Lab 3: Enzyme Kinetics

Background
Catalysts are agents that speed up chemical processes. The majority of catalysts produced by living cells that speed up biochemical processes are called enzymes. Enzymes are proteins. Each cell produces hundreds or thousands of them every second. Enzymes accelerate the velocity of virtually all reactions that occur in biological systems including those involved in the breakdown, the synthesis and chemical transfers between compounds. In so doing, they are responsible for performing essentially all the changes associated with life processes. The general expression frequently used to describe an enzyme reaction is \( \text{Enzyme} + \text{substrate} \Leftrightarrow \text{enzyme/substrate complex} \Rightarrow \text{enzyme/product complex} \Rightarrow \text{Enzyme} + \text{product} \).

Three important features of enzyme reactions that you should be familiar with:

1. The term substrate refers to the compound that is acted upon by the enzyme. In general, enzymes exhibit a high degree of specificity in that they usually catalyze only a single chemical reaction. Indeed, enzymes are often denoted by the name of the substrate that is acted upon and the suffix “-ase”. Thus, protein-splitting enzymes are proteases and enzymes that hydrolyze lipids are lipases.
2. The enzyme binds to the substrate to form an enzymes-substrate complex. This interaction is responsible for the specificity of enzyme action since only those compounds that “fit” into the substrate-binding site (the “Active site”) can be acted upon by the enzyme. The binding of the substrate to the enzyme also serves to alter the substrate in such a way that the conversion of substrate to product is facilitated.
3. The enzyme is not destroyed during the reaction but rather is set free after the formation of the end product. Thus, the liberated enzyme is available to combine with more substrate to produce more product. This feature makes enzyme molecules exceedingly efficient in catalysis and explains how very small quantities of enzymes are sufficient for cellular processes.

Measurement of the rate of Enzyme Reactions

The velocity or rate of the enzyme reaction can be determined by measuring the decrease in substrate concentration with time, or more commonly, by measuring the rate of appearance of product. During the early part of the reaction, the amount of product formed increase, linearly with time. However, in the latter part of the reaction, the rate of product appearance diminishes. This is seen in the “leveling off” of the product curve at the far right of figure 1.

Figure 1. Product formed vs. time of reaction
The far right of the [product] line on top shows a “leveling-off compared to the rate near the beginning of the reaction.
A number of reasons can account for the decline in reaction rate with time including depletion of substrate or the breakdown of the enzyme. In any case, in order to describe this reaction, its velocity must be determined during an early time interval when the amount of product is increasing in a linear manner. The rate measured during this time is the slope of a straight line. It is called the initial velocity of the reaction (Vo).

The initial velocity is determined by the slope of the line...remember the definition of slope is $\Delta Y/\Delta X$, or $Vo = (\text{nmoles of product at time 2} - \text{nmoles of product at time 1})/\text{(time 2} - \text{time 1})$. It can also be determined from the slope value (“m”) of the straight line generated during a linear regression analysis of the data points. The Vo is proportional to the amount of active enzyme molecules. The more enzyme, the faster the initial velocity value. This is a linear relationship. This feature is important and enables one to determine the amount of an enzyme in an unknown sample. For example, if 1ug of a pure enzyme gives a Vo (a slope value or “m” in $y=mx+b$) of 10 nmoles of product/minute, a cell extract that yields a Vo of 20 nmoles product/minute contains 2 ug of active enzyme (or twice the amount).

Remember, all enzymes operate at optimal temperature and pH values. Since enzymes are proteins, they are usually denatured and inactivated by temperatures above 50-70 degrees C. On the other hand, increased temperature also speeds up chemical reactions. With a typical enzyme, the predominant effect of increase temperature up to about 45 degree C is to increase the enzyme catalyzed reaction rate. Above 45 degrees C, thermal denaturation becomes increasingly important and destroys the catalytic function of the enzyme. When the temperature is lowered, the kinetics or rate of reaction of the enzyme is slowed. Typically at low temperatures, the enzyme is NOT denatured (enzymes are often frozen to maintain their activity). The decrease in rate is due to slower kinetics of substrate encountering enzyme during the reaction.

With regard to pH, the activity of enzymes is also greatly influenced by acidity or alkalinity. Excess acidity or alkalinity generally causes denaturation and inactivation of enzymes, just as high temperature causes heat denaturation. Most enzymes in plants and animals operate effectively near neutral pH. However, the pH optimum of the enzyme that you will study today is 4.5. In fact, the name of the enzyme, acid phosphatase, is derived in part from its low pH optimum.

Substrate Concentration –

The binding of an enzyme to its substrate is an essential part of the enzyme catalyzed reaction. At low substrate concentration, the active site on the enzyme is not saturated by substrate and thus the enzyme is not working at full capacity. As the concentration of the substrate increases, the sites are bound to a greater degree until at saturation, no more sites are available for substrate binding. At this saturating substrate concentration, the enzyme is working at full capacity and the maximum velocity ($V_{max}$) of the reaction is seen. Recall that a plot of Rate vs. substrate concentration, shows that initial velocity of a reaction increases in a hyperbolic manner as the substrate concentration is increased. This increase in reaction rate is proportional to the concentration of the enzyme-substrate complex. Thus, the $V_{max}$ occurs because the enzyme becomes saturated with substrate. The substrate concentration required to yield half the maximal velocity ($V_{max}/2$) can also be determined from the previously described plot and is an important constant in describing an enzyme. This constant is
known as the **Michaelis constant** and is abbreviated **Km**. Under conditions of defined temperature, pH, and ionic strength, the Km approximates the dissociation constant of an enzyme for its substrate (how well the substrate binds to the enzyme’s active site). The Km reflects the affinity of the enzyme for the substrate. For example, a Km of 0.2 mole/liter of substrate would indicate that the substrate binding site would be half saturated when the substrate is present at that concentration. Such an enzyme would have a LOW affinity for its substrate. In contrast, a Km of $10^{-7}$ mole/liter indicates that the enzyme has a HIGH affinity for its substrate since it is half-saturated at this very low substrate concentration. **The Higher the Km value, the weaker and less efficient the binding of substrate.** Of course, the opposite is true…very small Km values indicate a tight, efficient binding of substrate.

The effects of substrate concentration on the velocity of an enzyme catalyzed reaction is described quantitatively by the Michaelis-Menten equation given in Figure 2.

![Figure 2](image)

**Enzyme Inhibitors** –

Enzyme inhibitors are compounds that slow down the rate of an enzyme reaction. Two classes of inhibitors are competitive inhibitors and noncompetitive inhibitors. Competitive inhibitors are compounds that usually mimic the substrate’s structure. These inhibitors bind in the enzyme’s active site, thus blocking the actual substrate from binding. Hence, the reaction rate is diminished because substrate is blocked from binding to enzyme. Graphically, **Competitive inhibitors have little effect on the Vmax value of the enzyme BUT drastically affect the Km value.** They cause the Km value to significantly increase, indicating the enzyme is having a hard time finding the correct substrate. Noncompetitive inhibitors interact with the enzyme outside of the active site. They usually lack structural similarities to the substrate. Even though they don’t bind active site, they still reduce the rate. The enzyme binds the correct substrate the same way it does without the inhibitor, but some other factors cause the reaction to slow. Graphically, **Noncompetitive inhibitors have little effect on the Km value of the enzyme, BUT drastically affect the Vmax.** They cause the Vmax value to significantly decrease compared to the standard reaction.
Computer graphing programs, such as “Prism” can accurately generate a kinetics curve using reaction rate and substrate concentration data. However, before computers that make the process simpler, an alternative process was developed. For experimental determination of the Km and Vmax, the Michaelis-Menten equation is usually rearranged to a form that is equivalent to the straight line equation (y=mx+b). Once such rearrangement, first described by Lineweaver and Burk, involves taking the reciprocals of each side of the Michaelis-Menten equation as shown in Figure 3.

![Figure 3](image)

**Figure 3.** The Michaelis-Menten equation from Figure 2 is rearranged to the form on the right which yields the straight line equation.

If a **double reciprocal plot** is made with $1/Vo$ on the ordinate (Y-axis) and $1/[S]$ on the abscissa, a straight line relationship exists where the slope is $Km/Vmax$, the intercept on the Y axis is $1/Vmax$, and the intercept on the X axis is $-1/Km$ as seen in Figure 3. Graphing programs usually give you the value of the Y intercept in linear regression analysis (remember it is the “b” value in $y=mx + b$). The X intercept is also sometimes given depending on the program you use. You can also determine it mathematically by using the equation of the straight line and setting the Y value equal to zero and solving for “X”.

**Extraction of Wheat Germ Acid Phosphatase –**

The wheat kernel consists of three parts: (1) the embryo or germ that produces the new plant (2) the starchy endosperm which serves as a food source for the embryo and (3) covering layers which protect the grain. The endosperm is the raw material for flour production, while the wheat germ is used in food products as a source of vitamins. Wheat germ is also frequently used in the molecular biology laboratory as a source of plant cell proteins and nucleic acids. In today’s laboratory, you will extract and analyze wheat germ **acid phosphatase**.

The study of enzymes frequently begins with the extraction of these proteins from tissue. Enzyme extraction procedures usually require a method that destroys the integrity of the cell. The broken cells then release their molecular constituents including enzymes. One method that can be used to lyse cells involves treating tissue with a mild detergent that breaks or dissolves cell membranes. However, it can not be too strong so that it denatures the proteins in the process. An alternative process, called sonication, mechanically disrupts the cells leading to lysis. This method eliminates the possibility of
denaturing proteins due to excess detergent. In the procedure described below, you will sonicate a wheat germ/buffer suspension in order to prepare an enzyme extract from wheat germ containing acid phosphatase.

**Acid phosphatase** catalyzes the removal of phosphate groups from macromolecules and smaller molecules that are stored in the wheat seed. The free phosphate is then used by the growing embryo to make nucleic acids, for example. In the exercise described below, you will measure the velocity of the reaction catalyzed by purified acid phosphatase (from the “enzyme store”) and by the acid phosphatase that is found in the wheat germ extract that you prepare. A synthetic phosphatase substrate called para-nitrophenol phosphate (p-nitrophenol phosphate) will be used in the experiment. P-Nitrophenol phosphate is colorless but is broken down to free phosphate and nitrophenol as shown below. Nitrophenol is yellow in alkaline solution so that the appearance of yellow color is indicative of the amount of product formed in the reaction.

![Substrate and Products Diagram]

**Description of This Laboratory –**

Today’s experiments are timed experiments and require accurate pipetting skills! In order to obtain good data, your group MUST work like a “well-oiled” machine. There are several solutions, each of which does something different. DEVELOP A GAME PLAN AND BE ORGANIZED. WORK AS A TEAM SO THAT YOU DON’T HAVE TO REPEAT THE EXPERIMENTS!

**OBJECTIVES OF TODAY’S LAB**

1. Prepare dilutions of yellow nitrophenol (the product of the reaction) in order to generate a standard curve relating absorbance of nitrophenol (at 405nm) to its concentration.

2. Set up a standard reaction using pure acid phosphatases enzyme and p-nitrophenol phosphate (substrate) in increasing concentration. Obtain the amount of product produced/min (the rate) for each condition (by measuring the produced absorbance and the standard curve generated in objective 1). Use Prism to generate a Michaelis Menton curve describing your data. Record values for Vmax and Km for this standard reaction.
3. Repeat objective #2, using a buffer system containing an enzyme inhibitor (phosphate ion). As above, generate a Michaelis Menton curve and record values for Vmax and Km. This inhibitor WILL affect the reaction rate. Compare Vmax and Km values of this reaction to the standard reaction and see how the values change. These changes will indicate what type of inhibition is being displayed by phosphate ion.

4. Repeat objective #3, but this time use a buffer system containing a different enzyme inhibitor (NaF). Generate a graph and compare Vmax and Km values to the standard reaction. Make conclusions about the type of inhibition present.

5. Graph your data as a double-reciprocal plot (as a Lineweaver Burk plot). Calculate Km and Vmax values from the Lineweaver plot, compare to the values generated from the Michaelis/Menton curve in Prism.

6. Extract acid phosphatases from raw wheat germ and run a timed reaction in parallel with pure enzyme to deduce how much enzyme you were able to extract. This is done by collecting data from each experiment, generating a linear regression curve for each, and comparing slope values, through ratio and proportion.

WORK IN GROUPS OF 4 (or by table)

I. Preparation of the para-nitrophenol standard curve

1. You need to prepare 1 ml (final volume) dilutions of p-nitrophenol. These will be used to generate a standard curve, relating absorbance at 405nm to their concentration.

2. Prepare 10ml of 0.01M NaOH from a 1M NaOH stock. This diluted NaOH will be used to dilute p-nitrophenol to produce your standards.

3. Using the 0.01M NaOH and a 1000uM stock of p-nitrophenol. Prepare 7 standards at concentrations of:

<table>
<thead>
<tr>
<th>p-nitrophen. stand. conc(uM)</th>
<th>ul of stock p-nitrophenol</th>
<th>ul of dil.NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
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</tr>
<tr>
<td>50</td>
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<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td></td>
<td></td>
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<tr>
<td>500</td>
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</tr>
</tbody>
</table>

PREPARE THESE STANDARDS CAREFULLY!!!!!! YOUR SUCCESS HERE WILL AFFECT ALL OTHER DATA COLLECTED DURING THIS LAB!

4. Transfer 100 uL each standard to the row A1-A7 of a microtiter plate.

5. Determine the absorbance of each standard using the plate-reader in LS216. The program should be Abs 405 0.1sec.
6. Generate a Standard Curve using linear regression of the data using prism. **WHEN EXITING PRISM, MAKE SURE YOU CHOOSE FILE...EXIT. DO NOT JUST “X” OUT OF THE PROGRAM OR IT WILL INTERFER WITH OTHER PRISM PROGRAMS RUNNING ON THE NETWORK!!!!**

7. Obtain the equation of the line of best fit using the data obtained from the graph analysis.

8. Rearrange the equation to the term of X (so that you are solving for X instead of Y). This equation will now allow you to determine the amount of product (p-nitrophenol) produced during an enzyme reaction from its absorbance value. This conversion will be **essential** to accurately do the rest of the experiments!

II. The Enzyme Assays (Both Standard and Inhibited)

1. Each group needs the following:
   i. A small tube of pure acid phosphatases enzyme (Conc. is 1mg/ml)
   ii. Prepare a working dilution of Sodium Acetate Buffer (NaOAc) pH 4.5 Each group needs 10mL of a 50mM dilution using a 500mM concentrated stock. Dilute the stock with dH2O.
   
   10mL                    10mL

   iii. A small tube of p-nitrophenol phosphate (the substrate) (1 mg/ml)
   iv. A tube of 1M NaOH

2. In a Row combine the following in this order:

<table>
<thead>
<tr>
<th>Well</th>
<th>uL buffer</th>
<th>uL substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>B-2</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>B-3</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>B-4</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>B-5</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>B-6</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>B-7</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>B-8</td>
<td>30</td>
<td>70</td>
</tr>
</tbody>
</table>

3. Notify the “timer” in the group!

4. Quickly yet accurately add **10 ul of pure enzyme** to each of the wells. The repeat pipettor will help you do this accurately and quickly. Ask me for help when you are ready to do this and I will show you how the pipettor works. **Work as fast as you can…this is a timed reaction!**

5. Once the enzyme has been added to the last well, start the timer to countdown from 5 minutes.

6. After 5 minutes, add 50 ul of 1M NaOH to each well to stop the reaction.

7. Read and record the absorbance of each well as you did with the standards.
8. For the phosphate inhibitor reactions, repeat steps 2 – 7 BUT USE THE PHOSPHATE INHIBITOR BUFFER (50mM NaOAc, pH 4.5 with 1mM Na₃PO₄) IN PLACE OF THE BUFFER USED IN THE STANDARD REACTION. IT HAS ALREADY BEEN DILUTED TO THE CORRECT CONCENTRATION SO USE IT AS IT IS PROVIDED TO YOU.

9. For the fluoride ion inhibitor reactions, repeat steps 2 – 7 BUT USE THE FLUORIDE ION INHIBITOR BUFFER (50mM NaOAc, pH 4.5 with 1mM NaF) IN PLACE OF THE BUFFER USED IN THE STANDARD REACTION. IT HAS ALREADY BEEN DILUTED TO THE CORRECT CONCENTRATION SO USE IT AS IT IS PROVIDED TO YOU.

10. Using your standard curve, calculate how much product (nitrophenol) was produced at each substrate concentration by substituting the absorbance values for each well into the standard equation and solving for “X”. This value is the umoles/5 minutes. YOU NEED TO TAKE THIS NUMBER AND DIVIDE BY 5 TO GET Umoles/min. These values are the rates (Y values in the Michealis/Menten curve). To obtain the X value, plot the molar concentration of substrate used in each well. You can determine this by multiplying the volume of substrate added to each well by the concentration of the substrate (1 mg/ml or 1 ug/ul) to give you a mass. Paranitrophenylphosphate (the substrate) has a formula weight of 275 g/mole. The substrate is diluted in 100uL in each well. Using these values, calculate the molar concentration of substrate in each well across the plate. These are the X values. You are now ready to plot the kinetics curve. (Graphing instructions for prism are found at the end of this handout!)

11. To plot the kinetics curve in Prism: FOR THE UNINHIBITED REACTION, THE REACTION INHIBITED WITH PHOSPHATE ION, AND THE REACTION INHIBITED WITH FLUORIDE ION. ALL ON THE SAME GRAPH! Make sure you use the key to indicate which line is which.

14. WHEN EXITING PRISM, MAKE SURE YOU CHOOSE FILE...EXIT. DO NOT JUST “X” OUT OF THE PROGRAM OR IT WILL INTERFER WITH OTHER PRISM PROGRAMS RUNNING ON THE NETWORK!!!!

III. Extraction and Quantification of Acid Phosphatase Enzyme from Wheat Germ

1. Obtain a 15 mL conical centrifugation tube and add 0.5 grams of raw wheat germ to the tube.
2. Add 5 mL of 0.05M Sodium Acetate buffer, pH 4.5. Cap and shake the tube to disperse the wheat germ into the buffer
3. Using the sonicator, disrupt the suspended wheat germ by sonicating for 30 seconds.
4. Shake the tube again and with a plastic transfer pipet, remove about 1 mL of the suspension into a new microcentrifuge tube.
5. Centrifuge on high for 30 seconds and place tube in a tube rack. You will be using the supernatant (which should contain acid phosphatase) in the next experiment.
6. We need two rows of 6 wells on your plate. Mark one as pure enzyme and the other as extract.
7. Into both rows add 50 uL of 1M NaOH to each well.
8. Obtain two clean microcentrifuge tubes, label one pure enzyme and the other as extract.
9. Into each tube, add 600 uL of 0.05M Sodium Acetate buffer, and 100 uL of the substrate solution.
10. Notify your timer to pay attention!
11. Quickly but accurately add 60 uL of pure enzyme to the pure enzyme tube and 60 uL of the extracted enzyme to the extracted enzyme tube.
12. Immediately mix well by shaking, start the timer counting up, immediately remove 100 uL of each tube and put it in the first wells of each row, combining it with the NaOH already in the well. This is a time=0 time point.
13. Repeat taking out 100uL samples from each tube and putting it in the appropriate wells at time=2 minutes, =4 minutes, =6 minutes, =8 minutes, and finally, time=10 minutes.
14. On the plate reader, record absorbance values for each well at 405 nm.
15. Plot and X-Y plot using the absorbance (on the Y) vs. time (on the X) and do linear regression through the points.
16. Analyze the linear regression data to obtain the slope value (m) for each line. This tells how fast the reaction was proceeding. The more enzyme, the faster the rate. We can use these values to quantify how much enzyme is present in the reaction tubes.
17. We added 50 uL of pure active enzyme to the tube to produce the rate observed in the pure enzyme timed reaction. The concentration was 1 mg/ml. How much active enzyme was in this tube?
18. By setting up a ratio between the slope/enzyme ug to the slope of the extract reaction, we can determine how much active enzyme was in the extract.

Homework problems

1. Print out nice Michaelis Menten graphs for the standard reactions and both inhibitor reactions.
2. What are the Km and Vmax values for all?
3. Print out nice Lineweaver-Burk graphs for the standard reactions and both inhibitor reactions.
4. What are the Km and Vmax values for all? How do they compare to the Michaelis values?
5. What kind of enzyme inhibitor is phosphate ion? Justify it by your data.
6. What kind of enzyme inhibitor is fluoride ion? Justify it by your data.
7. How much (ug) of acid phosphatase enzyme did you extract from the wheat germ? Justify your answer.
HOW TO GRAPH KINETICS DATA IN PRISM!!!

Equation: Michaelis-Menten model
Introduction

The most common kind of enzyme experiment is to vary the concentration of substrate and measure enzyme velocity. The goal is to determine the enzyme's Km (substrate concentration that yield a half-maximal velocity) and Vmax (maximum velocity). If your goal is to determine the turnover number kcat, rather than the Vmax, use an alternative version of the equation.

Step by step

Create an XY data table. Enter substrate concentration into X, and enzyme velocity into Y. If you have several experimental conditions, place the first into column A, the second into column B, etc.

You can also choose Prism's sample data: Enzyme -- Michaelis-Menten.

After entering data, click Analyze, choose nonlinear regression, choose the panel of enzyme equations, and choose Michaelis-Menten enzyme.

Model

Y = Vmax*X/(Km + X)

Interpret the parameters

Vmax is the maximum enzyme velocity in the same units as Y. It is the velocity of the enzyme extrapolated to very high concentrations of substrate, so its value is almost always higher than any velocity measured in your experiment.

Km is the Michaelis-Menten constant, in the same units as X. It is the substrate concentration needed to achieve a half-maximum enzyme velocity.

Create a Lineweaver-Burk plot

Before nonlinear regression was available, investigators had to transform curved data into
If you create a Lineweaver-Burk plot, use it only to display your data. Don't use the slope and intercept of a linear regression line to determine values for Vmax and Km. If you do this, you won't get the most accurate values for Vmax and Km. The problem is that the transformations (reciprocals) distort the experimental error, so the double-reciprocal plot does not obey the assumptions of linear regression. Use nonlinear regression to obtain the most accurate values of Km and Vmax.

To create a Lineweaver-Burke line corresponding to the nonlinear regression fit, follow these steps:

1. Create a new XY data table, with no subcolumns.
2. Into row 1 enter X = -1/KM, Y = 0 (previously determined by nonlinear regression).
3. Into row 2 enter X = 1/Smin (Smin is the smallest value of [substrate] you want to include on the graph) and Y = (1/Vmax)(1.0 + KM/Smin).
4. Note the name of this data table. Perhaps rename it to something appropriate.
5. Go to the Lineweaver-Burke graph.
6. Drag the new table from the navigator and drop onto the graph.
7. Double-click on one of the new symbols for that data set to bring up the Format Graph dialog.
8. Choose to plot no symbols, but to connect with a line.