Experiment #7
Tem-1 β-Lactamase Activity Assay

Introduction

Once you have purified an enzyme, it is valuable to characterize its steady-state kinetic properties by determining its K_m, v_{max}, and the inhibition constants for various substances, including its products. These studies provide information about how the enzyme responds to substrates and inhibitors. It allows you to compare its kinetic properties to those of other enzymes catalyzing similar reactions. From this you may deduce whether they utilize similar mechanisms. From steady-state kinetic analyses, you can frequently determine the order of substrate binding and release of products. Kinetic inhibition studies are important for evaluating the efficacy of pharmaceutical inhibitors as well as their mode of action on enzymes.

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

There are two ways to derive kinetic equations appropriate for describing the above reactions:

1. By assuming **ES** is in rapid equilibrium with **E** and **S** (Michaelis-Menten hypothesis)
2. By assuming a steady-state condition. For example, the rate of formation of **ES** equals the rate of breakdown of **ES** (Briggs-Haldane hypothesis)

For both conditions, **[ES]** is considered to remain essentially constant. The simple equilibrium treatment of Michaelis and Menten is a special case of the general steady-state theory.

Assumptions of the steady-state theory are:

1. **[S] >> [E]** so that the amount of substrate bound to the enzyme is negligible compared to the Michaelis constant (see definition below) during the reaction.
2. Only the initial velocity of the reaction is measured. Thus, the velocity measured is at **t = 0**, at which time **[P] = 0** and the **[S]** ≈ $\mathbf{[S]_{initial}}$.

Derivation of Michaelis-Menten Equation

Refer to the above scheme. Let's define **$[E]_t$** as a total enzyme concentration in the reaction. The concentration of free enzyme is then **$[E]=[E_t]-[ES]$** at any time during the reaction. Rate of enzyme-substrate complex (**ES**) formation is then:

$$\frac{d[ES]}{dt} = k_1[E][S] = k_1([E_t] - [ES])[S]$$

Similarly, rate of ES breakdown is:

$$\frac{d[ES]}{dt} = k_{-1}[ES] + k_2[ES] = (k_{-1} + k_2)[ES]$$

Under steady-state conditions, the rates of formation and breakdown of **ES** are equal; therefore:

$$k_1([E_t] - [ES])[S] = (k_{-1} + k_2)[ES]$$

After rearranging:

$$\frac{([E_t] - [ES])[S]}{[ES]} = \frac{k_{-1} + k_2}{k_1}$$

If we define Michaelis-Menten constant K_m,

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

equation for **[ES]** further simplifies:

$$\frac{[E_t][S]}{[ES]} - [S] = K_m$$

$$[ES] = \frac{[E_t][S]}{K_m + [S]}$$

The velocity of the catalyzed reaction at any time is:

$$v = k_2[ES]$$

When the enzyme is saturated with substrate so that all of it exists as the **ES** complex, the velocity is maximal ($\mathbf{v_{max}}$) and can be expressed as:

$$v_{max} = k_2[E]$$

Multiplying both sides of the equation for **[ES]** by $\mathbf{k_2}$ and substituting for **v** and $\mathbf{v_{max}}$ results in:

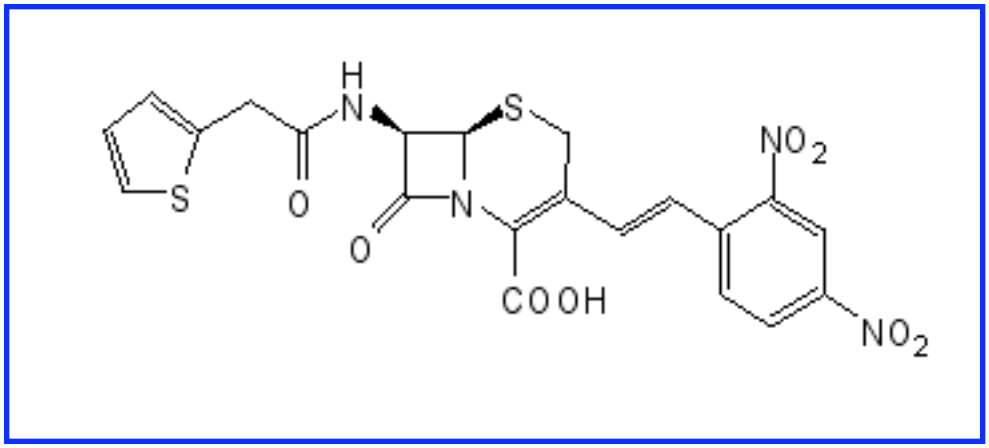
$$v = \frac{v_{max}[S]}{K_m + [S]}$$

This is the Michaelis-Menten equation where K_m is the Michaelis constant and v_{max} is the maximal velocity of enzymatically catalyzed reaction.

The $\mathbf{K_m}$ represents the concentration of substrate that produces $\mathbf{v_{max}/2}$ for the catalyzed reaction, expressed in the units of [M]. The value of $\mathbf{K_m}$ is independent of the amount of enzyme used but does depend on the assay conditions, such as pH, presence of inhibitors or activators, temperature, ionic strength, and so on. This is because $\mathbf{K_m}$ is made up of pure rate constants. $\mathbf{v_{max}}$ for a particular substrate is also a useful constant, but it should be realized that observed values for $\mathbf{v_{max}}$ (micromoles per minute) depend on the amount of enzyme used for the assay. However, for homogenous enzyme, if $\mathbf{v_{max}}$ is defined as micromoles per minute per milligram of enzyme protein, it will be a constant for a particular set of conditions (e.g., pH and temperature) for the enzyme. If the molecular weight is known and pure enzyme is available, one can calculate how much enzyme is being used in the essay; then $\mathbf{v_{max}}$ can be expressed as a fundamental constant

for the enzyme that is called **the maximum turnover number** or the catalytic constant $\mathbf{k_{cat}}$. The constant $\mathbf{k_{cat}}$ tells us how many moles of substrate per minute (or per second) will be converted to product by one mole of the enzyme under a particular set of environmental conditions when all substrates are saturating (i.e., at $\mathbf{v_{max}}$). For Michaelis-Menten kinetic scheme, $\mathbf{k_{cat} = k_2}$.

β-lactam antibiotics, such as the penicillins and the cephalosporins, are among the most commonly used antimicrobial agents. The production of β-lactamases, which catalyzes β-lactam hydrolysis, is the predominant mechanism of bacterial resistance to these antibiotics. β-lactamases are grouped into 4 classes (A, B, C, and D) based on primary sequence homology. Classes A, C and D are serine hydrolases and class B is made up of zinc metalloenzymes. Tem-1 β-lactamase is a plasmid encoded, class A enzyme that can efficiently cleave the penicillins and some cephalosporins. In this experiment you will utilize the chromogenic cephalosporin Nitrocefin as a substrate, which undergoes a color shift from yellow to red when it is hydrolyzed by Tem-1.



Experiment

For all subsequent assays you will be using a total volume of 2 mL. You will need 5 cuvettes; 50mM sodium phosphate, pH 7.0 buffer; the substrate Nitrocefin; and enzyme Tem-1 β-lactamase. For the first assay dilute the substrate 5-fold to a total volume of 2mL. Place the sample in the Ultrospec 1000 and use it as a blank at 486 nm. Dilute your enzyme 10-fold and add 2 μL of this dilution to the cuvette. Quickly and carefully cover the cuvette with parafilm and mix gently. Place the sample into the spectrophotometer, and read the absorbance at 486 nm for 10 minutes, recording the absorbance every 30 seconds. After this reading, remove the cuvette and prepare for the next assay.

The following table indicates the dilution of substrate for each experiment. You should follow each experiment as explained above.

Trial	Substrate dilution	Enzyme Dilution
#1	5-fold	10,000-fold
#2	10-fold	10,000-fold
#3	20-fold	10,000-fold
#4	40-fold	10,000-fold
#5	80-fold	10,000-fold

Data analysis

1. From the stock concentration of Nitrocefin (500 μg/mL) and its molecular weight of 516.5 g/mol, you should be able to calculate the substrate concentration in each assay. Secondly, you earlier calculated the enzyme concentration, and should be able to calculate the enzyme concentration in each experiment.
2. Plot absorbance versus time for each trial. The slope of this plot is the enzymatic rate (**v**).
3. Next, plot the rate (**v**) versus the substrate concentration for the 5 experiments.
4. In order to calculate $\mathbf{K_m}$, $\mathbf{v_{max}}$, and $\mathbf{k_{cat}}$ prepare a Lineweaver-Burke plot of **1/v** versus **1/[S]**. From this plot you should be able to calculate $\mathbf{K_m}$ and $\mathbf{v_{max}}$ and from this $\mathbf{k_{cat}}$.

   You may want to consult your previous Biochemistry text or other references for help with enzyme kinetics.