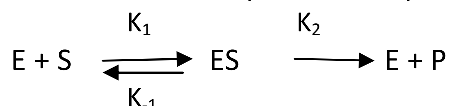
## BT 290 : Biomolecular Analysis Laboratory

**Determination of the $K_M$ for alkaline phosphatase enzyme using spectrophotometry**

Enzymes are a group of robust, versatile and ubiquitous molecules which perform the task of catalysts inside every living organism. Their presence is known to enhance the efficiency of chemical reactions by several orders of magnitude. Sweet potato β-amylase for example achieves a rate enhancement of more than 100,000,000,000,000,000 fold compared to the uncatalysed reaction [1—4]. Most enzymes are large proteins, a few comprise of several identical or non-identical polypeptide chains bound together in a complex. Multienzyme complexes involved in a sequence of biochemical reactions of a metabolic pathway (like glycolysis for example) also exist [5].

In general, enzyme activity is dependent on the pH and temperature. Higher enzyme activity is usually evident at the physiological temperature (37 $^{o}$C ), higher temperatures might denature the protein and lead to loss of enzyme activity. Solvent properties like ionic strength, solvent polarity and presence of denaturants like urea, which directly affect the protein conformation, also influence enzyme activity.

Enzymes are specific to their target substrates. The substrate is transiently bound to the enzyme through non-covalent interactions at the active site. The product once formed is released by the enzyme. The enzyme is then ready to bind to the next substrate molecule. The reaction can be represented by the following scheme:

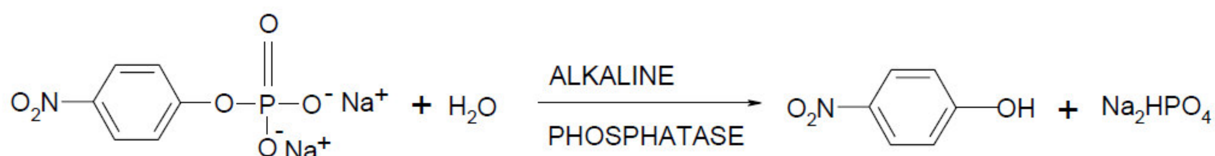



$$K_M = \frac{K_2 + K_{-1}}{K_1}$$

Here E, S and P represent the enzyme, substrate and product respectively. ES denotes the enzyme-substrate complex and $K_M$, the Michaelis constant is the dissociation constant for the [ES] complex. From the Michaelis-Menten equation, the rate of product formation ($v$) can be written as,

$$v = \frac{[E]_0[S]k_{cat}}{K_M + [S]} \qquad \text{where} \quad k_{cat}\,[E]_0 = V_{max}$$

In this laboratory, we shall investigate the kinetics of the enzyme, ‘alkaline phosphatase’ (AP). AP catalyses the hydrolysis of phosphoester bond.



For our experiment, we shall be using p-nitrophenyl phosphate as the substrate. On hydrolysis in an alkaline medium, p-nitrophenol is released as a yellow product from the enzyme. This product can be easily quantitated by spectrophotometry since it has a distinct absorbance around 450 nm.

Enzyme Unit (U) : One U is defined as the amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute.

Our objective is to determine the Michaelis-Menten parameter, KM and Vmax for the above enzymatic reaction.

**Methodolgy**

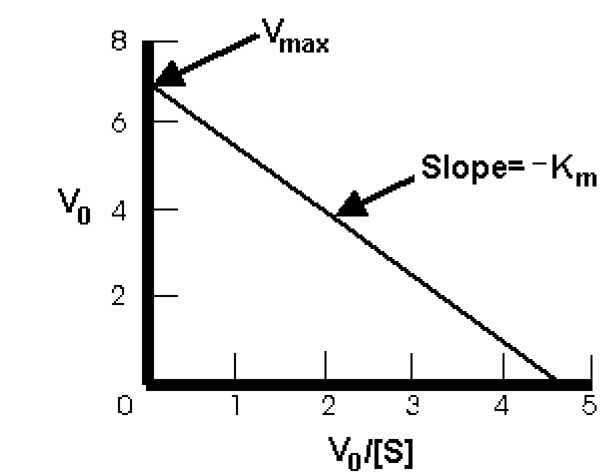
Materials required : 1. UV-Vis spectrophotometer and a pair of glass cuvettes

2. Alkaline Phosphatase (6mg/ml)
3. pH 9.5, 100mm glycine buffer as reaction medium
4. p-nitrophenyl phosphate (disodium salt) as substrate. Stock conc is 100mM.

Procedure :

1. Dissolve disodium salt of p-nitrophenyl phosphate in the pH 9.5, glycine buffer so as to prepare the substrate in different concentrations namely 0, 0.7, 1.25, 2.5, 5 and 10mM, each for a volume of 5 ml. All substrate solutions must have equilibrated to the room temperature.
2. Take 67µl of the enzyme in each of the spectrophotometer cuvettes. Add 2 ml of the pH 9.5, glycine buffer to each of them. Mix them well. Place them in the spectrophotometer and use them as solvent blank for 450nm.
3. Now start the reaction by mixing 67µl of the enzyme with 2ml of the substrate conc.0.7mM. Make sure that mixing is complete. Immediately start recording the absorbance of this reaction mixture against time in seconds. Continue the observation for 200 seconds.
4. Repeat the above procedure for rest of the substrate concentrations.
5. Plot the profile of absorbance against time, for each substrate concentration in one graph.
6. Calculate the rate of the reaction $v_o$ by taking the slope of the reaction
7. Plot the reaction rate, $v_o$ against substrate concentration, [S].
8. Generate a Eadie-Hofstee Plot. Calculate the $K_{M.}$

| S,No | Volume of PNPP(μl) | Volume of buffer (ml) | Substrate Conc (mM) | Enzyme volume (μl) | Spectra ( $Abs_{450}$ vs time upto 200sec) |
|---|---|---|---|---|---|
| 1 | 0 (Blank) | 2 | 0 | 67 | |
| 2 | 14 | 1.986 | 0.7 | 67 | |
| 3 | 25 | 1.975 | 1.25 | 67 | |
| 4 | 50 | 1.950 | 2.5 | 67 | |
| 5 | 100 | 1.900 | 5 | 67 | |
| 6 | 200 | 1.800 | 10 | 67 | |




References:

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