

EXPERIMENT 4

DETERMINATION OF K_m AND V_{max} USING LINEWEAVER – BURK GRAPH

Structure

4.1	Introduction	4.5	Determination of K_m and V_{max} Using Lineweaver – Burk plot/graph - Protocol
	Expected Learning Outcomes		
4.2	Principle	4.6	Precautions
4.3	Reagents	4.7	Summary
4.4	Preparation of Enzyme Extract		

4.1 INTRODUCTION

You have learned in core course BBCCT-107 of Enzymes that various factors e.g., substrate availability, pH of medium, temperature, presence of activator/inhibitor etc. can affect the rate of an enzyme catalyzed reaction. Among these, substrate concentration is very important factor affecting the rate of reaction. E and S combine to form [ES] complex which is converted to [EP] from which finally the product P is released. With the increase in substrate concentration, velocity of reaction is increased in a hyperbolic manner (See Fig. 4.1). At low substrate concentration, not all the active sites of enzyme are occupied by substrate molecules and the rate of reaction will be proportional to the substrate concentration exhibiting first order reaction kinetics. When the substrate concentration is very high, enzyme is saturated i.e., the number of substrate molecules exceeds the number of active sites of the enzyme. Under this condition, increase in substrate concentration will not affect the velocity of enzyme catalyzed reaction i.e., velocity will be independent of the substrate concentration. At this stage velocity is at a maximum (V_{max}), and zero order kinetics is observed.

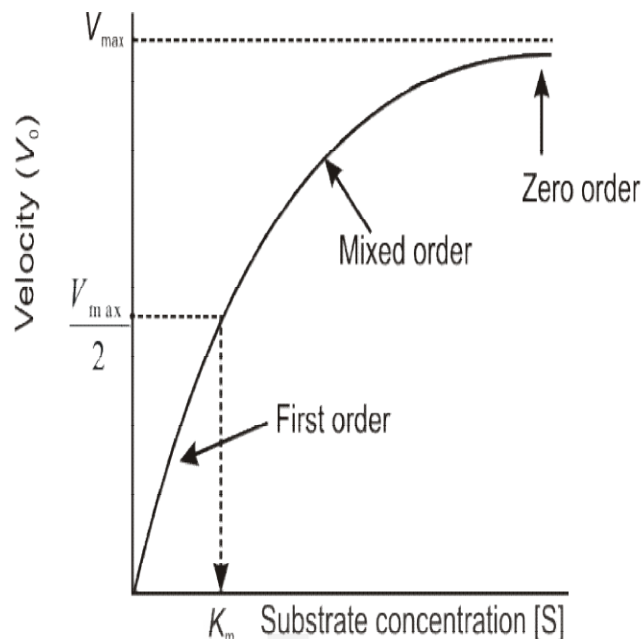


Fig. 4.1: Effect of substrate concentration on enzyme activity.

The mathematical expression of the hyperbolic shape curve is given by Michaelis-Menten equation:

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

It shows relationship between initial velocity (V_o) with maximum velocity (V_{max}), initial substrate concentration $[S]$ and the Michaelis constant (K_m).

In this equation, if $[S] = K_m$, the $V_o = V_{max}/2$

Therefore, Michaelis constant (K_m) can be defined as the substrate concentration at which the initial velocity is half of the maximum velocity. It is expressed in the units of substrate (M or mM).

K_m is a measure of affinity of an enzyme for its substrate. A large K_m means low affinity while a low K_m value indicates high affinity for the given substrate.

V_o vs. $[S]$ curve gives only an approx. value of K_m as V_{max} is not clearly determined.

Lineweaver-Burk plot is obtained by transformation of Michaelis-Menten equation and is a very common method to determine K_m and V_{max} .

Taking reciprocal on both sides of equation;

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

It is a straight line equation. By plotting a graph $1/V_0$ vs. $1/[S]$, V_{\max} on Y-axis and K_m from X-axis can be obtained. It is also called double reciprocal plot as $1/V_0$ vs. $1/[S]$ are plotted (Fig. 4.2).

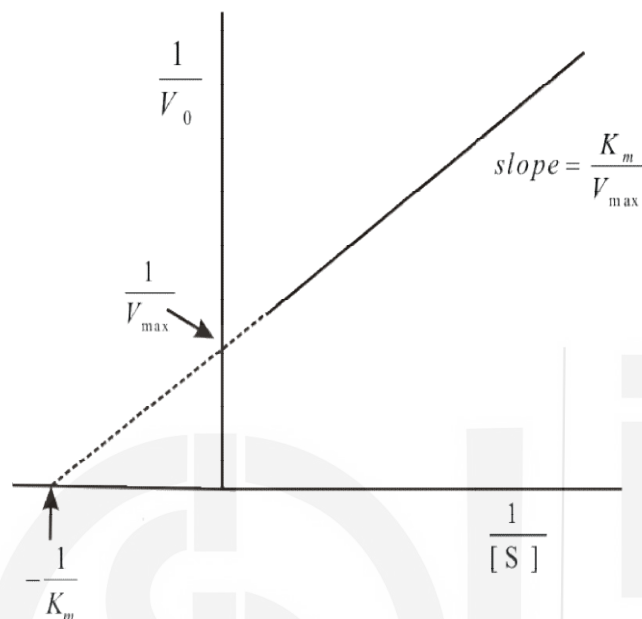


Fig. 4.2: Lineweaver-Burk plot (Double Reciprocal plot).

Expected Learning Outcomes

After studying this unit, you should be able to:

- ❖ understand how substrate level affects the rate of an enzyme catalyzed reaction; and
- ❖ perform the experiment to study the effect of substrate concentration on activity of acid phosphatase and determine K_m and V_{\max} using Lineweaver-Burk plot/graph.

4.2 PRINCIPLE

The hyperbolic curve (initial velocity V_0 vs. substrate concentration) is not entirely satisfactory for determination of K_m and V_{\max} for an enzyme catalyzed reaction. The Michaelis-Menten equation can be rearranged by taking reciprocals on both sides of equation to a form that is equivalent to the straight-line equation. By plotting $1/V_0$ on Y-axis (ordinate) and $1/[S]$ on X-axis (abscissa), a straight line relationship is observed which is known as Lineweaver-Burk plot, where the slope is K_m/V_{\max} and the intercept on the Y-axis is $1/V_{\max}$. The X-intercept is $-1/K_m$ where K_m is the Michaelis-Menten constant. In acid phosphatase catalyzed reaction using *p*-nitrophenyl phosphate as substrate, the inverse values of different concentrations of *p*-nitrophenol (i.e., $1/V_0$) vs. the inverse values of *p*NPP concentrations (i.e.,

$1/[S]$ are plotted to determine the K_m and V_{max} from the Lineweaver and Burk plot.

Note:

1. Only approximate values of V_{max} and K_m can be obtained from velocity (V_o) vs. $[S]$ graph.
2. Reactions for substrate conversion etc. are given in Expt. 1.

4.3 REAGENTS

- i) Buffer: 0.1M Sodium citrate buffer (pH 4.8) as described in experiment 1.
- ii) Substrate solution: 5 mM *p*-nitrophenyl phosphate (*p*NPP), dissolved in 0.1 M sodium citrate buffer (pH 4.8).
- iii) 0.2N Sodium hydroxide solution

4.4 PREPARATION OF ENZYME EXTRACT

Follow the steps as already explained in Ex. 1.

4.5 DETERMINATION OF K_m AND V_{max} USING LINEWEAVER-BURK PLOT/GRAPH-PROTOCOL

Take clean test tubes numbered from 1 to 9. Pipette out different volumes of 5 mM *p*NPP solution and the citrate buffer as follows (in duplicate). Prepare another set also and mark as Blank e.g. B1, B2, B3, B4 B5, B6, B7, B8, B9.

Reagent	Tube								
	1	2	3	4	5	6	7	8	9
5 mM <i>p</i> NPP solution (mL)	0.025	0.05	0.10	0.15	0.20	0.25	0.3	0.6	0.9
0.1M Citrate buffer (pH 4.8) (mL)	0.875	0.85	0.80	0.75	0.70	0.65	0.6	0.3	-
	Mix properly and equilibrate at 37°C for ~3 min								

- Start the reaction by adding 0.1 mL of enzyme extract in each tube (assay tubes only). Note down the time of adding the enzyme extract in first tube.
- Immediately mix properly and incubate at 37°C for 10 min.
- Stop the reaction by adding 4.0 mL of 0.2N NaOH in each tube.
- In blanks, add enzyme extract (0.1 mL in each tube) at the end after addition of 0.2N NaOH.
- Record the absorbance of each tube (assay) against the corresponding blank (prepared for each *p*NPP concentration), at 410 nm using a spectrophotometer.

Calculations and plotting the graph

- Determine the amount of *p*-nitrophenol produced in each tube from the standard curve prepared as explained in experiment 1.
- Calculate the μmoles of *p*-nitrophenol produced/min (termed as velocity, V_0) for each concentration (mM) of *p*NPP, represented as [S].
- Plot V_0 vs. [S] and note the shape of curve.
- For the Lineweaver-Burk plot (double reciprocal plot), you will have to find out the reciprocal of velocity ($1/V_0$) and substrate concentration ($1/[S]$).

Plot $1/V_0$ on Y-axis and $1/[S]$ on X-axis, and determine the value of K_m and V_{max} of the acid phosphatase catalyzed reaction.

Note: i) Prepare blank tube corresponding to each substrate concentration.

ii) The velocity of enzyme catalyzed reaction is expressed as product formed per unit time e.g. $\mu\text{moles } p\text{-nitrophenol/min}$.

4.6 PRECAUTIONS

- i) The substrate concentration should cover a wide range.
- ii) Pipetting of volumes should be very accurate.
- iii) Always wear gloves while working with *p*-nitrophenylphosphate and *p*-nitrophenol, avoid contact, inhalation etc.
- iv) Ensure equilibration at incubation temperature before adding the enzyme extract.

4.7 SUMMARY

In an enzyme catalyzed reaction as the substrate concentration is increased, the velocity of reaction increases till it reaches the maximum value (V_{max}). The substrate concentration at which the velocity of enzyme catalyzed reaction is half maximal is defined as the Michaelis constant (K_m). K_m is a measure of affinity of the enzyme for its substrate and its units are same as that of substrate. A double reciprocal plot ($1/V_0$ vs. $1/[S]$), known as Lineweaver-Burk

plot, is a common method to determine the K_m and V_{max} . Activity of acid phosphatase can be measured by using a chromogenic synthetic substrate, *p*-nitrophenylphosphate. The velocity is calculated as μ moles *p*-nitrophenol formed/min and K_m is expressed as mM. For an enzyme assay, the reaction is carried out under optimal conditions of pH, temperature, substrate concentration etc. To set up the enzyme assays, substrate should not be limiting i.e., enzyme should be saturated with the substrate.



ignou
THE PEOPLE'S
UNIVERSITY