Biochem 385 **Enzyme Kinetics: Kinetic Properties of β-Glucosidase***

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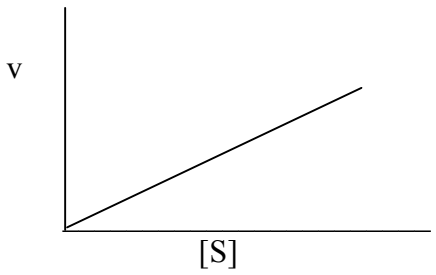
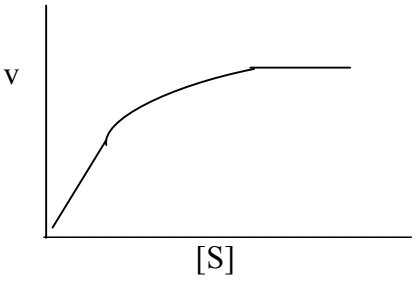
Background:

A reaction S ----->P (substrate -----> product) can be a chemical conversion or an enzyme catalyzed reaction. For a chemical conversion one gets graph A and for an enzymatic reaction one gets graph B.

Graph A



Graph B



The chemical reaction, graph A, is said to be a first order reaction because the velocity increases proportionately with substrate concentration. In the case of the enzymatic reaction, graph B, you initially get a first order reaction (at low substrate concentration), then a mixed order reaction. Finally, you get zero order kinetics, where the velocity of the reaction is no longer dependent on substrate concentration (at very high substrate concentration).

An enzyme forms a complex with its substrate. This complex then breaks down to give enzyme, E, and product, P,

$$\mathbf{E + S} \underset{\mathbf{k_{-1}}}{\overset{\mathbf{k_1}}{\rightleftharpoons}} \mathbf{ES} \xrightarrow{\mathbf{k_2}} \mathbf{E + P}$$

where $\mathbf{k_1}$ is the rate constant for the forward reaction and $\mathbf{k_{-1}}$ is the rate constant for the backward reaction. $\mathbf{k_2}$ is the rate constant for of **ES** into **E** and **P**. $\mathbf{k_2}$ is also known as $\mathbf{k_{cat}}$, or the *turnover number*.

The velocity of an enzyme catalyzed reaction can be defined as:

$$\mathbf{v = k_2\,[ES]} \quad \text{.....(1)}$$

When all the enzyme **[Et]** added to the reaction has complexed with the substrate and is producing the product, you reach maximum velocity, $\mathbf{V_{max}}$. This happens at very high substrate concentration. Therefore:

$$\mathbf{V_{max} = k_2\,[Et] = k_2\{\,[E] + [ES]\,\}} \quad \text{.....(2)}$$

Dividing equation (1) by (2) we get:

$$\frac{\mathbf{v}}{\mathbf{V_{max}}} = \frac{\mathbf{[ES]}}{\mathbf{[E] + [ES]}} \quad \text{.....(3)}$$

*Revised edition from Dr. M. Sugumaran's lab procedure and handout

At equilibrium, the rate of the forward reaction is going to be equal to the rate of the backward reaction:

$$k_1 [E] [S] = k_{-1} [ES]$$

Rearranging this equation one gets:

$$\frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1} = k_S$$ where k_S is the dissociation constant.

From this one can write:

$$[ES] = [E] [S] / k_S \quad \text{.....(4)}$$

Substituting this in equation (3) we get:

$$\frac{v}{V_{max}} = \frac{[E][S] / k_S}{[E] + [E][S] / k_S}$$

Dividing the numerator and denominator by $k_S / [E]$ we get:

$$\frac{v}{V_{max}} = \frac{[S]}{k_S + [S]} \quad \text{or} \quad v = \frac{V_{max} [S]}{k_S + [S]}$$

This is the Michalies-Menten equation: It correlates the velocity of an enzyme catalyzed reaction with the substrate concentration.
Using steady state approximation of Briggs-Haldane one can derive the same equation.
In this case one has to replace k_S in the denominator with **Km**. **Km** is the Michaelis constant and is equal to: $Km = \{ k_{-1} + k_2 \} / k_1$

At very low substrate concentration, $k_S >> [S]$, the equation can be simplified to:

$$v = V_{max} [S] / Km$$

Therefore, velocity is proportional to the substrate concentration under these conditions.

At very high substrate concentration, when $[S], >> k_S$, the equation simplifies to:

$$v = V_{max}$$

In other words, the velocity becomes constant at high substrate concentration. The substrate concentration required to produce ½ V_{max} is called "the **Km**". Km gives us another clue about how an enzyme behaves biologically.

The accurate value of $\mathbf{V_{max}}$ and **Km** can be calculated from a Lineweaver-Burk plot, which is obtained by the inversion of a Michaelis-Menten graph:

$$\mathbf{v = \frac{V_{max}\,[S]}{Km + [S]}} \qquad \textbf{'Michaelis-Menten equation}$$

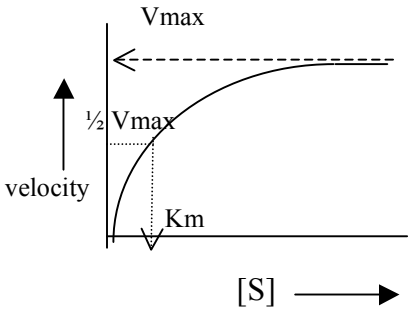
$$\mathbf{1/v = \frac{Km}{V_{max}\,[S]} + \frac{[S]}{V_{max}\,[S]}}$$

or

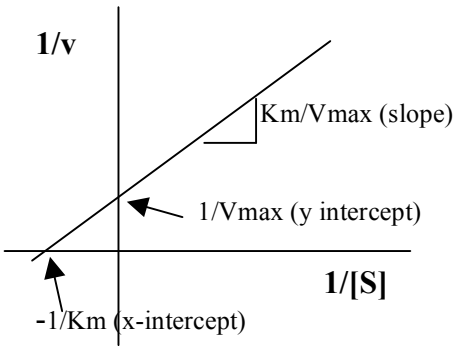
$$\mathbf{1/v = \frac{Km}{V_{max}\,[S]} + \frac{1}{V_{max}}} \qquad \textbf{'Lineweave-Burk equation}$$

y = mx + b, where: y=1/v, m = slope = Km/Vmax, x = 1/[S], b = y-intercept = 1/Vmax

If we plot 1/v on the y axis and 1/[S] on the x-axis we will get a straight line whose slope, *m* will be $\mathbf{Km / V_{max}}$ and intercept, *b,* on the y-axis will be $\mathbf{1/ V_{max}}$. The intercept on the negative side of the x-axis is **–1/Km.**



Michaelis-Menton Plot



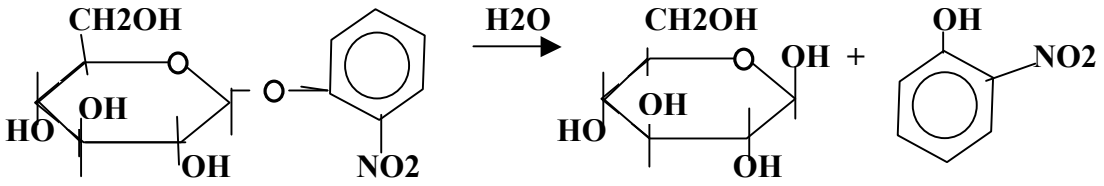
Lineweaver-Burk Plot

Introduction:

In this laboratory we will study some kinetic properties of B-glucosidase. B-glucosidase <3.2.1.21> belongs to the class of enzymes known as hydrolases, and catalyzes the conversion of glucosides into glucose and alcohol.

β-D-glucoside + water ⟶ β- D-glucose + alcohol

We will use the enzyme from almonds. This enzyme has two active forms, which differ in their molecular weight (66,500 and 117,000). It is optimally active at around pH 5. This enzyme is strongly inhibited by mercuric and other heavy metal ions. Sulfhydryl compounds and polyols also inhibit the enzyme. The enzyme activity can be measured either by monitoring the release of glucose or by following the release of alcohol. Since the enzyme shows wide substrate specificity, substrates which release chromogenic alcohols are excellent choices for quantifying the activity. We will use o-nitrophenyl-β-D-glucopyranoside as the substrate and measure the release of o-nitrophenol at 420 nm using a Spectronic 20 Colorimeter.



o-nitrophenyl-β-D-glucopyranoside β- D-glucose o-nitrophenol

The substrate o-nitrophenyl-β-D glucopyranoside is colorless; but when hydrolyzed by the enzyme, the resultant o-nitro phenol is yellow and hence the reaction can be easily monitored by the appearance of yellow color in the test tube containing enzyme and the substrate.

Materials:

Enzyme: B-glucosidase, 0.02 mg/ml in Buffer A and water
Substrate: o-nitrophenyl-β-D-glucopyranoside in water, concentrations from 0-20 mM.
Buffer A: 0.05 M sodium acetate buffer, pH5.2
Buffer B: 0.2 M sodium carbonate buffer

Spec 20 test tubes
30^{o} C water bath
Spec 20
P 200 and P 1000 pipettes

Methods:

Note: Accurate pipetting is a MUST throughout this experiment! If you have any questions on the proper use and technique of the pipettes, please ask!

A. Time course of the Reaction

In any enzyme-catalyzed reaction, the velocity is calculated based on a linear relationship between product formation and time for the initial phase of the reaction. We will check this assumption in the following exercise. This will also verify that in part B, when we test the effect of substrate concentration on the reaction, we incubate the reactions for a valid period of time, i.e. where the

product formation is linear. Some reasons for nonlinearity of an enzyme-catalyzed reaction include substrate depletion, inhibition of the enzyme by the product or enzyme inactivation.

1. Place seven Spec20 test tubes, labeled 0, 6, 12, 18, 24, 30, 36 (i.e. 6 minute intervals), in a test tube rack.
   Place flask of 0.05 M sodium acetate buffer at 30 °C to equilibrate
2. Add **200 ul** of 10 mM substrate solution to each tube.

3. Add **700 ul** of 0.05 M sodium acetate buffer, pH5.2 to each tube.

4. Place the test tube rack in a 30°C water bath for 10 minutes to equilibrate the tubes.

5. Add **4 ml** of 0.2 M sodium carbonate buffer followed by **100 ul** enzyme solution (2 ug) to the test tube marked '0 min'. Mix well. This is your **Blank** for setting the Spec20 to 0% absorbance (100% transmittance). By stopping the reaction before adding enzyme, this tube should show no enzymatic product formation, which means no yellow color!!

   Note: Each tube will receive 2 ug of enzyme.
   (0.020 mg β-glucosidase / ml) * 0.100ml = 2 ug β-glucosidase

6. For steps 7 and 8, keep tubes at 30°C during addition of enzyme…lift only briefly to mix. You may remove tubes from water bath after addition of 0.2 M Na Carbonate buffer.

7. Add **100 ul** of enzyme solution to the tube marked '6 min' and mix gently. Incubate at 30°C for six minutes before adding **4 ml** of 0.2 M sodium carbonate buffer to arrest the reaction.

8. After exactly one minute of starting the above reaction in tube labeled '6 min', start the reaction in the tube marked '12 min' by the addition of **100 ul** of enzyme. Let reaction proceed for 12 minutes before adding **4 ml** of 0.2 M sodium carbonate buffer to arrest the reaction.

9. Similarly start all the reactions at one minute intervals. Arrest the reactions at appropriate time intervals. Your time schedule for this experiment is shown in Table 1.

10. After stopping all the reactions, mix each tube well and read the absorbance of each tube at 420nm. Remember to set the Spec20 to zero absorbance with the tube labeled 'Blank'.
    [If the absorbance of any sample is too high, dilute that sample 1:3 by mixing 1 ml sample + 2 ml 0.2 M sodium carbonate buffer and measure the absorbance again. Remember to correct for the dilution factor in your calculations!]

Table 1, B-glucosidase, product vs. time

Test tube labeled:	**Start** each reaction by addition 100ul enzyme: (your start time)		**Stop** reaction by addition of 4 ml Na carbonate buffer: (your stop time)	
6	0 min	(_____)	6 min	(_____)
12	1 min	(_____)	13 min	(_____)
18	2 min	(_____)	20 min	(_____)
24	3 min	(_____)	27 min	(_____)
30	4 min	(_____)	34 min	(_____)
36	5 min	(_____)	41 min	(_____)

11. Calculate the formation of o-nitrophenol in umoles/ml:
    a) The millimolar extinction coefficient of o-nitrophenol at 420nm under these conditions is 17.7. This means that 1 umole o-nitrophenol in 1ml will have an absorbance of 17.7 OD.
    b) If your sample has an absorption of X units then the amount of o-nitrophenol in the sample is: X / 17.7 umoles
    c) You diluted 1 ml of your reaction mixture (0.2 substrate + 0.7 buffer + 0.1 enzyme) up to 5 ml total volume by addition of the 4ml Na Carbonate. This is a 1:5 dilution. Therefore the original 1 ml solution will have 5 times the above value of umoles.
    Umoles o-nitorphenol / 1ml reaction mixture = (X / 17.7) x 5
    d) If you made additional dilutions, multiply the result of (c) by the dilution factor.

Data Table 1, B-glucosidase, product vs. time

Test tube	Incubation Time, x	A420nm	Product Formation, y umoles/ml (see calculations in step 10)
0	0 min	_____	_____
6	6 min	_____	_____
12	12 min	_____	_____
18	18 min	_____	_____
24	24 min	_____	_____
30	30 min	_____	_____
36	36 min	_____	_____

12. Plot the time course of product formation, **o-nitrophenol (umoles /ml) vs. time (minutes).** Check whether it is linear or not, especially at 15 minutes incubation time.

B. EFFECT OF SUBSTRATE CONCENTRATION ON THE VELOCITY OF THE REACTION

In this part of the experiment, we will study the effect of different concentrations of substrate ion the initial velocity of the reaction. The velocity versus substrate concentration, Michaelis-Menton plot, is plotted first to get approximate values of Vmax and Km. They are then plotted as the double reciprocal plot, Lineweaver-Burk, to extract more exact values for Vmax and Km.

1. Place thirteen Spec 20 test tubes, labeled 0 mM, 1 mM, 1.33 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 10 mM, 15 mM and 20 mM in a test tube rack.
   Place flask of 0.05 M sodium acetate buffer at 30 °C to equilibrate
2. Add **200 ul** of the corresponding substrate solutions to the respective tubes.
3. Add **700 ul** of 0.05M sodium acetate buffer, pH 5.2 to each tube.
4. Place the test tube rack in a 30°C water bath for 10 minutes to equilibrate the tubes.
5. For steps 6 -10, keep tubes at 30°C during addition of enzyme…lift only briefly to mix. You may remove tubes from water bath after addition of 0.2 M Na Carbonate buffer.
6. Add **100 ul** of β-glucosidase solution (2 ug) to the tube marked '0mM' at 0 time. Mix gently. Keep the tube at 30°C; do not take outside of water bath. This tube will be used as your Blank.
7. After exactly one minute of starting the above reaction, start the reaction in the tube marked '1mM' by the addition of **100 ul** of enzyme. Mix gently. Keep the tube at 30°C; do not take outside of water bath.
8. Start all the other reactions at one minute intervals in exactly the same way. When you start the last reaction (20 mM) the time will be 12 min.
9. At exactly 15 minutes stop the reaction in the tube marked '0 mM' by addition of **4 ml** of 0.2 M sodium carbonate buffer.
10. At one minute intervals, stop the reactions in the remaining tubes by the addition of **4 ml** of 0.2 M sodium carbonate buffer. The time schedule is shown in Table 2.

Table 2, B-glucosidase, v vs. [S]

Test tube Labeled:	**Start** each reaction by addition 100ul enzyme:	**Stop** reaction by addition of 4 ml Na carbonate buffer:
0 mM	0 min (_____)	15 min (_____)
1 mM	1 min (_____)	16 min (_____)
1.33 mM	2 min (_____)	17 min (_____)
2 mM	3 min (_____)	18 min (_____)
3 mM	4 min (_____)	19 min (_____)
4 mM	5 min (_____)	20 min (_____)
5 mM	6 min (_____)	21min (_____)
6 mM	7 min (_____)	22 min (_____)
7 mM	8 min (_____)	23 min (_____)
8 mM	9 min (_____)	24 min (_____)
10 mM	10 min (_____)	25 min (_____)
15 mM	11 min (_____)	26 min (_____)
20 mM	12 min (_____)	27 min (_____)

13. After stopping all the reactions, mix each tube well and read the absorbance of each tube at 420nm. Remember to set the Spec20 to zero absorbance with the tube labeled '0 mM'.
    [If the absorbance of any sample is too high, dilute that sample 1:3 by mixing 1 ml reaction sample + 2 ml 0.2 M sodium carbonate buffer and measure the absorbance again. Remember to correct for the dilution factor in your calculations!]

14. Repeat the experiment once or twice if time permits. Average the duplicate absorbance units before proceeding to the calculations.

15. Calculate the Velocity of the reaction, which is *usually defined* as:

    umoles product / minute / mg enzyme.

    a) The millimolar extinction coefficient of o-nitrophenol at 420 nm under these conditions is 17.7. This means that 1 umole o-nitrophenol in 1ml will have an absorbance of 17.7 OD.

    b) If your sample has an absorption of X units then the amount of o-nitrophenol in the sample is: X / 17.7 umoles

    c) You diluted 1 ml of your reaction mixture (0.2 sub + 0.7 buffer + 0.1 enz) up to 5 ml total volume by addition of the 4ml Na Carbonate. This is a 1:5 dilution. Therefore the original 1 ml solution will have 5 times the above value of umoles.

Umoles o-nitorphenol / 1ml reaction mixture = (X / 17.7) x 5

d) If you made additional dilutions, multiply the result of (c) by the dilution factor.

e) You are interested in the **velocity** of the reaction. First calculate the umoles product / minute. Because each reaction was incubated for 15 minutes, divide the number of umoles obtained in step (c) by 15.

umoles / min = (X / 17.7) * (5 / 15 min)

f) Each assay (tube) contained 2 ug (0.002 mg) of enzyme protein. It is a standard custom to give velocity for 1 mg protein. Therefore, divide by 0.002mg.

Velocity = umoles / min / mg = (X / 17.7) * (5 / 15 min) * (1 / 0.002 mg)

= umoles o-nitrophenol formed / min / mg β-glucosidase

Data Table 2, B-glucosidase Kinetics, velocity vs. [S]

[S]	A420	velocity	1 / [S]	1/v
0 mM	_____	_____	___N/A__	___N/A__
1 mM	_____	_____	_____	_____
1.33 mM	_____	_____	_____	_____
2 mM	_____	_____	_____	_____
3 mM	_____	_____	_____	_____
4 mM	_____	_____	_____	_____
5 mM	_____	_____	_____	_____
6 mM	_____	_____	_____	_____
7 mM	_____	_____	_____	_____
8 mM	_____	_____	_____	_____
10 mM	_____	_____	_____	_____
15 mM	_____	_____	_____	_____
20 mM	_____	_____	_____	_____

RESULTS:

1. Plot **velocity vs. substrate concentration** in a standard Michaelis-Menton plot.
   label the Vmax, ½ Vmax and Km in the graph. Make sure to label x and y axis. Find out the values of Vmax, ½ Vmax and Km and present the values in the graph itself.

2. Plot the double reciprocal plot called Lineweaver-Burk plot, (**1 / v) vs. (1 / [S]). Find, draw** Remember to extend the x-axis so that you can draw the x-intercept.
   label 1/Vmax, and -1/Km in the graph. Also determint the slope of the line. Make sure to label x and y axis. Find out the values of Vmax, and Km and present it in the graph itself.

QUESTIONS: (If you answered any of these questions in your introduction, please write "see Introduction" so I can be sure you understand)

1. Write the equations for the M-M and L-B plots
2. Determine and/or calculate the **Vmax and Km** from each type of plot**.**
3. Compare the results from each method.
4. What are the advantages or disadvantages of each method of analysis?
5. What is the significance of Vmax and Km for someone studying enzyme kinetics.