Instructor's Note: Students will be required to use Excel to plot absorbance data and fit a line for each substrate concentration in order to calculate initial velocity. In addition, students will graph data to make Michaelis-Menten and Lineweaver-Burke plots.

Lab 3: Enzyme Kinetics: CHARACTERIZATION OF THE ENZYME ALKALINE PHOSPHATASE

READING:

The following article are available on the Bio 126 lab web page. Please read through the article before coming to lab. You will not understand everything in this article, but you should read it anyway – just do the best you can, and see the help link on the lab web page. For tips on reading journal articles such as this, refer to pages 19-24 in Knisely's "A Student Handbook for Writing in Biology, 2nd ed."

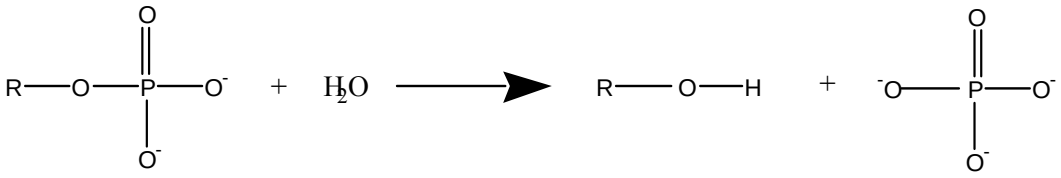
I. INTRODUCTION

The activity of enzymes is important for the proper functioning of cells. In the context of energy flow in living organisms, enzymes catalyze most reactions in metabolic pathways. Not only do enzymes make most reactions possible in an intracellular environment, enzymes allow for the control and stabilization of these reactions. It is not a stretch to say that without enzymes life itself would be impossible.

The behavior of enzymes in response to different concentrations of the reaction chemicals (both substrates and products) comprise the basic characteristics of each type of enzyme. This behavior, referred to as enzyme kinetics, is responsible for much of the reaction control in biological systems. In order to learn about enzymes and enzyme behavior, in this lab we will examine the kinetics of the enzyme alkaline phosphatase.

II. ALKALINE PHOSPHATASE ENZYME

Alkaline phosphatase is a ubiquitous enzyme that can be isolated from bone, kidney, intestine, plasma, liver, spleen, plants and microorganisms. It catalyzes the removal of a phosphate that is attached to an alcohol, resulting in the generation of a free phosphate (P_i) and an alcohol. Alkaline phosphatase can act on a variety of specific substrates; the generalized reaction is shown below:



substrate alcohol phosphate

Interestingly, the precise biological function of the enzyme is unknown. Clinical interest in alkaline phosphatase was inspired by the observation that certain pathological conditions, such as obstructive jaundice, rickets and other bone disorders were characterized by large increases in the plasma concentration of the enzyme. It is particularly abundant in tissues that are involved in the transport of nutrients. The fact that it is localized at the surface of absorptive tissues suggests a role in the transport of nutrients across the epithelial membrane. If rats are maintained on a

high fat diet, there is an increase in the amount of intestinal alkaline phosphatase, indicating a role in the transport/processing of the fats (Young et al., 1981). Another hypothesized function for intestinal alkaline phosphatase is protection from bacterial infection, whereby alkaline phosphatase removes phosphate groups from endotoxin, a lipopolysaccharide (lipid-carbohydrate) molecule that makes up the cell wall of some types of bacteria (Poelstra *et al,* 1997; Kopojos *et al.* 2003; Verweij *et al.* 2004). Endotoxin induces an inflammatory response in the host that can be fatal (septic shock), but there is no toxic response when the phosphate groups have been removed from the endotoxin.

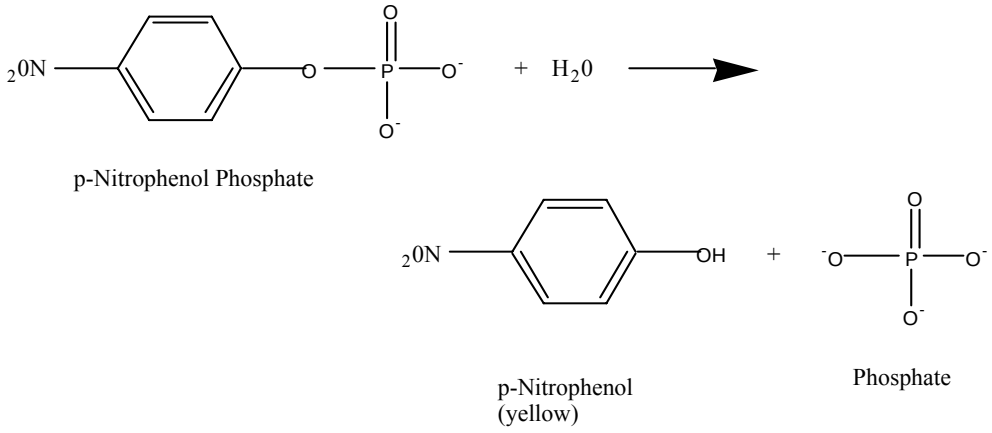
In this laboratory, we will be using alkaline phosphatase from bovine (cow) intestine. This particular alkaline phosphatase has a molecular weight of 138,000 daltons (a dalton is a unit of mass approximately equal to the mass of a hydrogen atom). It is a dimer (i.e., has two subunits), so the molecular weight of each monomer is 138,000/2 or 69,000 daltons. The enzyme requires 4 atoms of Zn^{++} per dimer for activity. The Zn^{++} atoms are said to be cofactors for the enzyme, meaning that they are necessary for the enzyme to be active. An enzyme with its cofactors is called a holoenzyme; without the cofactors, it is an apoenzyme.

apoenzyme + cofactors = holoenzyme

Typically, apoenzymes are not active, so when we refer to enzymes, we usually mean holoenzymes.

III. THE ENZYME-CATALYZED REACTION

The particular reaction that we are going to examine is the removal of a phosphate from the molecule p-nitrophenolphosphate. This is not a natural substrate for the enzyme, but it has properties which make it particularly useful to us. The enzyme will remove the phosphate and generate free phosphate (P_i) and p-nitrophenol. While the substrate, p-nitrophenolphosphate, is colorless, the product p-nitrophenol is yellow, so we can follow the reaction progress by measuring the generation of yellow color.



Substrates that change color when acted upon by an enzyme are called chromogenic substrates and these make studying enzymes significantly easier. P-nitrophenol absorbs light at 410 nm, so we can follow the rate of the reaction by following the increase in absorption at 410 nm by using a spectrophotometer.

Alkaline phosphatase activity can be tested by adding a certain amount of substrate and a certain amount of enzyme to a buffered aqueous solution. In this series of lab experiments, a 25 µl portion of a substrate solution and a 25 µl portion of enzyme will be added to a tube containing 3.0 ml of buffer. The absorbance at 410 nm then will be measured for a short period of time in a spectrophotometer as a way of determining the amount of p-nitrophenol formed. When the absorbance is graphed as a function of time, a plot like that shown Figure 3.1 will usually be obtained.

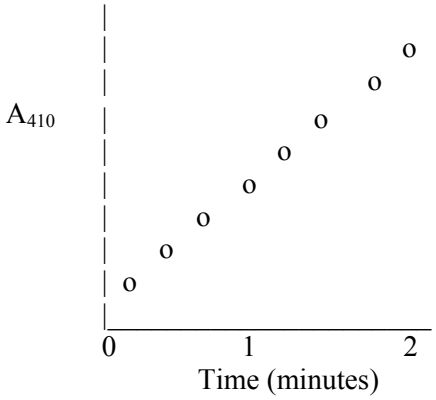


Figure 3.1. Absorbance of enzyme product (p-nitrophenol)

Notice that the absorbance increases at a linear rate as more and more p-nitrophenol is formed. However, the absorbance may eventually approach a plateau as the reaction slows down and less product is formed.

The initial linear rate of product formation is called the initial velocity, or $\mathbf{v_o}$. It is one of the most important characteristics of any enzyme-catalyzed reaction. One factor that affects the initial velocity is the substrate concentration, [S]. You will look at the relationship between v_o and [S] in lab this week.

IV. ENZYME KINETICS

Let's do a quick preview of enzyme catalysis. As mentioned above, one common kinetic study of enzymes is to examine how the velocity of the reaction changes when substrate concentration changes in the presence of a constant enzyme concentration. It is important to measure the initial velocity, or v_o . (Why is it important to measure *initial* velocity?) If we plot v_o vs. substrate concentration [S], we will see the following curve, called a Michaelis-Menten curve, shown in Figure 3.2 below.

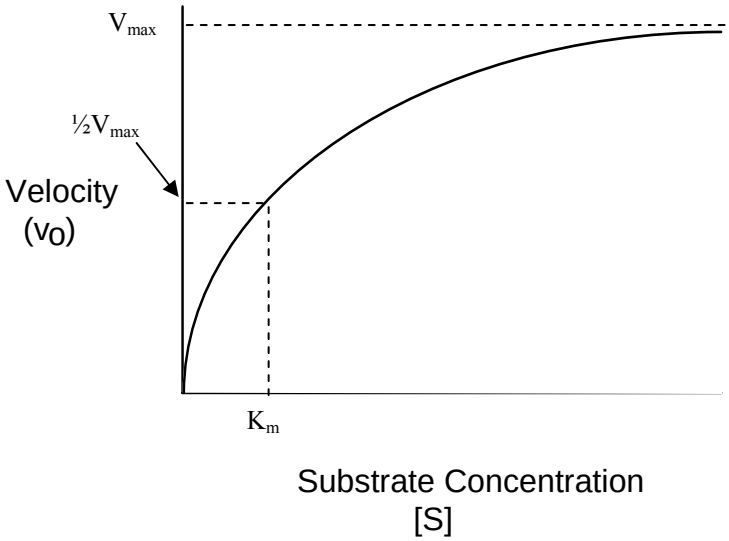


Figure 3.2. The Michaelis-Menten curve. This curve describes the relationship between an enzyme (at constant concentration) and the concentration of S, the enzyme's substrate. v_o is the initial rate of production of enzyme product. See the text on the following page for descriptions of V_{max}, $1/2V_{max}$, and K_m

As [S] increases, v_o eventually becomes independent of [S] (why?). The velocity at which this occurs is called V_{max}, and it is the fastest that the given amount of enzyme can operate. The [S] that yields 1/2 V_{max} is another important kinetic parameter called the Michaelis-Menten constant,

designated K_m. K_m is important in that it indicates the [S] at which the enzyme is most effective at altering the rate of the reaction. (Make sure that you understand why this is so.) Enzymes are tightly regulated to maintain homeostasis, and one handy mechanism for regulation is having your enzyme activity change based on substrate availability (Is more substrate around? - increase the rate at which the enzyme works to compensate). In fact, it is frequently found that [S] *in vivo* is near the K_m for an enzyme. You can think of this as a cell exploiting the Michaelis-Menten character of an enzyme. Furthermore, K_m is important in understanding many other kinetic parameters of enzyme activity that we will not discuss here due to National Security Considerations. K_m and V_{max} are characteristics of a reaction that help characterize the enzyme in question. (How would K_m and V_{max} change if you increased the amount of enzyme?)

To determine K_m and V_{max}, we could determine a set of v_0 values at various concentrations of S, make the graph above and read off the values. As you can imagine, this would not be very accurate since it is difficult to obtain an accurate value for a number that is approached asymptotically. The equation that describes the above Michaelis-Menten curve is the following:

$$v_0 = \frac{V_{max} \cdot [S]}{[S] + K_m}$$ Michaelis-Menten Equation

This is called the Michaelis-Menten equation. Two algebraically inclined fellows by the names of Lineweaver and Burke manipulated the Michaelis-Menten equation to yield the following:

$$\frac{1}{v_0} = \frac{1}{V_{max}} + \frac{K_m}{V_{max} \cdot [S]}$$ Lineweaver-Burke Equation

(Hang in there - we are almost done.) If you plot $1/v_o$ vs. $1/[S]$, you get the following Lineweaver-Burke plot or double-reciprocal plot:

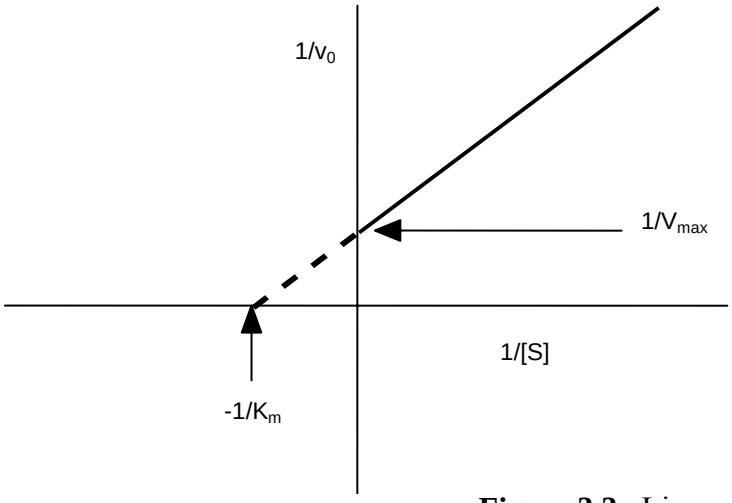


Figure 3.3. Lineweaver-Burke plot.

The slope of the line is K_m / V_{max}, the y-intercept is $1/ V_{max}$ and, if we extrapolate the line (i.e., set $y = 1/v_0 = 0$), the x-intercept is $-1/ K_m$. The use of the double reciprocal plot yields much more accurate values for K_m and V_{max} than an interpretation of the Michaelis-Menten curve. In this week's lab, we will determine K_m and V_{max} for the enzyme alkaline phosphatase.

V. DETERMINATION OF INITIAL REACTION RATE, V_0

To analyze the data you are collecting today, you will need to calculate initial velocity, $\mathbf{v_0}$. This initial rate of reaction can be expressed simply as a change in absorbance per unit of time: for p-nitrophenol formation this would be ΔA_{410}/min. This corresponds to the slope on your absorbance vs. time graph. For example, say the straightest portion of your graph is between 0 minutes and 0.75 minutes, and the absorbance changes from 0 to 0.375 during this time. This means your slope, v_0, is:

$$v_0 = \frac{(0.375 - 0)}{(0.75 - 0)}$$

$$= 0.5/\text{min}$$ (note that absorbance does not have any units).

However, it is more useful to express the rate in terms of the actual amount of p-nitrophenol formed per unit time; this allows researchers working under different experimental conditions to compare their results. This may be given as nanomoles per minute (nmol/min) or micromoles per minute (μmol/min). The absorbance value at 410 nm can be converted to an actual concentration using the Beer-Lambert law described in last week's lab. After a little rearrangement:

$$c = \frac{A}{\varepsilon \cdot \ell}$$

Remember that **c** is the concentration of the absorbing material (p-nitrophenol, in our case), **A** is the absorbance measured at 410nm, and $\boldsymbol{\ell}$ is the length of the light path (1.0 cm for our spectrophotometers). The extinction coefficient, **ε**, for p-nitrophenol at 410 nm is 18.5 $mM^{-1}cm^{-1}$. Therefore, using this value and the Beer-Lambert equation, you can convert an absorbance reading into an actual amount of product formed.

Let us work through an example. Suppose that the slope of your line was 0.5/min, as described above.

$$\text{Concentration} = \mathbf{c} = \frac{0.5/\text{min}}{(18.5/\text{mM}\cdot\text{cm})\cdot 1.0\,\text{cm}}$$

$$= 0.027\ \text{mM/min}$$

$$= 27\ \mu\text{M/min}$$

Now things get a little messy. The units, μM, are in terms of micromoles per liter, by definition. That is what the big M means. If we have a concentration of 27 μM, that means we have 27 micromoles per liter, or 27 nanomoles/ml (make sure you understand this conversion).

Moreover, our reaction mixture is 3.05 ml, and we want the actual total amount of product formed in our tube, not a concentration.

So: 27 nmoles/ml x 3.05 ml = 82 nmoles formed in one minute.

This then is how much p-nitrophenol was responsible for the yellow color, and represents how much p-nitrophenol was generated by the enzyme in one minute.

In summary, you'll need to follow three steps to get from your graph of absorbance vs. time to the amount of product formed per minute:

1. Determine the slope of the straight portion of your curve (units are ΔA_{410}/min).
2. Convert this slope from ΔA_{410}/min to μM/min by using the Beer-Lambert equation.
3. Convert this concentration to the total amount of product formed in the cuvette by taking the total volume into account. Report your result in terms of nmol/min.

VI. EXPERIMENTAL PROCEDURES

This week, you will need to develop your own data tables for recording your results. You should do this before coming to lab, to help things go smoothly and expediently (it is easy to do this by creating an Excel spreadsheet with columns and rows for your data). The first section below (part A) gives you a chance to practice the assay protocol and get some baseline data. An **assay** is (generally speaking) a test which tells you something about a substance, in this case the activity of an enzyme. Part B tells how to estimate K_m and V_{max}.

A. Time Course of Alkaline Phosphatase Activity

1. Turn on the spectrophotometer. **Allow the instrument to warm up for 30 minutes** before you use it.
2. The machine should be in "Absorbance mode" – i.e., the display should show some numbers followed by an "A". If not, press '**A/T/C**' to select the absorbance mode. .
3. Press  or  to set the wavelength to 410 nm.
4. To a cuvette add 3.0 ml of Tris-HCl buffer at pH 8.0. Then add 25 μl of the 100 mM stock solution of p-nitrophenolphosphate. Cover the tube with a piece of Parafilm and invert the tube several times to mix the solution. Insert the cuvette with the reaction mixture into the spectrophotometer and blank the machine by pressing the '**0 ABS/100%T**' button. (What is the purpose of this step?)
5. Add 25 μl of the stock enzyme solution to the tube while simultaneously marking the time (you might try to bring a watch to lab). Quickly mix the solution by inversion and put the tube back in the sample compartment of the Spectrophotometer. Note that you start timing when you add the enzyme solution, NOT when you finish mixing.
6. Beginning at 15 seconds, read the absorbance at 410 nm at 15 second intervals for 2 minutes.
7. Plot absorbance as a function of time on graph paper (available in lab) or on a computer in the adjoining computer lab. Determine the period over which absorbance increases linearly with time and calculate the initial velocity as ΔA_{410}/min. You can do this on the graph paper by choosing two readings in the linear region and calculating the slope (change in absorbance over change in time, remember?).

8. Repeat this procedure (including the blanking step, #4!) with the same amount of enzyme at least two more times to determine the accuracy and reproducibility of the assay method. Be sure to invert the cuvette the same number of times for each sample – you're going to be making comparisons later which depend on consistency. You can plot all three data sets on the same axes.
9. Once a good set of values for v_o has been obtained, calculate the average initial velocity in ΔA_{410}/min. Then, using the calculation given on pages 3-5 and 3-6 as a guide, convert the initial change in absorbance per unit time to an actual rate of product formation in nmol/min.

B. Protocol for the Determination of K_m and V_{max} Values

1. Blank the spectrophotometer: To a cuvette, add 3 ml of pH 8 buffer and 25 µl of the lowest substrate concentration (5 mM). Cover the tube with a piece of parafilm and mix by inverting. Blank the spectrophotometer at 410 nm with this solution.
2. Add 25 µl of the stock enzyme to the tube while starting a stop watch at the same time. Quickly mix the tube by inversion and place the tube in the sample compartment of the spectrophotometer.
3. Read the absorbance at 410 nm at 15 sec intervals for 2 min.
4. Repeat the assay at least once. For this particular experiment, it will help you to have duplicates at each substrate concentration; if they are very different, conduct the assay a third time.
5. Then carry out the same procedure with each of the other substrate concentrations (5 mM, 10 mM, 25 mM, 50 mM, and 75 mM). Use the data from part A for the 100mM substrate concentration. Remember to blank the spectrophotometer with each substrate concentration. (Why must you do a separate blank at each substrate concentration?)
6. For each substrate concentration, plot absorbance versus time and determine the initial velocity. (Again, why is it important to determine the <u>initial</u> velocity?). Then calculate the initial velocity as nmoles of p-nitrophenol generated/minute.

Now you are ready to summarize all those data in just a couple of graphs, which will allow you to look at patterns in the data and determine Km and Vmax. It is a good idea to work on this before you leave lab, in case you want to run any additional assays. There are computers available in the adjoining computer lab; your TA can help you with Excel.

Before graphing, you will need to first convert your [S] values from the initial concentration in the microfuge tubes to the concentration of substrate in your final reaction volume.

Make a simple hyperbolic plot of the data; that is, make a graph in which v_o (in nmol/min) is plotted as a function of [S]. Your graph should resemble the typical shape of the Michaelis-Menten plot on page 3-3. Don't forget to include the rate for the 100 mM substrate concentration from part A.

Then make a double-reciprocal plot of the data, that is, a graph of $1/v_o$ vs $1/[S]$. It should form a straight line, like the Lineweaver-Burke plot on page 3-4. Fit a line to the points and determine the K_m and V_{max} values for this reaction. Remember that the slope of the line is K_m / V_{max}, and the y-intercept is $1/ V_{max}$.

VII. LABORATORY WRITE-UP

A. Introduction

You will use the data from this week's lab to write two sections of a scientific journal article – Materials & Methods and Results. We encourage you to discuss your results among your group members, but the write-up should be your own individual work.

Articles in scientific journals are the main method of written communication among scientists. As such, we feel that it is important that students in the introductory biology classes gain skills in writing papers in the format of a journal article.

B. Contents of Lab Write-up

Your lab report should have two sections – Materials & Methods, and Results. In your experiment, you are attempting to answer one main question: "What are the characteristics of Bovine Alkaline Phosphatase?" For the Materials and Methods section, you should report how you conducted your experiment. In your results section you should present the information that is necessary for you (and your reader) to answer your experimental question. You should not include raw data – i.e., do not include the Absorbance vs. time graphs.

C. Report Format

Please refer to the report stylesheet on the lab web page for information on the format of the paper.

Literature Cited

Kapojos J., K. Poelstra, T. Borghuis, A. Van den Berg, H. Baelde, P. Klok, and W. Bakker. 2003. Induction of glomerular alkaline phosphatase after challenge with lipopolysaccharide. International Journal of Experimental Pahtology 84:135-144

Poelstra, K., W. Bakker, P. Klok, M. Hardonk, and D. Meijer. 1997. A physiologic function for alkaline phosphatase: Endotoxin detoxification. Laboratory Investigation 76:319-328.

Sakharov, I. Y., Makarova, I. E., and Ermolin, G. A. 1988. Purification and characterization of intestinal alkaline phosphatase from harp seal. Comparative Biochemistry and Physiology, B 90B, 709-714.

Verweij W., H. Bentala, A. Huizinga-van der, A. van Loenen-Weemaes, K. Kooi, D. Meijer, and K. Poelstra. 2004. Protection against an Escherichia coli-induced sepsis by alkaline phosphatase in mice. Shock 22:174-179

Young, G., S. Friedman, S. Yedlin, and D. Alpers. 1981. Effect of fat feeding on Intestinal alkaline-phosphatase activity in tissue and serum. American Journal of Physiology 241:G461-G468