Bioengineering Laboratory I

Enzyme Assays

Part II: Determination of Kinetic Parameters

2016-2017 Fall Semester

1. Theoretical background

There are several mathematical models to determine the kinetic constants for enzymatic reactions. One of the simplest and most useful is that developed by Michaelis and Menten. Consider the following set of reactions for an enzyme-catalyzed process:

$$\mathbf{E} + \mathbf{S} \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} \mathbf{ES} \underset{k_{-2}}{\overset{k_2}{\leftrightarrows}} \mathbf{E} + \mathbf{P} \quad (1)$$

where **E** is the free enzyme; **S** is the free substrate, the substance for which the enzyme serves as a reaction catalyst; **ES** is the enzyme-substrate complex; **P** is the product of the reaction; and the kn's are the individual forward and backward rate constants. Note that the equilibrium constant for the first step in the reaction is $K_{eq} = k_1/k_{-1}$.

Usually the reaction is studied only in its early stages so that there is no significant buildup of the product **P**. Therefore, very little of the back reaction represented by k_{-2} occurs. In this case, Equation (1) can be simplified to

$$\mathbf{E} + \mathbf{S} \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} \mathbf{ES} \xrightarrow{k_{cat}} \mathbf{E} + \mathbf{P} \quad (2)$$

where k_{cat} is the catalytic rate constant for the conversion of the enzyme-substrate complex **ES** to the product **P** and regenerating the free enzyme **E**, which is then able to react with another substrate molecule.

Under these conditions, the reaction rate or reaction velocity v is linearly proportional to the enzyme concentration [**E**], assuming it remains at a low, catalytic level. However, a plot of v as a function of [**S**] shows a linear dependence and first-order kinetics only during the initial stages of the reaction, about the first 10% or so, before the rate of the back reaction becomes significant. The plot then becomes curvilinear downward and approaches an asymptomatic value of v as shown in Figure 1. Therefore enzyme-catalyzed reaction kinetics can be studied by varying the concentration of substrate S and measuring the amount of product P formed by the enzyme per unit time. Without going into the details of its derivation, the Michaelis-Menten equation was developed to explain the observed kinetic behavior:

$$v = \frac{V_{max}[\mathbf{S}]}{K_m + [\mathbf{S}]} \quad (3)$$

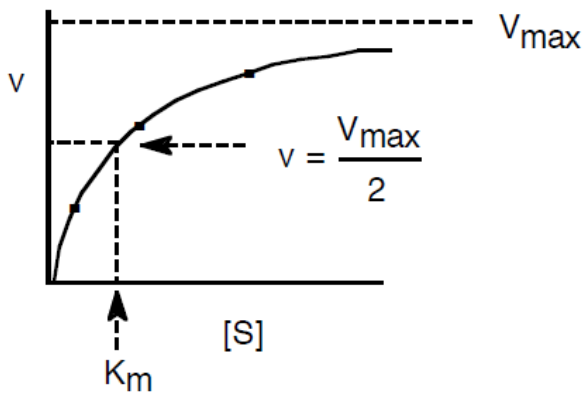


Figure 1. Change in reaction rate as a function of substrate concentrations

where v is the velocity of the reaction, V_{max} is the maximum (theoretical) velocity, and K_m is the Michaelis constant $(k_{-1} + k_{cat})/k_1$. The maximum theoretical velocity, V_{max}, is the velocity when the substrate binds to all of the active sites on all the enzymes, when it is totally "saturated". This is impossible because there will always be some free **E** available; the reaction to produce product and free **E** is always going on. The kinetic parameters can be estimated by fitting the experimental data (points) to the Michaelis-Menten equation using non-linear regression. This generates a hyperbolic curved line of best fit through the data points. The Michaelis constant K_m turns out to be numerically equal to the substrate concentration [**S**] that produces a velocity $v = V_{max}/2$ (Figure 1)

The features of the v vs [S] plot can be summarized as;

i) At low substrate concentrations ($[S] << K_m$) the observed product formation rates v are directly proportional to substrate concentrations (v is 1st order with respect to substrate; double the substrate concentration doubles the rate of product formation). In this region, a constant percentage of substrate is cleared from solution per unit time.

ii) At high, saturating concentrations of substrate ($[S] >> K_m$) the observed product formation rates are independent of substrate concentrations (v is zero order with respect to substrate; double the substrate concentration, no change in rate). In this region a constant amount of substrate is cleared from solution per unit time.

iii) At substrate concentrations in the region of the K_m ($[S]=K_m$) the reaction order is approximately 0.5 (double the substrate concentration increase v by 50%.

K_m and V_{max} provide very important information about an enzymatic reaction, and are among the very first things that scientists try to determine or verify for an enzyme they are using.

Important aspects of K_m

i) K_m values are reported in units of substrate concentration (molar (M), millimolar (mM), micromolar (μM), etc).

ii) The K_m is a measure of the affinity of a particular substrate for a particular enzyme. The lower the K_m the higher the affinity of the substrate for the enzyme.

iii) K_m is not dependent on enzyme concentration and is a constant for a given substrate enzyme pair under standard conditions.

When we know the amount of the enzyme itself we can calculate a k_{cat} or **turnover number**, the number of substrate molecules transformed to product per unit time by a single enzyme molecule under maximal conditions. This provides a good measure of the speed and efficiency of an enzyme. The kcat is a constant for a given substrate enzyme pair under standard conditions.

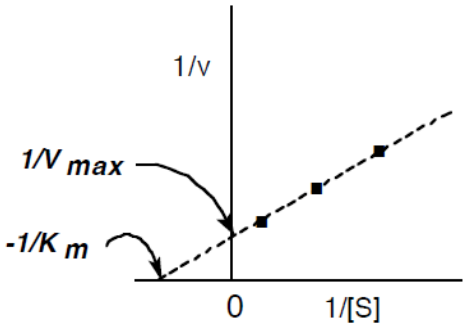
The ratio of V_{max}/K_m or (enzyme catalytic efficiency when we know the amount of enzyme) is a useful parameter.

i) Prediction of whole body hepatic or tissue clearances from in vitro incubation data using pharmacokinetic models and scaling factors.

ii) Comparing the effects of amino acid changes on enzyme function.

iii) Predicting the relative contribution of different enzymes to the clearance of a drug.

Another model to determine the kinetic constants for enzymatic reaction is Lineweaver-Burke Plot (a double reciprocal plot of the data (1/v vs 1/[S])). We can calculate the reciprocal values of the velocities and substrate concentrations and plot each pair of reciprocal values. If the points lie on a straight line we can draw or calculate that line and calculate V_{max} and K_m from the intercepts.



Lineweaver Burke Plot
of a hyperbolic function
is a straight line.
A double reciprocal plot

Figure 2. Lineweaver-Burke Plot

Eadie Hofstee Plot is also third model.(a reciprocal plot (v vs v/[S]). Again data points should lie on a line. A very sensitive and discriminating plot.

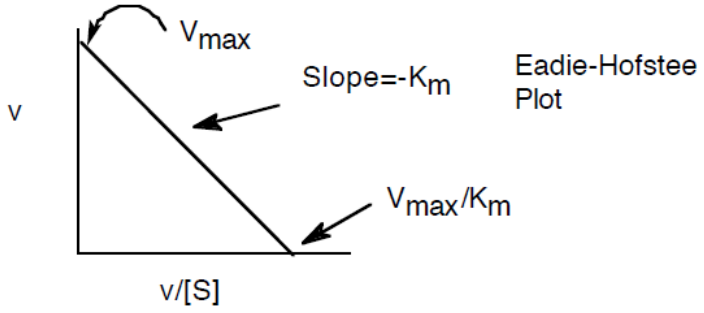


Figure 3. Eadie Hofstee Plot

The reciprocal plots are very useful for detecting non-Michaelis-Menten behavior. Three major types of non-classical behavior are:

i) When multiple enzymes in liver microsomes catalyze the formation of the same product.

ii) Allosteric behavior when more than one substrate binding site exists on a single enzyme.

iii) Non-specific binding of substrates to protein or lipid. When free substrate concentrations available to the enzyme vary significantly from the nominal concentrations or you have messed up on your serial dilutions.

2. Objective

The aim of this experiment is to introduce the important concepts in enzyme kinetics using tyrosinase enzyme as an example. Another scope is to familiarize students with the important terms and assumptions. They improve their "kinetic intuition" (does this make sense?) and pattern recognition (plots). The students also learn to determine parameters and verify mechanism whether or not the enzyme-catalyzed reaction follows Michaelis Menten kinetics.

3. Materials

Chemicals: Tyrosinase from mushroom (Sigma-Aldrich T3824 -25KU), sodium dihydrogen phosphate monohydrate "$NaH_2PO_4*H_2O$" (98%, Merck), di-Sodium hydrogen phosphate dihydrate "$Na_2HPO_4*2H_2O$" (99%, Merck), pyrocathechol (Sigma-Aldrich C9510). All solution are prepared with ultra pure water (18.2 MΩ) .

Materials: Micropipettes, UV quartz cuvettes (3 ml), stirrer bar, erlenmayer, beaker, spoon, graduated cylinder.

Instruments: Thermo scientific evaluation 201 UV-Visible Spectrophotometer, Thermo Scientific Peltier System, pH meter (WTW), analytical balance (Sartorius), magnetic stirrer.

4. Experimental procedure

Preparation of solutions

a- Prepare 50 mM 50 ml $NaH_2PO_4*H_2O$ and $Na_2HPO_4*2H_2O$ solution by using ultrapure water

b- Measure the pH of these two buffer solution by pH meter.

c- Adjust pH of the phosphate buffer solution (PBS) as 6.5 by mixing these two buffers.

d- Prepare a stock of 2 mM catechol in 30 ml PBS.

e- Prepare 0.1, 0.3, 0.5, 0.7, 1 and 1.5 mM catechol solution by using the 2mM one (dilution)

f- Prepare 700 µl, 350 U enzyme solution from stock enzyme solution (25 KU/ml PBS).

g- Use catechol and enzyme solutions within 1 or 2 hours. Because these solutions are rather unstable at room temperature.

Activity measurement

a- Open rate mode.

b- Set wavelength at maximum absorbance for the product (390 nm), temperature to 25 ºC, stirrer speed at 7 level (approximately 700 RPM) and time 70 sec.

c- Fill both UV quartz cuvette with 3 ml PBS.

d- Put the cuvettes in UV instrument for base line correction.

e- Cleanout one of your cuvette and fill with 3 ml 0.1 mM catechol solution.

f- Start measurement while pipetting 100 µl (includes 50 U) tyrosinase solution which provides in-situ analysis. Formation o-benzoquinone at early time points can be observed, when it is linear with time.

g- According to the max peak of o-quinone (determined as 390 nm from previous experiment), enzymatic activity is determined by monitoring its formation during 70 sec.

h- Repeat step from e to h for each concentration of catechol (from 0.3 to 2 mM).

i- Therefore velocity of the enzyme reaction could be determined for each substrate concentration.

5. Data evaluation

5.1. Determination of Initial Velocity (from previous experiment): In this part tyrosinase activity is measured by evaluating the velocity (rate) at which substrate (catechol) is converted to product (o-quinone). A change in absorbace with time provides an accurate estimation of the initial velocity of the reaction. For this aim, plot absorbance of o-quinone formation versus time graph. It is significant to collect the optimal data. The increase in absorbance should be linear with time. Be sure that the graph is a linear portion at least 30 seconds and the linear region of your time trace does not exceed 0.8 absorbance value so that you can trust your measurement of the initial product formation rate.

The reaction slope: dA/dt (change of absorbance ΔA over the time interval Δt: 30 sec.) gives the initial steady-state reaction condition

Use the Beer–Lambert relationship; [28].

$$A = \varepsilon p l$$

where "A" is absorbance, "p" is product concentration, "ε" is extinction coefficient (for o-quinone formation from catechol at maximum absorbance was taken as ε=1417 $M^{-1}cm^{-1}$) and "l=1 cm" is path- length. The slope of the kinetic curves is converted to the tyrosinase activity (expressed as μmol/min).

$$\frac{dP}{dt} = \frac{dA}{dt}\frac{1}{\varepsilon l}$$

5.2.Determination of kinetic parameters (V_{max} and Km):

✓ Fill in the following TABLE

[S] (mM)	v (μmoles/min)	v/[S]	1/v	1/[S]
0.1				
0.3				
0.5				
0.7				
1				
1.5				
2				

✓ Determine the kinetic parameters (V_{max} and K_m) by using both Michaelis-Menten plot Lineweaver-Burke Plot (a double reciprocal plot of the data (1/v vs 1/[S])) and Eadie Hofstee plot a reciprocal plot of the data (v versus v/[S]).

Michaelis-Menten method: Plot the rate of catechol conversion (v) (y-axis) and substrate concentration (x-axis). (Michaelis-Menten plot). Then solver program can be used to find out the kinetic parameters (V_{max} and K_m)

Lineweaver-Burke method: Plot a double reciprocal of the values that is, 1/S versus 1/v. (Note that the x intercept is $-1/K_m$, from which the Michaelis-Menten Constant (K_m) may be determined. The y intercept is $1/V_{max}$ and the slope equals K_m/V_{max}.)

Eadie Hofstee method: Plot a reciprocal of the values that is, v versus v/[S]. (Note that the x intercept is V_{max}/K_m, from which the Michaelis-Menten Constant (K_m) may be determined. The y intercept is V_{max} and the slope equals $-K_m$)