

BL 4820 Biochemical Techniques -- Lecture 2 - Characterizing an Acid Phosphatase

Text Reading:

- General Introduction to Amino Acids, Proteins and Enzymology, pages 67-77
- Enzymology and Enzyme Kinetics, pages 77 to 85
- Most relevant this week, pages 82 to 85
- Expt #9 Kinetics of Acid Phosphatase

Expt #2 - Characterization of Wheat Germ Acid Phosphatase

This Week you will:

1. Establish Assay Method
2. Determine valid [Phosphatase], ie find best enzyme concentration for the assay conditions being used.
3. Determine the pH optimum

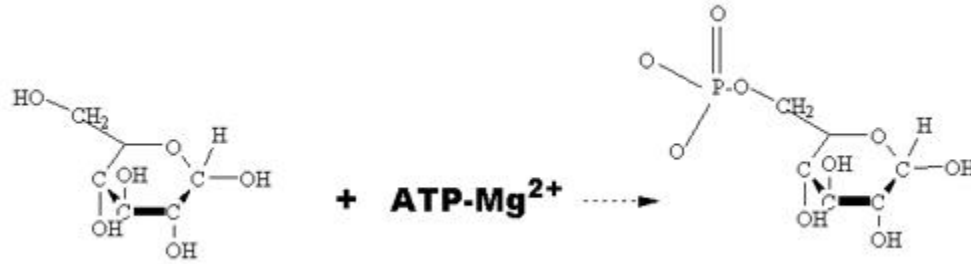
Next Week you will:

- Analyze the Kinetics of Phosphatase using Initial Velocity Measurements
- Find K_m and V_{max} in absence of Inhibitors
- Find K_m' and V_{max}' in presence of 2 Inhibitors
- Use Competitive Inhibitor (inorganic phosphate, a reaction product)
- Use a Non-Competitive Inhibitor (fluoride ion)

1. General Principles Underlying Enzyme Assays

First, to analyze the properties of an enzyme, you need to establish a valid assay method and identify the conditions to use to get the maximum activity using a specific substrate. To illustrate these general principles of enzymology, I will use the enzyme hexokinase as an example.

Hexokinase catalyzes the conversion of glucose to 6-phospho-glucose using Mg-ATP as the phosphate donor:



Glucose + Mg-ATP = Glucose-6-phosphate + Mg-ADP

This is basically an irreversible reaction since a lot of free energy is released during the reaction. But of greater importance to our discussion is that it is a two substrate enzyme. To make things simpler we will add a lot of Mg-ATP so that it is in excess. This makes the concentration of Mg-ATP (ie. [Mg-ATP]) fixed and [glucose] an allowed variable.

To optimize the assay conditions, you need to determine:

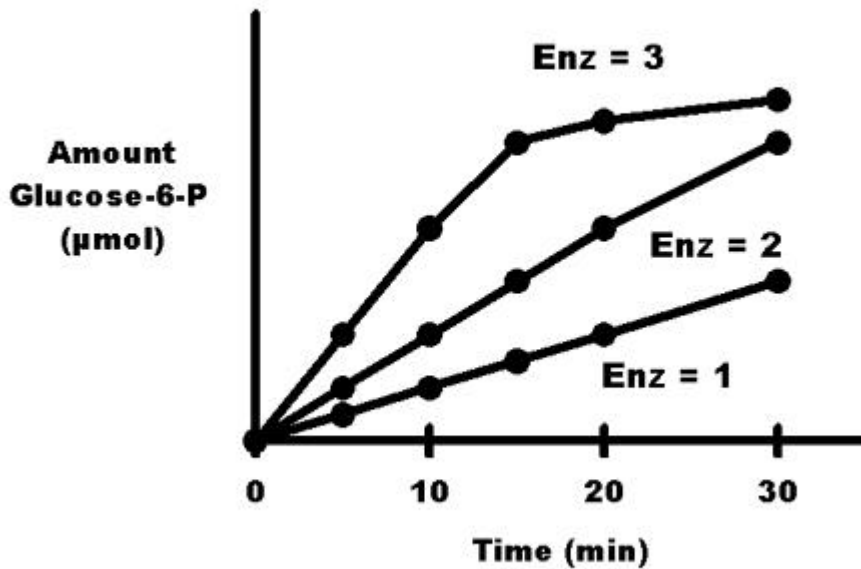
- Amount of Enzyme to use
- Optimum pH for the enzyme activity
- Optimum [Glucose]

To optimize these conditions for determining enzyme activity, you need a valid assay method. To do this, you can either measure the decrease in glucose during the enzyme catalyzed reaction or measure appearance of glucose-6-P. The assay will be much more sensitive if appearance of product is measured since you start the assay with no product present.

First, it should be determined in a control assay that in the absence of hexokinase, glucose and MgATP, the substrates and reactants in the reaction, do not form a significant amount of products (glucose-6-P and MgADP). Next, if you have a method available for measuring the amount of glucose-6-P formed during the reaction, then use it as the basis of the assay. The evaluation method for amount of glucose-6-P must be quantitative and give results in amounts of μmol glucose-6-P formed during measured periods of reaction time. This will allow you to calculate the reaction rate in terms of $\mu\text{mol}/\text{min}$. This will be called the enzyme catalyzed rate or enzyme activity.

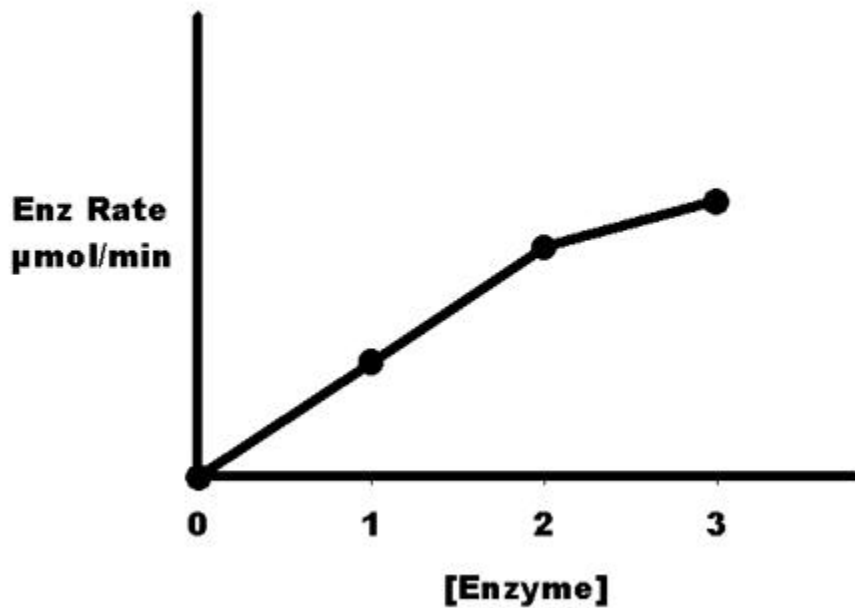
2. Finding a Valid Amount of Enzyme for Assay Conditions

To find the amounts of enzyme which will work well with the enzyme assay you are setting up for hexokinase, you must select a pH and [glucose] to start with. With these conditions set, you can then use different amounts of enzyme in the assay and find the conditions where you get a linear response in the assay:



The amount of glucose-6-P formed at different times in the reaction is measured using different concentrations of hexokinase. You can see in the above graphic that Enzyme concentrations of 1 and 2 are linear for the 30 min reaction time used, while at enzyme concentration 3, the amount of glucose-6-P falls off after 15 min. Clearly, only $[Enz] = 1$ and 2 give a valid assay; apparently at $[Enz] = 3$, the glucose is being used up or some other parameter is limiting production of the product.

When the amount of glucose-6-P produced in 30 min is used to calculate the rate of the reaction or the enzyme rate, a plot can be made to show which concentrations of enzyme are valid for the assay:

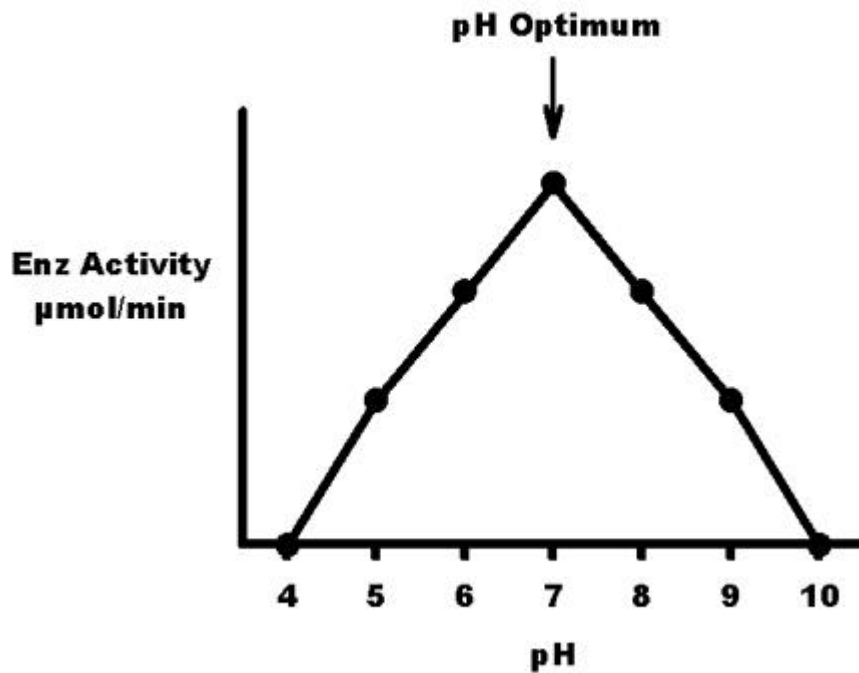


From the linear portion of this plot, you can then easily choose the valid assay conditions with respect to the amount of enzyme which can be used and still get results that can be compared to one another.

3. Finding Optimum pH and Temperature

A. pH Optimum

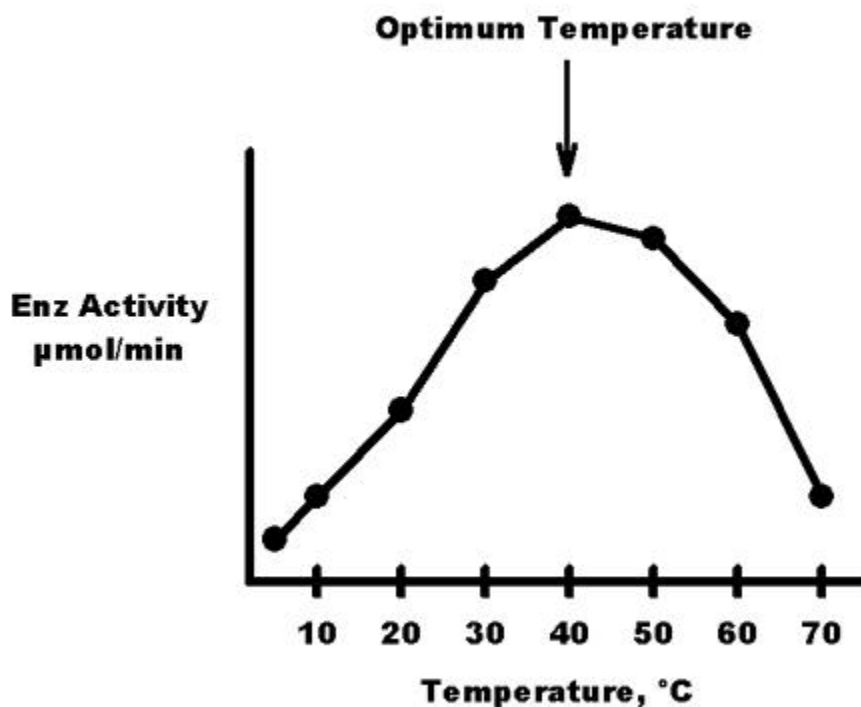
After the valid amount of enzyme to use is found, the next step is to optimize the pH. This is done by assaying the enzyme activity in buffers of different pH, calculating the enzyme activity rates at each pH and then plotting enzyme activity versus pH:



The plot of enzyme activity Vs. pH is often "bell shaped" since two different amino acid groups of the enzyme are being titrated to different states of ionization at the different pH values. This means that only one of the two possible ionization states of the amino acid side chain is effective in enzyme catalysis - in other words, if an acidic side chain like Glu is involved in catalysis, it probably only works when it is ionized; since Glu side chain carboxylic acid groups have a pK around pH 5, at pH values below this pK, the enzyme will be less active; while at pH values above the pK, the enzyme will be most active. At higher pH values, another group, for example, the amino side chain group in a Lys may control the activity. So at pH values below the pK of the Lys amino side chain, which is about pH 9, the enzyme is active, while at pH values above the Lys side chain pK, the enzyme will be less active. In the end, the pH optimum lies somewhere between the pK values for these two groups that control the enzyme's activity.

B. Temperature Optimum

Most enzymes have an optimum temperature, which may be related to the type of organism from which the enzyme was isolated. Some organisms like plants grow well near room temperature and so their enzymes are most active at a temperature around 30 to 40°C. A general rule of thumb from chemistry is that a reaction's rate approximately doubles with a 10°C increase in reaction or assay temperature. However, since enzymes are held together by weak non-covalent bonds, at higher temperatures, the enzyme catalyzed rate slows down rather than increases:



Of course, at low temperatures, all the molecules in the solution slow down and so does the enzyme catalyzed reaction. At high temperatures, the thermal movement of the molecules become too great for the enzyme to hold its shape or conformation and so it begins to denature and lose its activity. Animal enzymes often have temperature optima near 37°C (especially human's since this is body temperature). For thermophilic organisms, like bacteria or blue-green algae which grow well in near boiling hot springs, much higher temperature optima are found; some enzymes even have temperature optima near 90°C.

In the end, the temperature you use for an enzyme assay may depend on the equipment available to you in the lab. While we have water baths in the lab, it is much easier to assay enzymes at room temperature.

4. Acid Phosphatase - Assay System

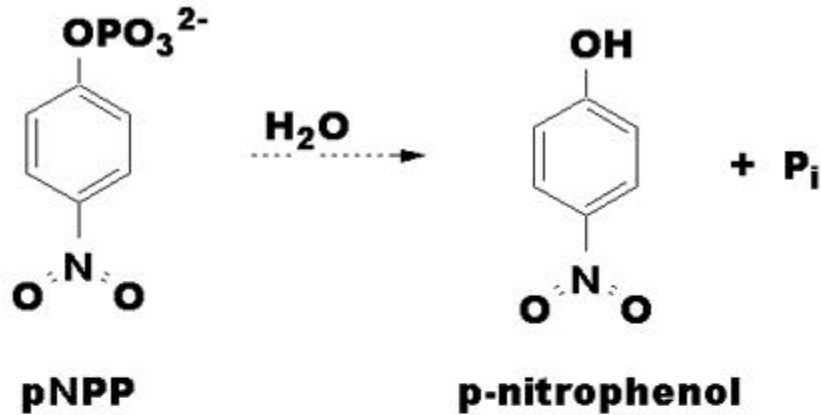
In Expt #2, you will be analyzing wheat germ acid phosphatase as an example enzyme. The physiological function of this acid phosphatase is to provide inorganic phosphate to the growing wheat seedling during germination. Many different phosphate esters of sugars and substrates are stored in the wheat seed and these need to be hydrolyzed during germination, which makes the carbohydrates available as an energy source and the phosphate to be used as building blocks in making new cells (new RNA and DNA all need phosphate in their backbones).

So while we could use a phosphate ester of a natural compound like glucose-6-P which was discussed in the general discussion of this lecture, it is also possible to use artificial phosphate esters since the enzyme is rather non-specific and will catalyze phosphate ester hydrolysis on

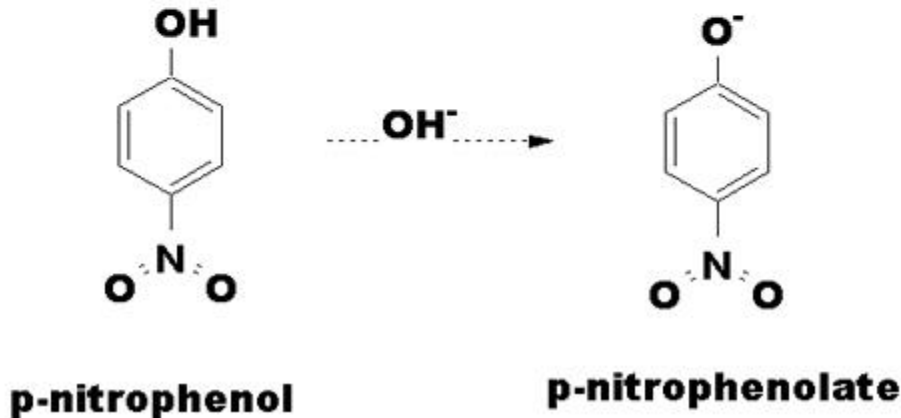
many different substrates. Phosphate ester of p-nitrophenol is a good substrate to use since the product formed after ester hydrolysis, p-nitrophenol, can easily be measured.

Here is the reaction catalyzed by wheat germ phosphatase with p-nitrophenylphosphate (pNPP):

1. Phosphatase Catalyzed Reaction



2. Color Reaction (add NaOH)



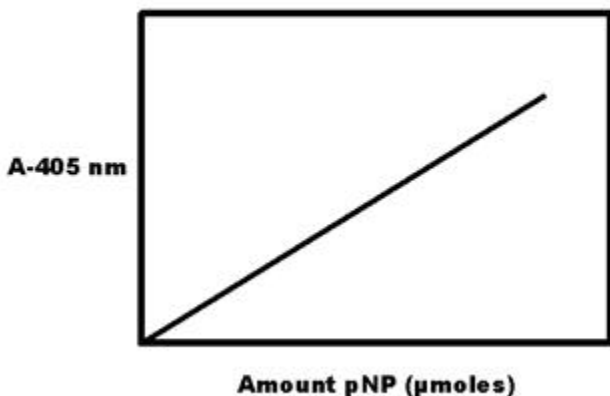
The first step is the enzyme catalyzed part of this graphic. In this reaction, the catalyst is the phosphatase and the products are p-nitrophenol and inorganic phosphate or P_i .

The second part of the graphic illustrates the way to detect the p-nitrophenol formed as product in the phosphatase catalyzed reaction shown in part 1. In part 2, NaOH is added to end the phosphatase assay after a given reaction time of say 10 min. The hydroxide reacts with the p-nitrophenol to remove the phenolic proton and p-nitrophenolate is formed, which is a yellow colored compound absorbing at 405 nm. Since nothing much else in the reaction mixture absorbs light at this wavelength, it is easy to measure in a quantitative way the amount of p-nitrophenol formed in the enzyme catalyzed reaction.

This type of enzyme assay is called an "end-point" assay since the activity of the enzyme is evaluated after a given time of reaction. The other type of enzyme assay is a continuous assay where the appearance of product or disappearance of substrate is measured all the time during

the reaction. End-point enzyme assays are often used in clinical and other analytical biochemical assays since they are easy to do and often are useful for detecting the presence of an enzyme in a sample in a quantitative manner.

To quantify the amount of p-nitrophenol formed during the phosphatase catalyzed reaction, you need to make a standard curve using known amounts of p-nitrophenol:

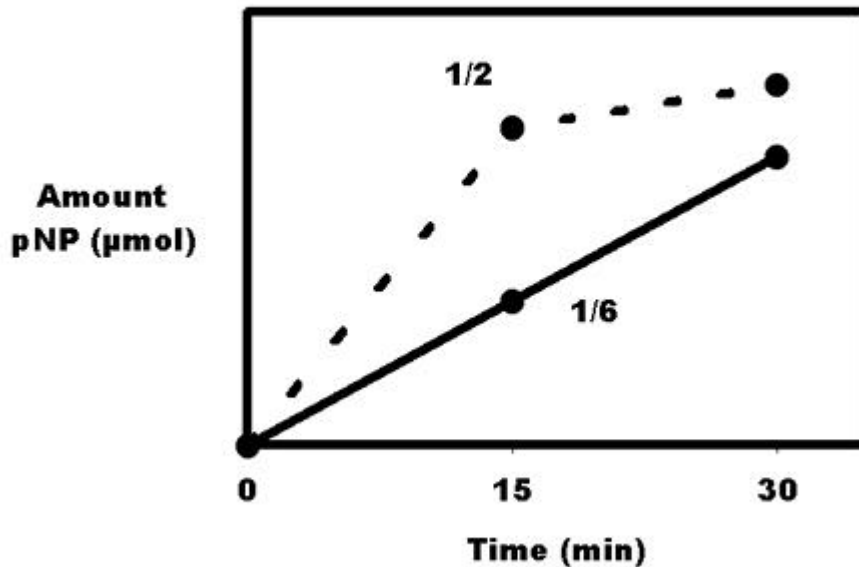


You will be provided with a standard solution of p-nitrophenol in 0.02 M NaOH, so it will already be yellow. From this stock p-nitrophenol (pNP) solution, you can prepare various dilutions of p-nitrophenolate (pNP) for constructing the standard curve. Subsequently, when you do a phosphatase assay with pNPP, you can determine the amount of p-nitrophenol made during the reaction time by measuring the A-405 nm and reading the amount of p-nitrophenol from the standard curve. The pNP standard curve should be very linear and so it works well to do a linear regression analysis on it and use the equation of the line to convert the A-405 nm values into amounts of pNP in terms of μmol or pmol .

It may be useful for you to review the concepts of the standard curve and linear regression in [Lecture 1](#) and the [Help on Units](#) (last part of this help page).

5. Optimizing the Phosphatase Assay

You will do 2 optimization procedures in the lab this week. First, you will find the optimum amount of enzyme to use in the assay. This procedure will be a little different than that described in the lab manual text since we will not be using a water bath. Here is an example of what your results may look like this week:



This plot illustrates results like those you should find in the lab this week. Since we will not be using a heated water bath for the phosphatase assay, we will use different concentrations of enzyme than are described in the lab manual text. A dilution of 1/6 of the phosphatase stock solution is shown here as being linear, while the 1/2 dilution is not. How do you make a 1/6th dilution? Take 1 part of stock phosphatase solution and add to it 5 parts of buffer (called 1:5 dilution).

Once you find the proper enzyme dilution to use for the phosphatase assay, then you will do a pH optimum. I will not illustrate it here. But if you [go back to Part 3](#) of this lecture you will see what a typical enzyme pH optimum plot looks like.

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