calculations and Questions

1. Determine the amount of p-nitrophenol produced in 5 minutes for each substrate concentration.

2. Average the data for all three trials and calculate the average velocity (μmoles of p-nitrophenol/minute) for each substrate concentration.

3. Plot velocity against substrate concentration (mmoles p-nitrophenyl phosphate) in the standard manner of Michaelis and Menten. Determine V_{max} and K_m for acid phosphatase.

4. Calculate the reciprocals of velocity ($1/v$) and substrate concentration ($1/[S]$) and present these data as a table.

5. Prepare the double-reciprocal plot of Lineweaver and Burke and determine the K_m and V_{max} from the x and y intercepts.

6. Investigate the literature and see how your values for K_m and V_{max} compare with published ones for acid phosphatase.

Kinetic Properties III.
The Inhibition of Acid Phosphatase
by Inorganic Phosphate

Introduction

Inorganic phosphate (Pi) is an inhibitor of acid phosphatase, and it is your task to determine whether it is a competitive, noncompetitive, or uncompetitive inhibitor. This experiment must be done in conjunction (preferably the same day) with the previous one because the kinetics for the uninhibited reactions must be compared with those of reactions run in the presence of the inhibitor. The setup is basically the same as in the previous experiment (Kinetic Properties II) except that a constant amount of phosphate (1.0 mM K$_2$HPO$_4$) will be present in each reaction tube. Run the reactions as before and compare Michaelis-Menten and Lineweaver-Burke plots in the presence (Kinetic Properties III) and absence (Kinetic Properties II) of the inhibitor. Determinations of V_{max} and K_m will determine the specific mode of inhibition.

Procedure

1. Use the same set of substrate dilutions prepared for Kinetic Properties II (0 to 0.05 M p-nitrophenyl phosphate).

2. Prepare eight reaction tubes labeled in accordance with the substrate concentrations to be used. To each tube add 0.5 ml of 1.0 M sodium acetate buffer (pH 5.7), 0.5 ml of 0.1 M MgCl$_2$, 2.3 ml of distilled water, and 1.0 ml of 0.005 M K$_2$HPO$_4$. To each tube add 0.5 ml of the appropriate diluted substrate (p-nitrophenyl phosphate). Note that each tube contains a different substrate concentration and the identical inhibitor concentration.

3. Place the tubes in a 37°C water bath for 5 minutes.

4. Begin the reaction in each assay tube at 2-minute intervals by adding 0.2 ml of the enzyme, let the reactions proceed for 5 minutes, and then stop them by adding 2.5 ml of 0.5 M KOH.

5. Determine the absorbance at 405 nm for each sample, using the first tube (0 mM p-nitrophenyl phosphate) as the blank.

6. Repeat the experiment two more times.

Calculations and Questions

1. Repeat the Calculations and questions (items 1 to 5) described at the end of Kinetic Properties II.

2. Prepare Michaelis-Menten and Lineweaver-Burke plots that compare the inhibited reaction with the uninhibited reaction.

3. Determine the K_m (K_i) and V_{max} in the presence of phosphate.

4. Is phosphate a competitive, noncompetitive, or un-
   competitive inhibitor? Justify your answer.
5. Investigate the literature to determine how your re-
   sults compare with those of previous workers.
6. What do you think would happen if you ran this
   same experiment substituting ATP for the inorganic
   phosphate?

Kinetic Properties IV.
The Effect of Different Enzyme Concentrations on Reaction Velocity

Introduction

This exercise illustrates the effect of increasing enzyme
concentrations on reaction rate. You will perform a se-
ries of 5-minute assays, in which a different enzyme
concentration is added each time the reaction is initi-
ated. The results should indicate the range of enzyme
concentrations that yield a linear response.

Procedure

1. Prepare a series of enzyme dilutions according to
   the protocol in Table 5-3. Notice that the enzyme
   is to be diluted with a solution of bovine serum al-
   bumin (BSA) at a concentration of 1 mg/ml. This
   added protein protects the enzyme against denatur-
   ation at low concentration. You must fill in the last
   column of Table 5-3 (enzyme concentration) based
   upon the actual concentration of your own sample
   of enzyme.
2. Prepare nine reaction tubes labeled according to the
   various enzyme concentrations (units/ml) and to
   each of these add 0.5 ml of 1.0 M sodium acetate
   buffer (pH 5.7), 0.5 ml of 0.1 M $MgCl_2$, 0.5 ml of
   0.05 M p-nitrophenyl phosphate, and 3.3 ml of
   distilled water.
3. Place all the tubes in a test tube rack situated in a
   37°C water bath and let the temperature equilibrate
   for 5 minutes.
4. Using a time schedule patterned after the one de-
   scribed in Table 5-1, start the reactions at 2-minute
   intervals by adding 0.2 ml of the different enzyme
   concentrations to each of the corresponding reac-
   tion tubes. Stop each reaction after 5 minutes by
   adding 2.5 ml of 0.5 M KOH.
5. Determine the absorbance for each reaction mixture

Table 5-3.
Protocol for the dilution of acid phosphatase

Tube	Undiluted enzyme (ml)	BSA (ml)	Dilution factor	Enzyme conc. (units/ml)
A	0	1.00	—	
B	0.01	0.99	1:100	
C	0.02	0.98	1:50	
D	0.04	0.96	1:25	
E	0.10	0.90	1:10	
F	0.20	0.80	1:5	
G	0.40	0.60	2:5	
H	0.80	0.20	4:5	
I	1.00	0	none	

at 405 nm using the tube containing no enzyme as
the blank.
6. Repeat the experiment two more times.

Calculations and Questions

1. Use the extinction coefficient for p-nitrophenol to
   determine the micromoles of product produced in 5
   minutes at each of the enzyme concentrations.
2. Average your data for the three trials and calculate
   the average reaction velocity (μmoles p-nitro-
   phenol/minute) for each enzyme concentration.
   Present these data in tabular form.
3. Plot velocity against enzyme concentration (units/
   ml). Describe the shape of this curve and discuss
   the reasons for its shape.
4. What is the valid range of enzyme concentrations
   for the acid phosphatase assay?

Kinetic Properties V.
Effects of Different Temperatures on Reaction Velocity

Introduction

As is the case with all chemical reactions, enzymatic
reactions are sensitive to changes in temperature. This

exercise will demonstrate that phenomenon in two different ways: (1) by showing the effect on enzyme stability of enzyme preincubation at a range of temperatures and (2) by showing the effect of different temperatures on the rate of the enzymatic reactions. For the first experiment, samples of the enzyme are maintained at eight predetermined temperatures (0, 10, 20, 30, 37, 50, 80, and 100°C) for 30 minutes, cooled in an ice bath, and then used in the standard 5-minute assay run at 37°C. The second experiment employs the standard stock of enzyme (no previous temperature treatment) in regular 5-minute assays performed at the eight different temperatures.

Procedure

1. One factor that is critical to the success of these experiments is the preparation and maintenance of water baths at different temperatures. Once a bath has been adjusted, its temperature should be continuously monitored and all temperature changes recorded. Baths prepared in the manner described in Table 5-4 can be shared by several laboratory pairs.

2. The effect on enzyme stability of enzyme preincubation at different temperatures
   a. Place a small sample (1.0 ml) of the enzyme in each of the water baths.
   b. After 30 minutes, return the samples to an ice bath.
   c. Prepare eight labeled enzyme assay tubes (one for each temperature) and add to each 0.5 ml of 1.0 M sodium acetate buffer (pH 5.7), 0.5 ml

Table 5-4.
Procedure for the preparation of water baths of different temperatures

Desired temperature (°C)	Method of preparation
0-4	Ice plus tap water in an ice bucket
10	Tap water and ice
20	Tap water at room temperature
30	Thermostatted water bath
37	Thermostatted water bath
50	Thermostatted water bath
80	Hot tap water
100	Boiling water bath

of 0.1 M MgCl$_2$, 0.5 ml of 0.05 M p-nitrophenyl phosphate, and 3.3 ml of distilled water.
   d. Place the tubes in a test tube rack situated in a 37°C water bath and let them equilibrate for 5 minutes.
   e. Start each of the enzyme reactions at 2-minute intervals by adding 0.2 ml of the appropriately treated enzyme to the corresponding reaction mixture. Let each reaction proceed at 37°C for 5 minutes, terminating it by the addition of 2.5 ml of 0.5 M KOH.
   f. Prepare a blank assay tube containing all the reaction components except the enzyme and incubate it at 37°C for 5 minutes along with the other tubes. After the KOH has been added to this tube, it may be used to zero the colorimeter at 405 nm.
   g. Determine the absorbance of each reaction mixture.
   h. Repeat the experiment two more times.
3. The effect of performing the reaction at different temperatures on the rate of the reaction
   a. Label four assay tubes (A, B, C, D) and into each of them pipette 0.5 ml of 1.0 M sodium acetate buffer (pH 5.7), 0.5 ml of 0.1 M MgCl$_2$, 0.5 ml of 0.05 M p-nitrophenyl phosphate, and 3.3 ml of distilled water.
   b. Place the tubes in a water bath maintained at 0 to 4°C and let the temperature equilibrate for 5 minutes.
   c. Add 0.2 ml of enzyme to tubes B, C, and D at 2-minute intervals and allow each reaction to proceed for 5 minutes before stopping it with the addition of 2.5 ml of 0.5 M KOH. Tube A, which serves as a reagent blank, should be treated in the same fashion except that 0.2 ml of distilled water should be added to the reaction mixture instead of enzyme.
   d. Place the tubes in a test tube rack at room temperature.
   e. Repeat steps a through d using all the water bath temperatures described in Table 5-4. When all of the reaction mixtures have returned to room temperature, determine the absorbance at 405 nm of each experimental tube against its own blank tube (A).

Calculations and Questions

1. Convert absorbance data to velocity data and compute the average velocity for all reactions at each temperature.

2. Report the data for the first experiment in a tabular format illustrating enzymatic activity remaining after enzyme preincubation at various temperatures. This is a measure of enzyme stability.

   a. Does it appear that in any case the enzymatic activity was increased or decreased by the temperature treatments?

   b. How do you explain these results?

   c. If you planned to store the enzyme for a prolonged period of time, what temperature would you suggest? Why?

3. Report the results of the second experiment in the form of a graph illustrating the effect of different temperatures on the rate of the reaction.

   a. Does this graph conform with your expectations? If not, why not?

   b. What is the optimum temperature for acid phosphatase?

   c. Why do you think it was necessary to prepare individual reagent blanks to be run at each of the temperatures?

4. Discuss the long- and short-term effects of different temperatures on acid phosphatase.

Additional Reading

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