

# Enzyme Activity

Commercially available enzymes are usually described in terms of units and the unit value is specific for each individual enzyme. One unit of alkaline phosphatase is defined as the amount of enzyme necessary to produce 1  $\mu\text{mol}$  of p-nitrophenol/minute.

When measuring the activity of your enzyme for each step, make serial dilutions (1:1, 1:10, 1:100) of your enzyme to make sure that it is readable in the spectrophotometer (Abs 0.2-0.8). Calculate the dilution factor needed for the following activity test.

Calculate the enzyme activity of each step or fractions using the following protocol.

In each well of a 96-well plate add:

50  $\mu\text{L}$  Buffer (1M boric acid pH 9.5, 0.5 mM  $\text{MgCl}_2$ , 20  $\mu\text{M}$   $\text{ZnCl}_2$ )

50  $\mu\text{L}$  Substrate (5 mg/mL pNPP in  $\text{H}_2\text{O}$ )

10  $\mu\text{L}$  Enzyme solution (in blank wells just add water – this will be the reference abs)

Mix and incubate at 37°C for exactly 10 minutes

Add 100  $\mu\text{L}$  of Stop Solution (2 M NaOH)

Mix and read absorbance at 405 nm

$$\text{Unit/mL} = \frac{(\text{Abs Sample} - \text{Abs Reference}) \times (\text{Total Reaction Volume in mL})}{(18.3^*) \times (\text{minutes}) \times (\text{enzyme volume in mL})}$$

\* 18.3 = molar extinction coefficient for p-nitrophenol

Don't forget to correct for your dilution factor!

# Enzyme Analysis



E = Enzyme S=substrate ES=enzyme-substrate complex P=product  
 K = rate constant for that part of the reaction

$$V_o = K_2[ES] \quad \text{Eq. 1}$$

$$\text{Rate of ES formation } K_1([E_t] - [ES])[S] \quad \text{Eq. 2}$$

$$\text{Rate of ES breakdown } K_{-1}[ES] + K_2[ES] \quad \text{Eq. 3}$$

Rate of ES formation = Rate of ES breakdown

$$K_1([E_t] - [ES])[S] = K_{-1}[ES] + K_2[ES] \quad \text{Eq. 4}$$

$$\text{Or } K_1[Et][S] - K_1[ES][S] = (K_{-1} + K_2)[ES]$$

$$\text{So if we isolate [ES] it looks like this } [ES] = \frac{K_1[Et][S]}{K_1[S] + K_{-1} + K_2} \quad \text{Eq. 5}$$

$$\text{This can be rewritten like this } [ES] = \frac{K_1[Et][S]}{K_1[S] + K_{-1} + K_2} \text{ or } = \frac{[Et][S]}{K_m + [S]} \quad \text{Eq. 6}$$

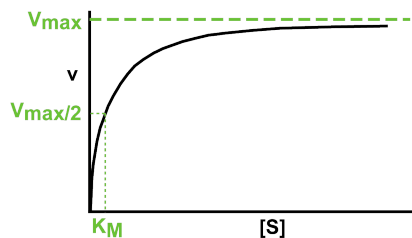
If we now substitute equation 6 for the [ES] of equation 1 and the equation looks like this.

$$V_o = \frac{K_2[Et][S]}{K_m + [S]} \quad \text{or}$$

The Michaelis-Menten Equation

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

So during an enzyme reaction where we hold the enzyme concentration constant and vary the substrate concentration, we will get a graph that looks like this.



The Michaelis constant ( $K_m$ ) is analogous to the dissociation constant of the Michaelis complex. (i.e. a measure of how sticky the enzyme is for the substrate)

$$K_m = \frac{k_2 + k_{-1}}{k_1} \quad \text{Eq.7}$$

$V_{\max}$  is the maximal velocity of an enzyme when it is fully saturated with substrate. The value of  $k_{cat}$  is often referred to as the turnover number or the number of times an enzyme can convert a substrate to product within a given amount of time.

$$V_{\max} = k_{cat}[E_t] \quad \text{Eq. 8}$$

How to calculate  $V_{\max}$  and  $K_m$

Once you have a purified sample of alkaline phosphatase, it is time to determine how fast the enzyme actually works. To do this you repeat the enzyme activity assay except you measure the same enzyme concentration against several different concentrations of substrate as outlined below.

In a 96-well plate add:

50 $\mu$ L Buffer (1M boric acid pH 9.5, 0.5 mM  $MgCl_2$ , 20 $\mu$ M  $ZnCl_2$ )

50 $\mu$ L Substrate dilutions in different wells(1:2, 1:4, 1:8, 1:16, 1:32)

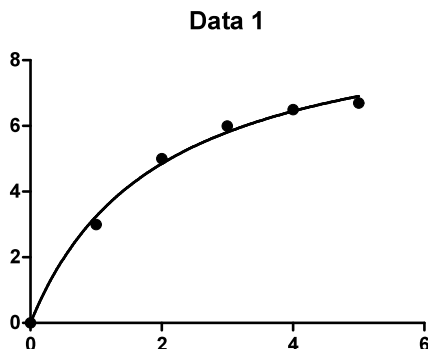
10  $\mu$ L Enzyme solution (in blank wells just add water – this will be there reference abs)

Mix and incubate at 37°C for exactly 5 minutes

Add 100 $\mu$ L of Stop Solution (2 M NaOH)

Mix and read absorbance at 405 nm

We will calculate  $V_{\max}$  and  $K_m$  using the program Prism 5 found on each of the lab computers. To use Prism 5 open the program and click to start a new file. Verify that you are entering data for an XY plot, start with an empty data table, under Y: enter “3” replicate values and plot “Mean and Error” and “SD”, and then click on the create button. When we enter the data into Prism 5, we place the substrate concentrations on the x-axis and absorbance values on the y-axis. Click “graph 1” on the left column and you should see a graph like this.



Click on the x and y axis to label appropriately and name the graph. Within the top tool bar is an analysis box. Within that box is a button for nonlinear regression. Click this button and then select “enzyme kinetics” and then the “Michaelis-Menten” equation and

enter. Click on the results in the left column and you should find the calculated values for  $V_{\max}$ ,  $K_m$  and  $R^2$ . Record these.

The only value left to determine is  $[Et]$ . This value is calculated by quantitating your enzyme fraction using the Bradford assay. The Bradford assay will give you a value in  $\mu\text{g/mL}$ . This must be converted to  $\text{g/L}$ . If you divide this value by the Fw of Alkaline Phosphatase (86,000 Daltons) you will calculate the molarity of the enzyme. This value is  $[Et]$ .

The factor  $K_{\text{cat}}/K_m$  is generally the best kinetic parameter to use in comparing catalytic activity. Molecules diffuse through an aqueous solution at a rate of  $1 \times 10^8$  to  $1 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ . What this means is that the rate in which an enzyme turns a substrate into a product is limited to the time it takes a substrate to float through the water to get to the enzyme. How does the rate of the enzyme you purified compare to diffusion through water?

## Enzyme Inhibition (Extra Credit)

Test the inhibition of alkaline phosphatase by an inhibitor of your choice. Read JBC Vol. 257 No. 8 (1982) pp 4141-4146 to get information about inhibitors for this experiment. Demonstrate what type of inhibition is taking place by fitting the data to equations in Prism 5 for each type of inhibition.