	YILDIZ TECHNICAL UNIVERSITY **BIOMEDICAL ENGINEERING DEPARTMENT** **BME2901- BIOCHEMISTRY COURSE** **2020-2021 FALL SEMESTER**

EXPERIMENT 5
ENZYME KINETICS

5.1. PURPOSE OF THE EXPERIMENT

To learn the key equations and graphing methods for explaining and examining enzyme activity. To learn methods for spectrophotometrically measuring the kinetics of an enzymatic reaction.

5.2. THEORETICAL KNOWLEDGE

The activity of enzymes is important for the proper functioning of cells since the organism must be able to catalyze chemical reactions efficiently and selectively. In the context of energy flow in living organisms, enzymes catalyze most reactions in metabolic pathways. Acting in organized sequences, they catalyze the hundreds of stepwise reactions that degrade nutrient molecules, conserve and transform chemical energy, and make biological macromolecules from simple precursors. Through the action of regulatory enzymes, metabolic pathways are highly coordinated to yield a harmony in the interplay of the many activities necessary to sustain life. Thus, enzymes not only make most reactions possible in an intracellular environment, enzymes allow for the control and stabilization of these reactions.

The behaviour of enzymes and reaction rates in response to different concentrations of the reaction chemicals (both substrates and products) comprise the basic characteristics of each type of enzyme. This behaviour, referred to as enzyme kinetics, is responsible for much of the reaction control in biological systems.

A key factor affecting the rate of a reaction catalyzed by an enzyme is the concentration of substrate, [S]. However, studying the effects of substrate concentration is complicated by the fact

that [S] changes during the course of an in vitro reaction as substrate is converted to product. One simplifying approach in kinetics experiments is to measure the **initial rate** (or **initial velocity**), designated $\mathbf{V_0}$**,** when [S] is much greater than the concentration of enzyme, [E].

The effect on V_0 of varying [S] when the enzyme concentration is held constant is shown in Figure 1. At relatively low concentrations of substrate, V_0 increases almost linearly with an increase in [S]. At higher substrate concentrations, V_0 increases by smaller and smaller amounts in response to increases in [S]. Finally, a point is reached beyond which increases in V_0 are very small as [S] increases. This plateau-like V_0 region is close to the maximum velocity, V_{max}.

Leonor Michaelis and Maud Menten explained the relationship between [S] and V_0 and derived an equation called Michaelis-Menten Equation:

$$V_0 = \frac{V_{\max}[S]}{K_{\mathrm{m}} + [S]}$$

Where V_0 is the initial velocity, V_{max} is the maximum velocity, [S] is the substrate concentration and K_m is the Michaelis constant. Substances that stick tightly to the stationary phase move very slowly, while those that stick loosely or do not stick at all move rapidly.

The curve expressing the relationship between [S] and V_0 (Figure 5.1) is also called as Michaelis-Menten curve and has the same general shape for most enzymes (it approaches a rectangular hyperbola).

An important numerical relationship emerges from the Michaelis-Menten equation in the special case when V_0 is exactly one-half V_{max}. Then,

$$\frac{V_{\max}}{2} = \frac{V_{\max}[S]}{K_{\mathrm{m}} + [S]}$$

If we solve the equation we obtain,

$$K_{\mathrm{m}} = [S], \qquad \text{when } V_0 = \frac{1}{2}V_{\max}$$

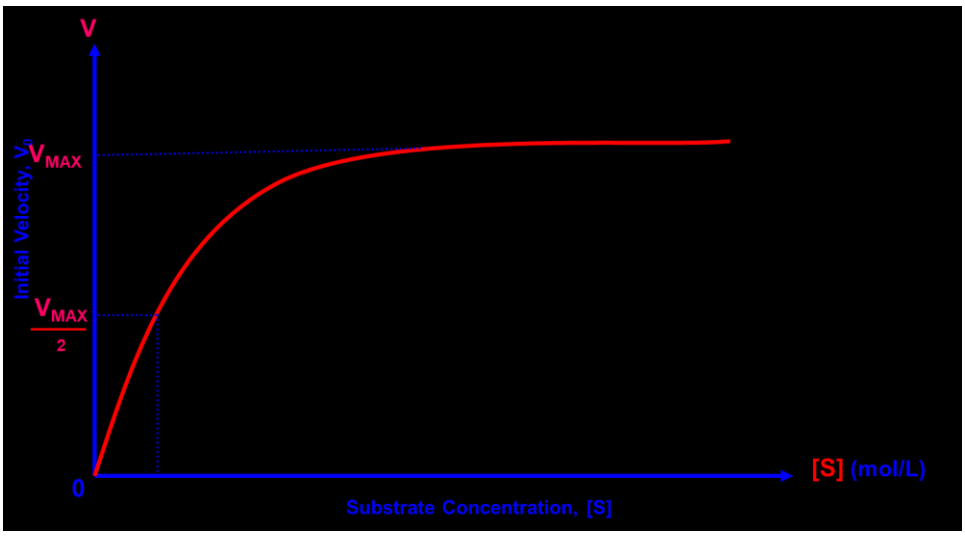

Figure 5.1. Michaelis-Menten Plot

It is not easy to determine V_{max} using the Michaelis Menten plot constructed with the experimental values obtained in an enzyme kinetics study. Only an approximation is possible.

Lineweaver and Burk simplified the Michaelis-Menten equation to:

$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

A plot of $1/V_0$ versus $1/[S]$ (the "double reciprocal" of the V_0 versus [S] plot we have been using to this point) yields a straight line. This line has a slope of K_m/V_{max}, an intercept of $1/V_{max}$ on the $1/V_0$ axis, and an intercept of $-1/K_m$ on the $1/[S]$ axis. The double-reciprocal presentation, also called a Lineweaver-Burk plot, has the great advantage of allowing a more accurate determination of V_{max}.

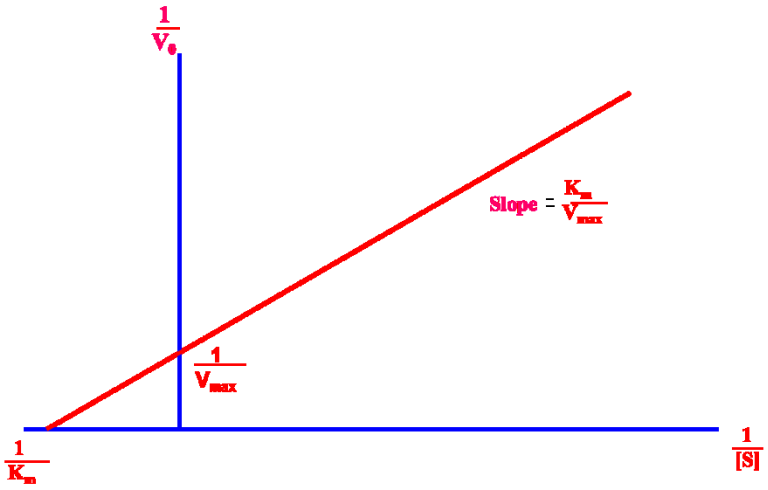

Figure 5.2. Lineweaver-Burk plot

In this experiment, we will study the kinetics of alkaline phosphatase (ALP) enzyme which is widely expressed in tissues like kidney, bone, liver and placenta. It cleaves phosphate from phosphate containing products and uses Zn^{++} and Mg^{++} as cofactor. It shows the best catalytic activity under alkaline conditions (pH = 9.0). In the experiment, we will use p-nitrophenly phosphate as the phosphate containing substrate of the ALP enzyme. The enzyme will convert p-nitrophenly phosphate into p-nitrophenol which is a yellow colored product (Figure 5.3). Product concentration can be calculated from the absorbance of the p-nitrophenol using Beer-Lambert's Law.

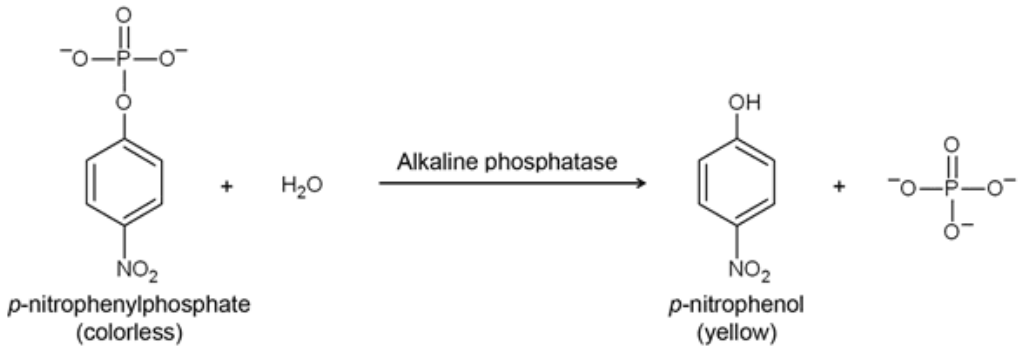

Figure 5.3. The reaction catalyzed by alkaline phosphatase.

5.3. MATERIALS AND METHODS

5.3.1. Materials

Alkaline Phosphatase from Human Placenta, p-nitrophenyl phosphate, Spectrophotometer, 96-well plates, Micropipettors, Pipette tips

5.3.2. Experimental Method:

Determination of V_0

1. Pipette 100 µL of the enzyme into a well on the 96-well plate.
2. Pipette 100 µL of the enzyme and 100 µL buffer solution to another well as the blank.
3. Add 100 µL of the substrate into the enzyme solution.
4. Start the timer.
5. Quickly mix the solution in the well by inversion and place in the spectrophotometer.
6. Read the absorbance at 405 nm beginning from the first 15th second after addition of substrate for 2 minutes with 15 seconds intervals.
7. Record the absorbance values and calculate the product concentration using Beer-Lambert's Law (Use $\varepsilon = 18.5\ mM^{-1}\ cm^{-1}$ and $l = 0{,}56$ cm).

8. Plot product concentration vs. time graph using Excel. Draw a best-line. Using the slope of the best-line determine the initial velocity (V_0).

Determination of K_m and V_{max}

1. Prepare the following substrate concentrations: 0.1 mM, 0.01 mM, 0.001 mM, 0.0001 mM, 0.0002 mM, 0.00001 mM, 0.00002 mM,
2. Pipette 100 µL of the enzyme into 7 wells on the 96-well plate.
3. Pipette 200 µL of the enzyme solution to another well as the blank.
4. Add 100 µL of each substrate concentration on the enzyme solutions in different wells.
5. Start the timer.
6. Quickly mix the solution in the well by inversion and place in the spectrophotometer.
7. Read the absorbance at 405 nm beginning from the first 15th second after addition of substrate for 2 minutes with 15 seconds intervals.
8. Record the absorbance values and calculate the product concentration using Beer-Lambert's Law (Use $\varepsilon = 18.5\ mM^{-1}\ cm^{-1}$ and $l = 0.56$ cm).
9. Plot product concentration vs. time graph using Excel. Draw a best-line. Using the slope of the best-line determine the initial velocity (V_0) for each substrate concentration.
10. Plot Michaelis-Menten and Lineweaver Burke plot curves using V_0 and [S] values.
11. Determine V_{max} and K_m values.