EXPLORING THE ACTIVE SITE TOPOLOGY OF SWEET ALMOND

β -GLUCOSIDASE

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ABSTRACT

Enzyme β-glucosidase is a glycoside hydrolase that hydrolyzes β-glucosidase into water and alcohol. The research focuses on exploring the topology of the active site of sweet almond β-glucosidase (EC 3.2.1.21) by testing the kinetics of the enzyme with and without inhibitors using a spectrophotometer. By comparing the activity of the enzyme with the presence of various inhibitors, information about how structural differences of inhibitors interfere with the binding of the inhibitor to the active site of the enzyme can be obtained. To achieve better understanding of the active site topology, pH profile of the enzyme and pH profile of the binding will be constructed by monitoring reaction kinetics under different pH. Through the pH profile, the binding form of both the inhibitor and the enzyme at the active site can be inferred. The research provides inspiration of designing inhibitors of other related enzymes including some disease-relating glycoside hydrolases.

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INTRODUCTION

Catalysts are chemicals that speed up reactions. Enzymes, function as biochemical catalysts, accelerating the rate of a reaction. Unlike traditional inorganic catalysts, enzymes function much more efficient in organisms. Without the enzyme present, the half-life of some specific reactions may be over one million years. However, with enzymes present, reactions taking place in organisms can be 10^{17} times faster than without enzymes present, showing that enzymes are crucial for living organisms [1].

The enzyme active site is the place on the enzyme where substrates bind to and undergo a chemical reaction. The substrate interacts with groups at the active site of the enzyme and by this way, the enzyme lowers the activation energy of the reaction to make the reaction take place.

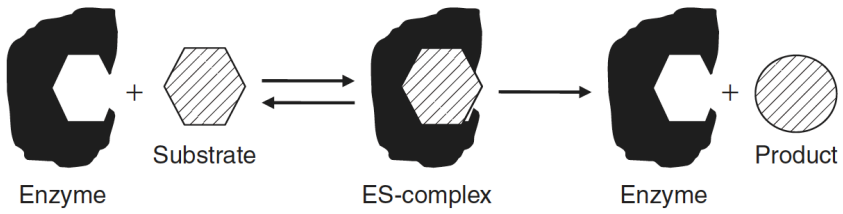


Figure 1: A simplified model of typical enzymatic reaction

Enzyme functions can be inhibited by various inhibitors. Inhibitors bind either to the active site to compete with substrate binding or to an allosteric site to change Inhibitors are widely used pharmaceutically, for example as anti-viral reagents. Inhibitors in these cases function by repressing virus from reproducing by targeting one of their enzymes that is necessary for their survival. For example, the iminosugars with glucose

stereochemistry target the glucosidases which are key for entry into the glycoprotein folding cycle, and thus prevent their proteins from folding into correct configuration. Not only can inhibitors be used pharmaceutically, it can also be used to explore the active site topology of the enzyme.

β-Glucosides are glycosides derived from glucose and are a group of substrates of our enzyme of interest, β-glucosidase. Sweet almond β-glucosidase (EC 3.2.1.21) is a glycoside hydrolase that acts on β-glycosidic bonds on different types of β-glucosides to terminal non-reducing residues in beta-D-glucosides and oligosaccharides, and release glucose.

Unlike many other enzymes, there is no crystal structure available for almond beta-glucosidase. To explore the topology of the active site of our enzyme, many inhibitors were used in experiments to see how those different functional groups contribute to their binding to the enzyme.

It is known from preliminary experiments that sweet almond β-glucosidase has a strong affinity for amine groups. Using this characteristic, good inhibitors can be selected by comparing inhibitors that contain an amine group.

Transition state analogs for the enzyme can also be used to inhibit enzyme activity. Many enzymes function by distorting substrates and bring reactive parts on substrates closer to boost reactions. In the transition state of β-glucosidase reaction, glucose assumes a half-chair conformation because the carbenium ion is stabilized by the adjacent hydrogen atom. As a result, if something is a stable analog of the transition state, then it will bind to

the enzyme much more tightly. By this way, the enzyme activity is inhibited by the transition state analogs.

There is pharmaceutical importance to unravel the kinetic characterizations of the enzyme and to explore the active site topology. Many diseases are caused by having higher activity of enzymes and a variety of drugs are good inhibitors of specific enzyme. The study of inhibitors of almond β-glucosidase leads to the design of drugs for other related glycohydrolases which are involved in a variety of diseases.

For the enzymatic reaction, in order to measure the kinetics of the reaction, para-nitrophenyl-glucoside (PNP-Glc) is used as a substrate. β-Glucosidase catalyzes the cleavage of PNP-glucoside to form the products glucose and para-nitrophenol. Para-nitrophenolate (the basic form of para-nitrophenol) is a yellow substance and has a maximum absorbance at around 400nm. The absorbance of the reaction mixture gives information of the amount of product formed.

Enzyme Kinetics

For general enzymatic reactions, there are several parameters used to measure the catalytic power of the enzyme. K_M is the Michaelis constant in the simple model below(Scheme1) is $K_M = \frac{(k_{-1}+k_2)}{k1}$, in which k_1 is the 2nd-order rate constant of the forward reaction of enzyme(E) and substrate(S) come together and produce the intermediate, the enzyme-substrate complex; k_{-1} is the 1st-order rate constant of its

reverse reaction; k_2 is the 1st-order rate constant for the ES intermediate to break down to yield product and regenerate the free enzyme. (Illustrated in the scheme below.)

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

Scheme 1: General Enzymatic Reaction

k_{cat} is the turnover number of an enzyme. It measures the maximal catalytic activity of an enzyme and is defined as the number of substrate molecules converted into product per enzyme molecule per unit time when the enzyme is saturated with substrate and can also represent the kinetic efficiency of the enzyme. In this simple model (Scheme 1), k_{cat} equals the k_2 in the reaction. In experimental conditions where the substrate used is far below saturating concentration, $\left(\frac{kcat}{K_M}\right)$ is used to show the catalytic efficiency of the enzyme.

$\frac{V_{max}}{K_M}$ is a pseudo-1st-order rate constant at fixed enzyme concentration. The reaction rate can be expressed by dP/dt which equals $\left(\frac{kcat}{K_M}\right) \times E \times S$ when $[S] \ll K_M$.

The enzymatic reaction kinetics is characterized by Michaelis-Menten equation:

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

Equation 1

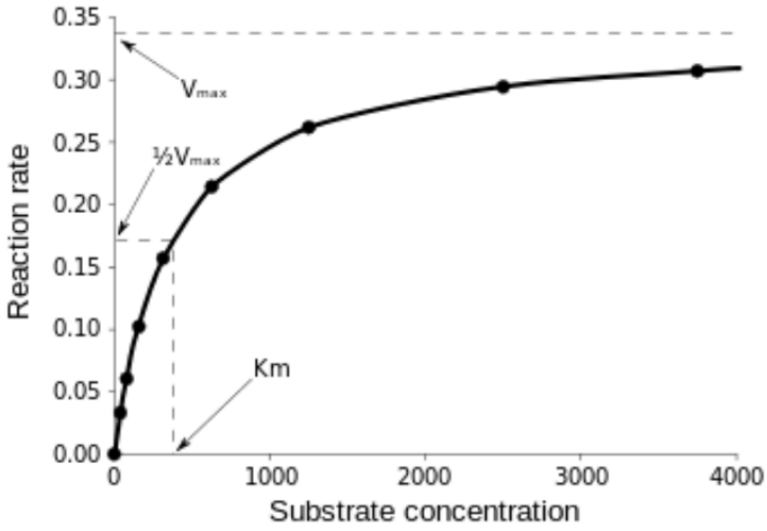


Figure 2: Reaction Kinetics

At a fixed enzyme concentration and a very low substrate concentration, v is linearly proportional to substrate concentration and show first order kinetics. With the increase of substrate concentration, v does not increase linearly any more. And at high substrate concentration $[S] \gg K_M$, the substrate concentration has no effect on v. By rearranging equation 1, the Michaelis constant can be expressed as

$$K_m = [S]\left(\frac{V_{max}}{v} - 1\right)$$

Equation 2

At $v = \frac{1}{2}V_{max}$, $K_M = [S]$. Thus, Km can be defined as the substrate concentration when velocity equals to a half of the maximal velocity. Also, when $k_{-1} \gg k_2$, the lower K_M indicates a tighter binding of the substrate to the enzyme active site.

Enzyme Inhibition

Inhibitors can interact with the enzyme either reversibly or irreversibly.

Reversible inhibitors interact with the enzyme in a non-covalent manner. They can associate and dissociate with the enzyme frequently and will not alter the structure of the enzyme or occupied active site permanently. There are 3 categories of reversible inhibitions: competitive, noncompetitive (mixed) and uncompetitive inhibition. Competitive inhibitors compete with the substrate for the enzyme active site, prevent substrate from binding, and thus inhibit the enzyme activity. Noncompetitive, or mixed inhibitors do not bind to the same site as substrate does but interact with both the enzyme and the enzyme-substrate complex. And the binding of mixed inhibitors to the enzyme affects the binding of substrate to the enzyme.

Irreversible inhibitors covalently modify the enzyme, rendering inactive enzyme whose activity cannot be recovered. Conduritol B epoxide is an irreversible inhibitor for the almond β-glucosidase. It covalently forms an ester structure with the carboxylic acid at the active site of the enzyme which is relatively stable. [16]

Several parameters are used to characterize the enzyme kinetics with competitive inhibitors present.

k_i is the pseudo-1st-order rate constant in the presence of the inhibitor and [I] is the concentration of the inhibitor. The ability of inhibitors to bind to the enzyme is measured by Ki, the inhibition constant. The lower the Ki, the more tightly the inhibitor binds to the enzyme. The Ki value of those inhibitors are calculated by the equation [3]:

$$\frac{k_0}{k_i} = 1 + \frac{[I]}{K_i}.$$

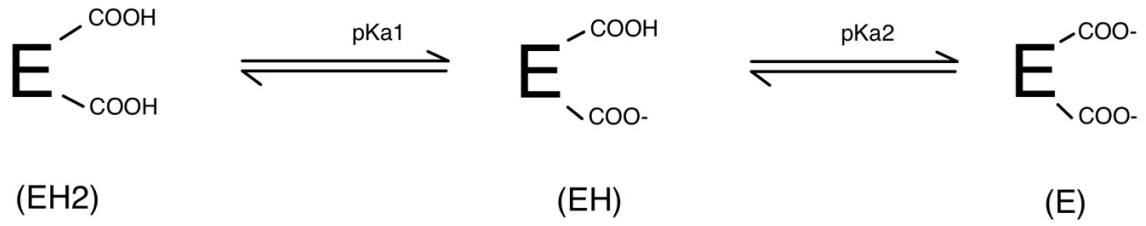
Equation 3

The equation is based on the competition between substrate and inhibitor for the enzyme. To compare the inhibitor structure and Ki, the contribution of different functional group to the binding can be understood. This provides much information for the enzyme active site topology.

pH Profile of Almond β-glucosidase

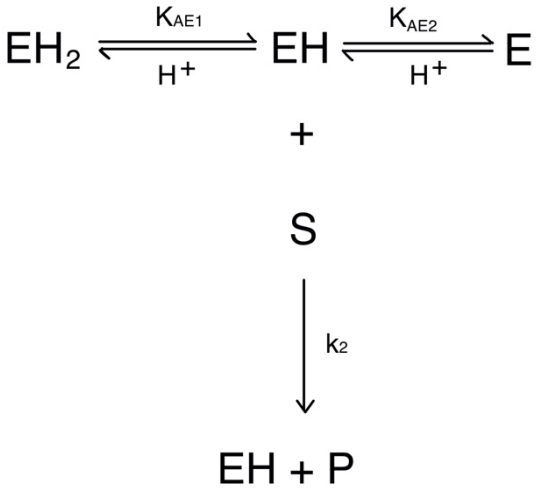
There are 2 different carboxylic acid catalytic groups on the enzyme which has different pKa values. When the pH is much lower than the pKa_1, both carboxyl groups are protonated (expressed by EH_2). When the pH is much higher than pKa_1 but lower than pKa_2, one of the carboxyl group will be deprotonated (expressed by EH). And when the pH is higher than pKa_2, both carboxyl groups will be predominantly deprotonated (expressed by E).

Between the two carboxylic acid groups, one of them functions as nucleophile and the other is involved in general acid-base reactions. When the nucleophilic carboxylic group is deprotonated and the general acid-base carboxylic acid group is protonated (in EH form), the enzyme will have catalytic activity.



Scheme 2: Protonated Form and Unprotonated Form of the Enzyme

The pH profile for the enzyme provides the 2 pKa value of the enzyme. The equation used to fit in the data is deduced by following steps.



Scheme 3: Enzyme kinetics

To conduct a pH profile for the enzyme, 2 parameters are important. k^{obs} is the data obtained from the experiment and the k^{lim} is the limit reaction rate constant in specific conditions. From the scheme above, and use k^{obs} obtained from the experiment, k^{lim} can be calculated using the equation [4]:

$$k^{obs} = \frac{k^{lim}}{1 + 10^{(pK_{AE1} - pH)} + 10^{(pH - pK_{AE2})}}$$

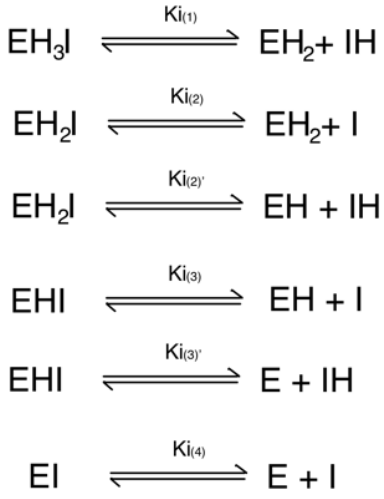
Equation 4

By plotting the data using equation 4, the maximum activity and the corresponding pH range can be inferred from the plot as well as the 2 pKa values. With the pH profile of the enzyme, pH profile for inhibitors binding to the enzyme can also be conducted.

pH Profile of the Binding

There are 6 possible binding forms of an ionizable inhibitors binding to the enzyme shown in the following scheme.

In order to figure out whether the protonated form or the unprotonated form binds to the enzyme, pH profile is needed for 1/Ki against pH. If the inhibitor binds to the enzyme at pH lower than the pKa of the inhibitor, the inhibitor binds in a protonated form to the enzyme. If the binding occurs at pH higher than inhibitor's pKa, the inhibitor binds to the enzyme at its unprotonated form. By studying the pH profile of inhibitors binding, the enzyme active site topology can be explored more.



Scheme 4: Enzyme-inhibitor binding forms

By monitoring the kinetics of β-glucosidase with inhibitor added under different pH, the pH profile of binding can be plotted and used to figure out the binding pattern of both inhibitors and the enzyme.

For protonated ionizable inhibitor in IH form to bind to the EH_2 form of the enzyme:

$$\frac{1}{K_i^{obs}} = \frac{1}{Ki_{(1)} \cdot (\frac{K_{AI}}{H} + 1 + \frac{K_{AE1}K_{AI}}{H^2} + \frac{K_{AE1}}{H} + \frac{K_{AE1} \cdot K_{AE2} \cdot K_{AI}}{H^3} + \frac{K_{AE2} \cdot K_{AE1}}{H^2})}$$

Equation 5

For protonated ionizable inhibitor in IH form to bind to the EH form of the enzyme:

$$\frac{1}{K_i^{obs}} = \frac{1}{Ki_{(2)} \cdot (\frac{K_{AI}}{K_{AE1}} + \frac{H}{K_{AE1}} + \frac{K_{AI}}{H} + 1 + \frac{K_{AE2} \cdot K_{AI}}{H^2} + \frac{K_{AE2}}{H})}$$

Equation 6

If the inhibitor is nonionizable, the equation will be transformed to

$$\frac{1}{K_i^{obs}} = \frac{1}{Ki_{(2)} \cdot (1 + \frac{K_{AE1}}{H} + \frac{K_{AE2} \cdot K_{AE1}}{H^2})}$$

Equation 7

For unprotonated ionizable inhibitor in I form to bind to the EH form of the enzyme:

$$\frac{1}{K_i^{obs}} = \frac{1}{Ki_{(3)} \cdot (\frac{K_{AE2}}{H} + \frac{K_{AE2}}{K_{AI}} + 1 + \frac{H}{K_{AI}} + \frac{H}{K_{AE1}} + \frac{H^2}{K_{AE1} \cdot K_{Ai}})}$$

Equation 8

If the inhibitor is nonionizable, the equation will be transformed to

$$\frac{1}{K_i^{obs}} = \frac{1}{Ki_{(3)} \cdot (\frac{H}{K_{AE1}} + \frac{K_{AE2}}{H} + 1)}$$

Equation 9

For unprotonated ionizable inhibitor in I form to bind to E form of the enzyme:

$$\frac{1}{K_i^{obs}} = \frac{1}{Ki_{(4)} \cdot (\frac{H^3}{K_{AE1} \cdot K_{AE2} \cdot K_{AI}} + \frac{H^2}{K_{AE1} \cdot K_{AE2}} + \frac{H^2}{K_{AI} \cdot K_{AE2}} + \frac{H}{K_{AE2}} + \frac{H}{K_{AI}} + 1)}$$

Equation 10

If the inhibitor is nonionizable, the equation will be transformed to

$$\frac{1}{K_i^{obs}} = \frac{1}{Ki_{(4)} \cdot (\frac{H^2}{K_{AE1} \cdot K_{AE2}} + \frac{H}{K_{AE2}} + 1)}$$

Equation 11

METHODS AND MATERIALS

The sweet almond β-glucosidase used in the experiment is from Calzyme Laboratories, Inc. The enzyme is found to be a dimer with each subunit weighing 65,000Da (Isozyme A)[8] after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The inhibitor 1-deoxynojirimycin was obtained from Carbosynth Ltd. Bisphenol A, Bisphenol S, Agmatine were obtained from Alfa Aesar. 3-Hydroxypiperidine, 2-piperidinemethanol, 4-hydroxypiperidine, 1-methyl-2-piperidinemethanol and delta-valerolactam were obtained from Acros Organics. The substrate para-nitrophenyl glucoside was obtained from Sigma-Aldrich, Inc..

Buffers were chosen according to their buffer range. MES buffer has a buffer range from 5.5 to 6.7. Sodium phosphate has a buffer range from 5.8 to 8.0. Sodium citrate has a buffer range from 3.0 to 6.2 [9].

The enzyme kinetics experiments were done using pH6.3 MES buffer. To conduct the pH profile for the enzyme and the pH profile for inhibitors binding, sodium citrate buffer was used at pH 3.2 to pH 6.2 and sodium phosphate buffer was used at pH higher than 6.2. All those buffers solutions were prepared with a 0.01M concentration and 0.01M NaCl were added to the solution. The product concentration and reaction kinetics were monitored using a HP 8452A Diode Array spectrophotometer.

Enzyme Kinetics

Absorbance measures the capacity of a substance to absorb light at certain wavelength. It can be measured by using a spectrophotometer. The absorbance of a substance is proportional to its concentration at a certain wavelength. It can be characterized using the formula:

$$A = \varepsilon \cdot c \cdot l$$

Equation 12

In the formula, ε is the extinction coefficient, c is the concentration of the substance and l is the path length which equals 1cm in all these experiment. Para-nitrophenolate has an extinction coefficient $\varepsilon = 18.3\ (mM^{-1} \cdot cm^{-1})$ at 400nm wavelength, pH10 [10].

Since the reaction releases para-nitrophenolate as a product, by measuring the absorbance change rate at pH6.3, the reaction rate can be monitored. Parameters used to characterize the enzyme catalytic ability are obtained by fitting the data with Michaelis-Menten equation.

Enzyme Inhibition

Different inhibitors were added to the enzyme buffer solution to test the effects of different functional groups on the inhibitors to its binding to the enzyme. Substrate para-nitrophenol-glucoside were used to initiate the reaction. Inhibitors 1-deoxynojirimycin, Bisphenol A, Bisphenol S, Agmatine, 3-hydroxypiperidine, 2-piperidinemethanol, 4-

hydroxypiperidine, 1-methyl-2-piperidinemethanol and delta-Valerolactam were used and first-order rates were measured under 400nm wavelength and pH6.3.

The active site of the enzyme is irreversibly inactivated by adding conduritol B epoxide to let the reaction progress for a day. Conduritol B epoxide was then separated from the modified enzyme by Sephadex G-25 gel filtration. The collected modified enzyme was assayed spectrophotometically to check the activity and to determine the rate of spontaneous regain of activity.

pH profile of the enzyme

The first order rates of the enzymatic reaction were measured at 348nm wavelength which is the isobestic point for para-nitrophenol and para-nitrophenolate. At the isobestic point, the absorbance of the solution will not change with pH change. The first order rates were measured between pH 3.2 and 7.6 with an interval of 0.2 to 0.5 pH change. The dependence of enzyme activity against pH was plotted and a curve is fitted to the data to give information about pK_{a1} and pK_{a2} of the enzyme.

pH profile of the binding

The first order rates of the enzymatic reaction were measured at 348nm wavelength. 1-Deoxynojirimycin, 3-hydroxypiperidine, 2-piperidinemethanol, 4-hydroxypiperidine, 1-methyl-2-piperidinemethanol and agmatine were used to study their pH profile of the

binding. Each inhibitors was added to achieve at least 50% inhibition of the enzyme activity. The first order rates were measured from pH 3.2 to 7.6 with an interval of 0.2 to 0.5 pH change. The equation containing all terms of equation 5, 6, 8, 10 was used to fit the raw data to figure out which binding form contributes to the enzyme-inhibitor binding at different pH. The equation used is the sum of equation 5, 6, 8, 10 and is shown below:

$$\frac{1}{K_i^{obs}} = \frac{1}{Ki_{(1)} \cdot (\frac{K_{AI}}{H} + 1 + \frac{K_{AE1}K_{AI}}{H^2} + \frac{K_{AE1}}{H} + \frac{K_{AE1} \cdot K_{AE2} \cdot K_{AI}}{H^3} + \frac{K_{AE2} \cdot K_{AE1}}{H^2})} +$$

$$\frac{1}{Ki_{(2)} \cdot (\frac{K_{AI}}{K_{AE1}} + \frac{H}{K_{AE1}} + \frac{K_{AI}}{H} + 1 + \frac{K_{AE2} \cdot K_{AI}}{H^2} + \frac{K_{AE2}}{H})}$$

$$+ \frac{1}{Ki_{(3)} \cdot (\frac{K_{AE2}}{H} + \frac{K_{AE2}}{K_{AI}} + 1 + \frac{H}{K_{AI}} + \frac{H}{K_{AE1}} + \frac{H^2}{K_{AE1} \cdot K_{Ai}})} +$$

$$\frac{1}{Ki_{(4)} \cdot (\frac{H^3}{K_{AE1} \cdot K_{AE2} \cdot K_{AI}} + \frac{H^2}{K_{AE1} \cdot K_{AE2}} + \frac{H^2}{K_{AI} \cdot K_{AE2}} + \frac{H}{K_{AE2}} + \frac{H}{K_{AI}} + 1)}$$

Equation 13

RESULTS

Enzyme Kinetics

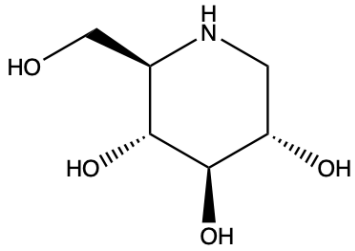
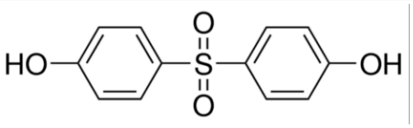
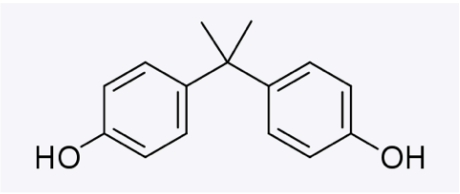
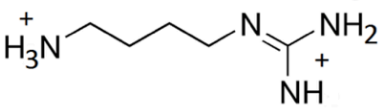
By measuring the pseudo-zero order kinetics (i.e. initial velocity) and fitting the data to the Michaelis-Menten equation, parameters were calculated.

V_{max} of the enzymatic reaction is $3.08 \times 10^{-5}(\pm 0.022)\ M/sec$ with an enzyme concentration of 1.03×10^{-3}mM. K_M is $3.00(\pm 0.08) \times 10^{-3}\ M$ and the k_{cat} value for the enzyme is $14.7(\pm 0.2)\ s^{-1}$, indicating 30 reactions in a second catalyzed by one molecule enzyme. The k_{cat} shows that the enzyme is relatively efficient in catalyzing the reaction. The catalytic efficiency $\frac{k_{cat}}{k_M} = 4907\ M^{-1}s^{-1}$ at pH 6.3 by calculation.

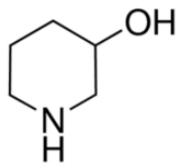
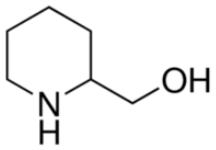
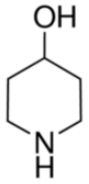
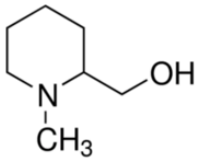
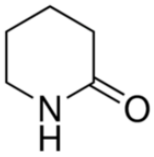
Enzyme Inhibition

The Ki of each inhibitor tested is listed in Table1 along with their structures and the enzyme used. Those inhibitors are ranked from low Ki value to high Ki value, corresponding to high affinity to low affinity for binding to the enzyme.

Table 1: Reversible Inhibition of beta-glucosidase

#	Name	Ki (mM)	Enzyme	Structure
1	1-deoxynojirimycin	0.0101(+-0.0007)	Calzyme	
2	Bisphenol S	0.31(+-0.08)	Calzyme	
3	Bisphenol A	0.62(+-0.11)	Calzyme	
4	Agmatine	1.45(+-0.26)	Calzyme	

(Continue Table 1)

5	3-hydroxypiperidine	1.49(+-0.18)	Calzyme	
6	2-piperidinemethanol	3.35(+-0.68)	Calzyme	
7	4-hydroxypiperidine	5.53(+-0.45)	Calzyme	
8	1-methyl-2-piperidinemethanol	16.65(+-1.72)	Calzyme	
9	delta-Valerolactam	67.12(+-6.86)	Calzyme	

In the case of irreversible inhibition of β-glucosidase, the retained enzyme activity was tested. The percentage activity was obtained by comparing the pseudo-zero order rate before and after the incubation of the enzyme with conduritol B epoxide. The corresponding result is shown in the following figure.

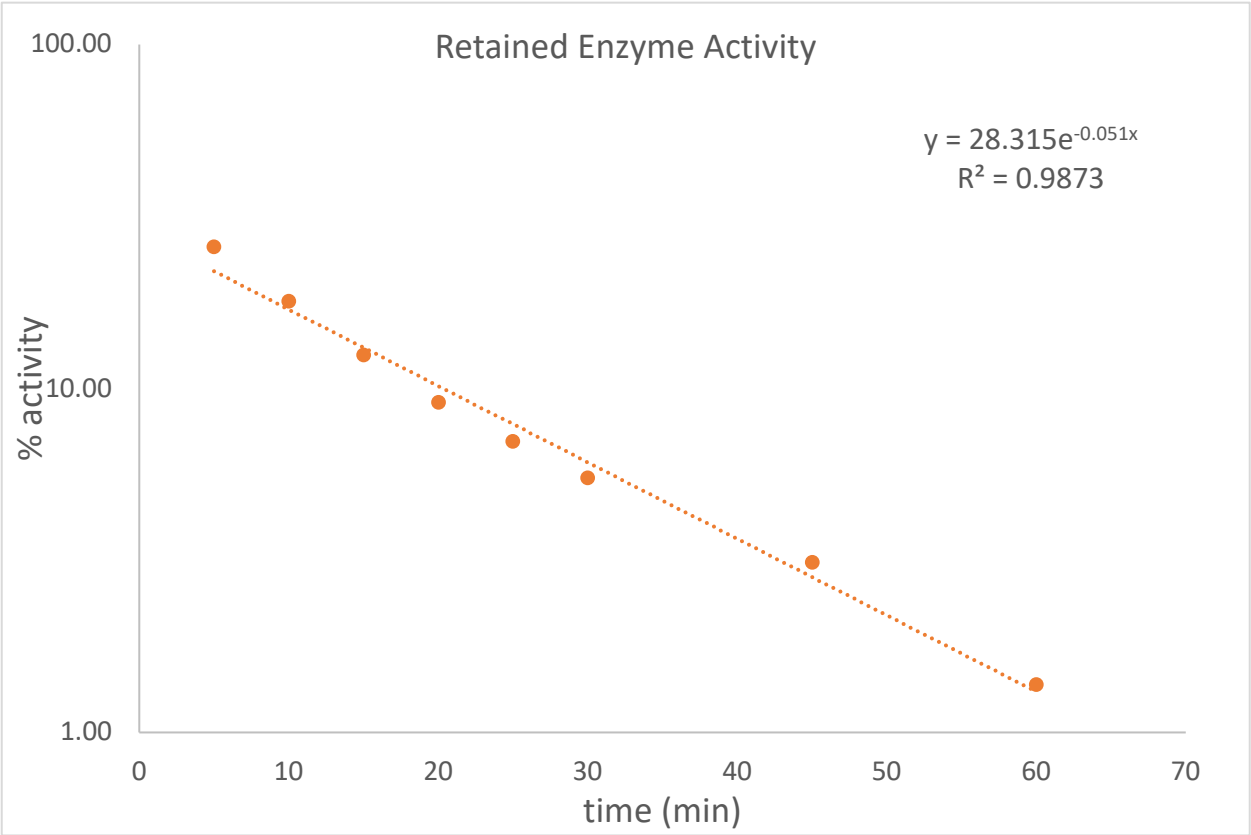


Figure 3: Retained enzyme activity after adding conduritol B epoxide

pH profile for the enzyme

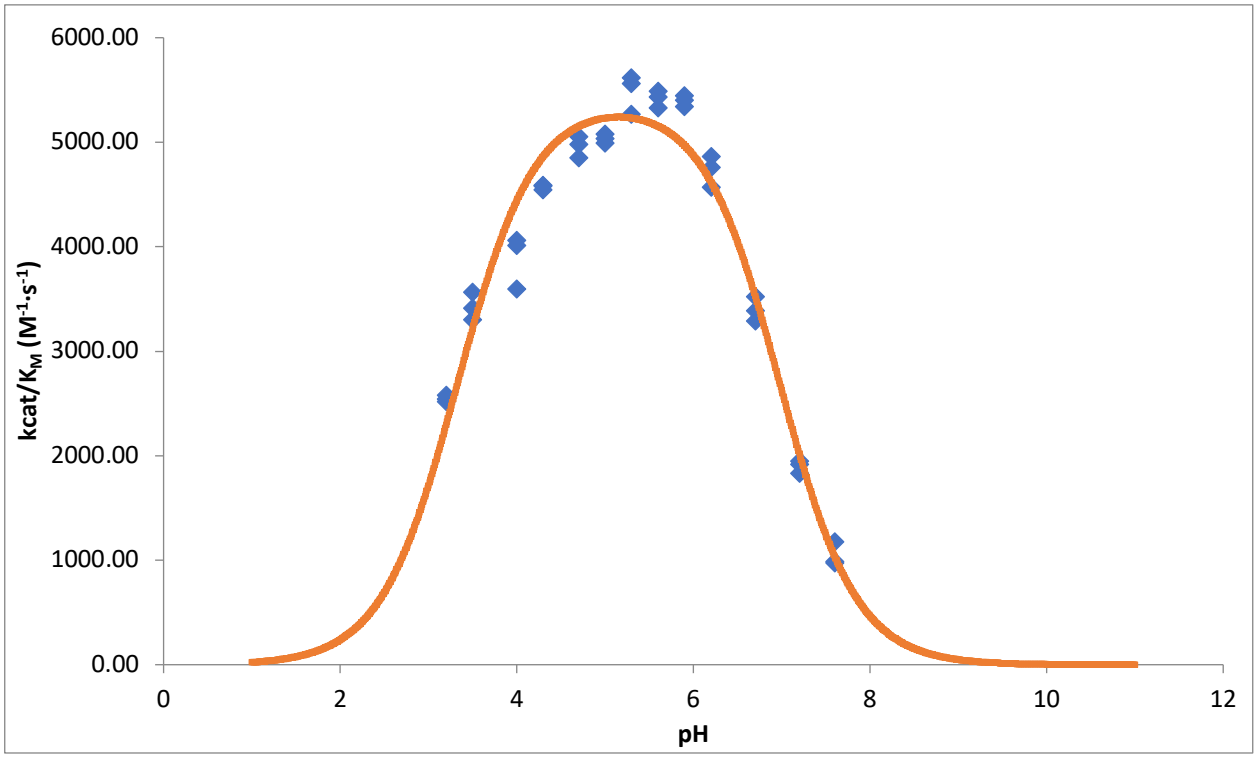


Figure 4: pH profile for beta-glucosidase

By fitting the equation

$$k^{obs} = \frac{k^{lim}}{1 + 10^{(pK_{AE1} - pH)} + 10^{(pH - pK_{AE2})}}$$

Equation 4

to the data using PSI-PLOT, a bell-shaped curve is the best-fit curve for the raw data.

For the β-glucosidase produced by Calzyme company, pK_{AE1}=3.68(±0.04), pK_{AE2}=6.98(±0.03) and k^{lim}=5.64(±0.02)×10^3 M^{-1}s^{-1}. The enzyme has maximum activity

in the range of pH5 to pH6 and the activity plummet at pH2~4 and pH6~8, indicating that the enzyme is in EH form when it shows maximum activity.

pH profile for the binding

The pH profile for inhibitor binding can be plotted as pH against 1/Ki, in which the larger the 1/Ki, the greater inhibition the inhibitor imposed on the enzyme.

Bisphenol A and bisphenol S are non-ionizable inhibitors. And 1-deoxynojirimycin, 1-methyl-2-piperidinemethanol, 2-piperidinemethanol, 3-hydroxypiperidine, 4-hydroxypiperidine and agmatine are ionizable and show similar pH profiles. All of them exert little inhibition on the enzyme at low pH and the inhibition upsurges at pH greater than 5 and reach maximum inhibition around pH 7.5. Inhibition at higher pH is hard to obtain because of the low stability of the enzyme at high pH.

In the following graphs, term 3 represents either the binding of the EH form of the enzyme to the I form of the inhibitor or the E form of the enzyme binds to the IH form of the inhibitor; term 4 represents the binding of the I form of the inhibitor to the E form of the enzyme.

The pH profile for inhibitor binding of all these inhibitors are shown below.

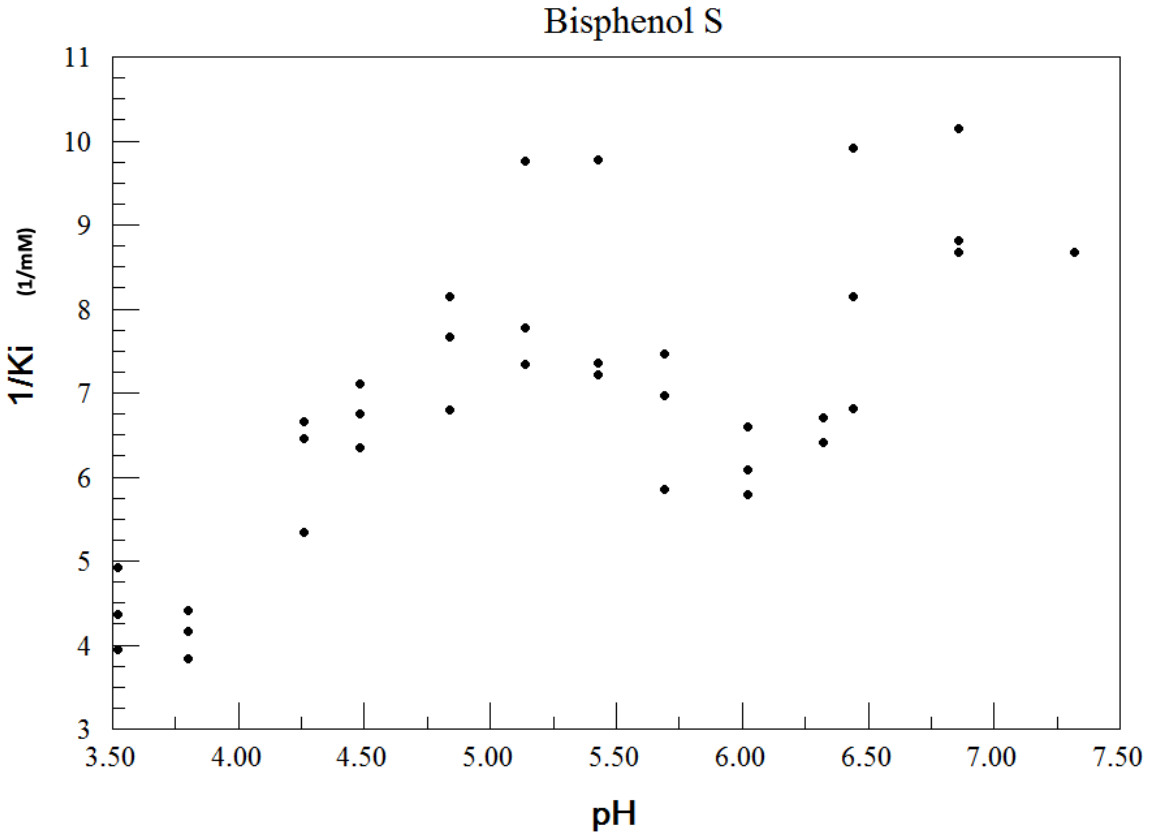


Figure 5: pH profile for Bisphenol S

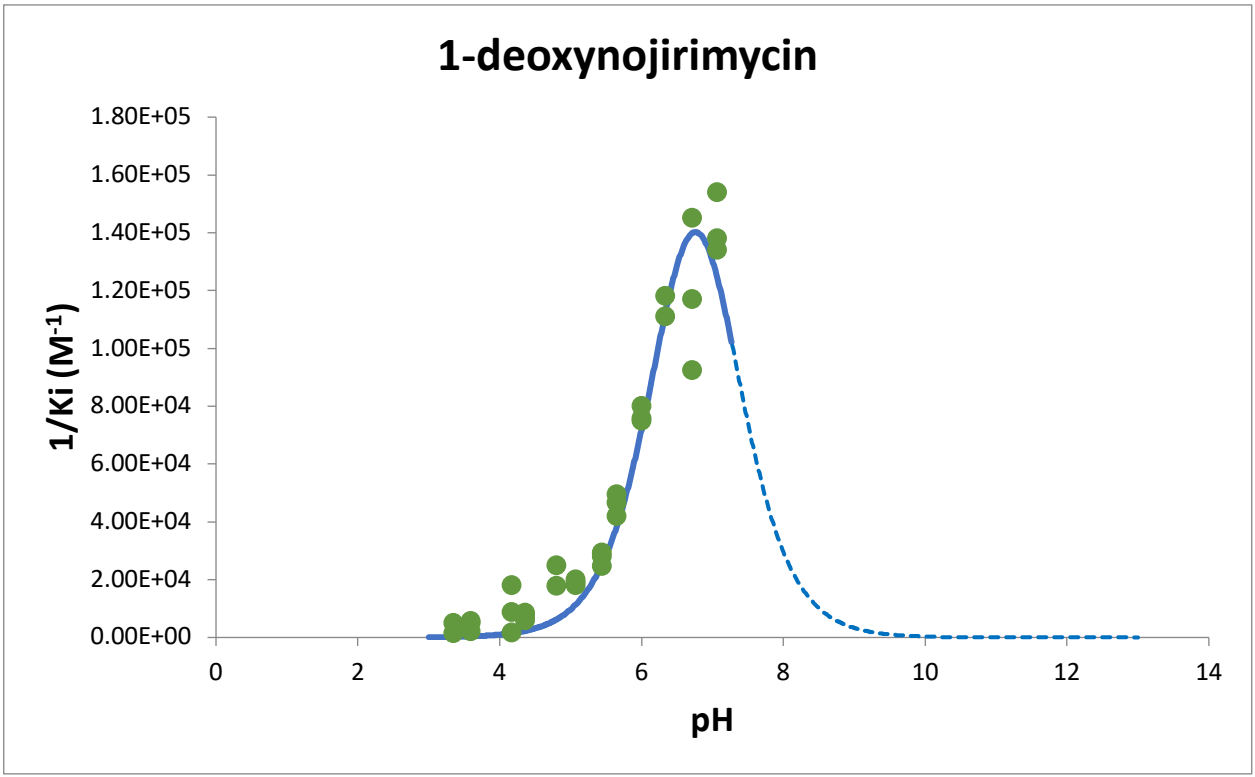


Figure 6: pH profile for 1-deoxynojirimycin binding (EH+I or E+IH)

For 1-deoxynojirimycin, it has a pKa=6.6, [11] $Ki_3 = 2.48(\pm 0.09)\times 10^{-6}$ M.

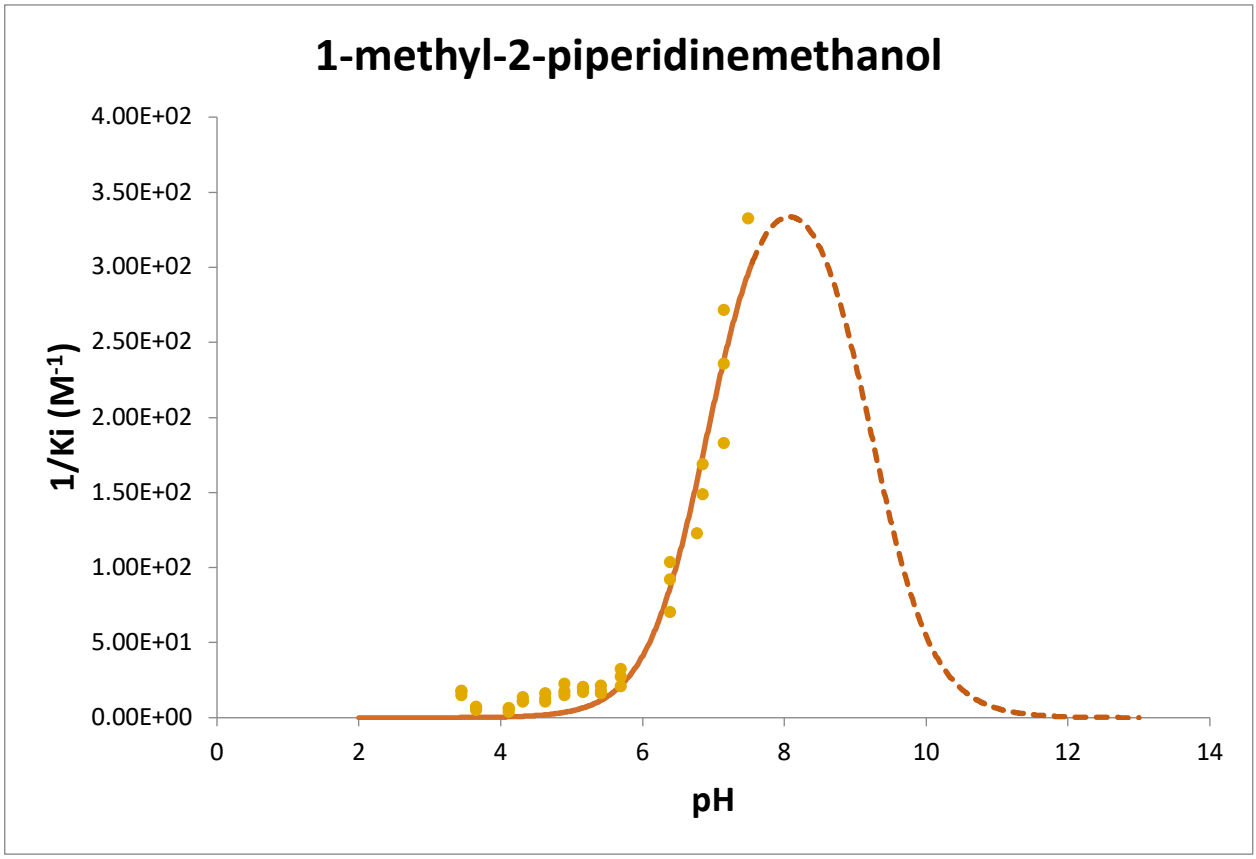


Figure 7: pH profile for 1-methyl-2-piperidinemethanol binding (EH+I or E+IH)

For 1-methyl-2-piperidinemethanol, the inhibition constants are $Ki_3 = 1.31(\pm 0.04)\times 10^{-5}$ M, and pKa value is 9.22 [12]. From the graph, it is obvious that term 3 contributes to the binding of the inhibitors to the enzyme.

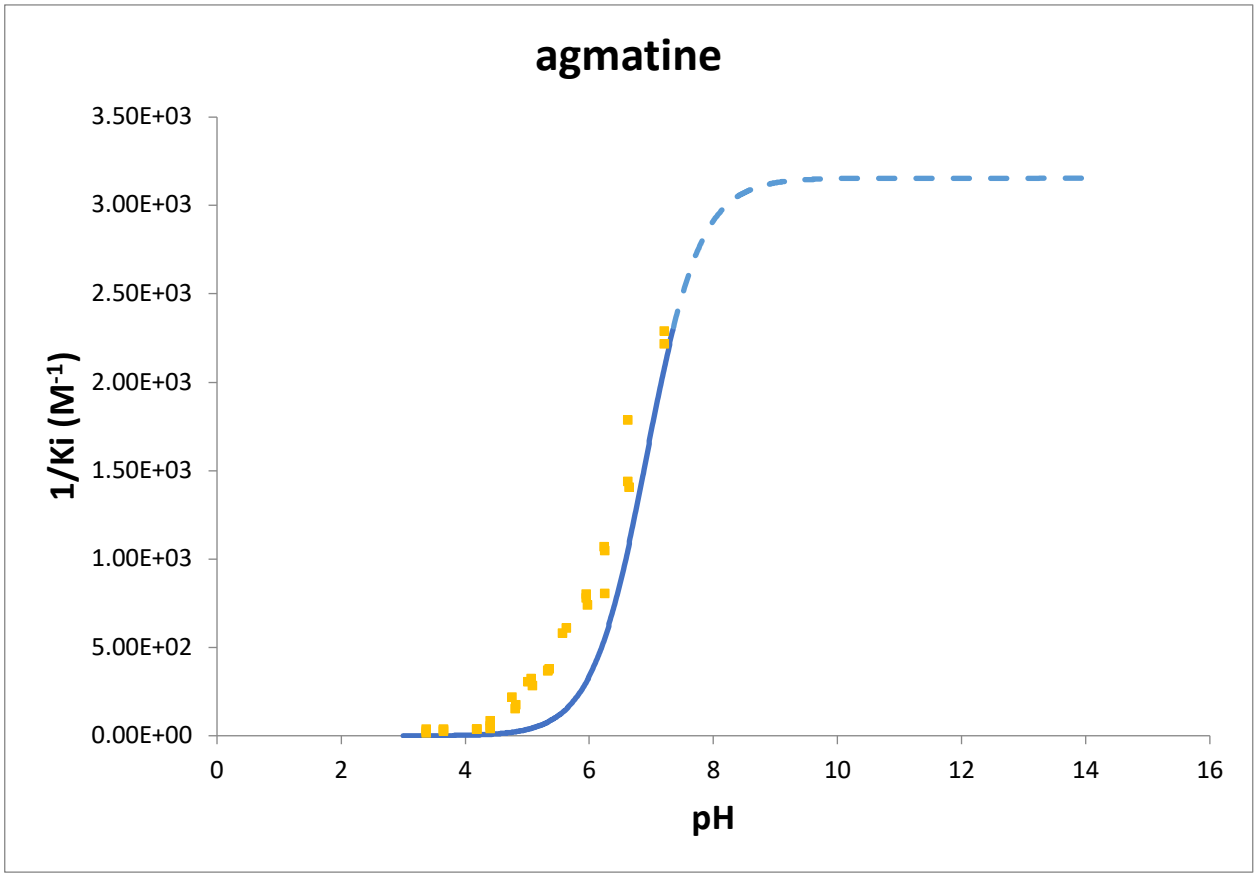


Figure 8: pH profile for agmatine binding ($E+IH_2^{2+}$)

The agmatine behaves as a non-ionizable inhibitor during the process of binding. The inhibition constants for agmatine is Ki = $3.17(\pm0.11)\times10^{-4}$ M. The two pK_as of agmatine are 8.9 (for the primary ammonium moiety) and 12.6 for the guanidinium moiety [13]. From the plot, it is the unprotonated enzyme (E form) that getting involved in the binding to the inhibitor (IH_2^{2+} form).

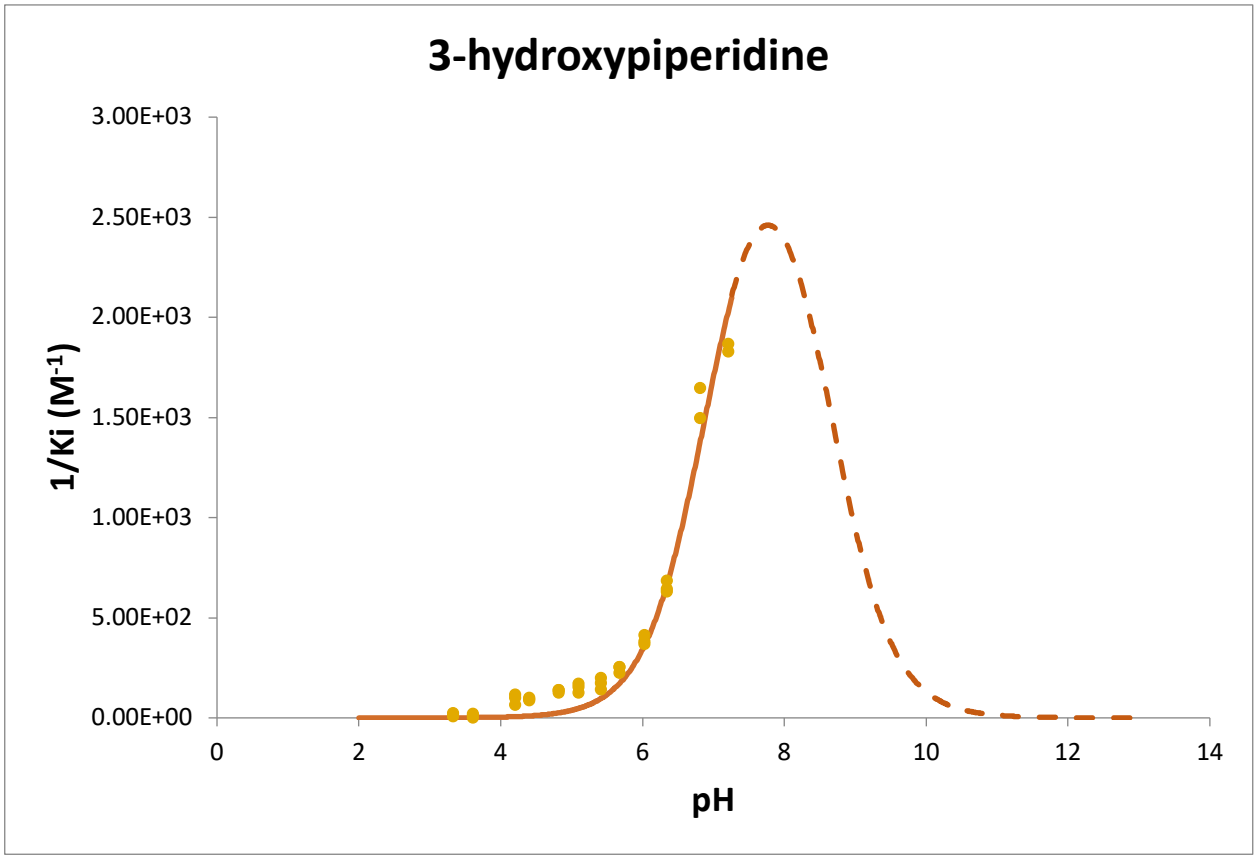


Figure 9: pH profile for 3-hydroxypiperidine (EH+I or E+IH)

For 3-hydroxypiperidine, it has an $Ki_3 = 5.98(\pm 0.17)\times 10^{-6}$ M. The pKa is predicted to be 8.64. The binding occurs mainly between the single protonated enzyme (EH form) and the unprotonated inhibitor (I form) or between the unprotonated enzyme (E form) with the protonated inhibitor (IH form).

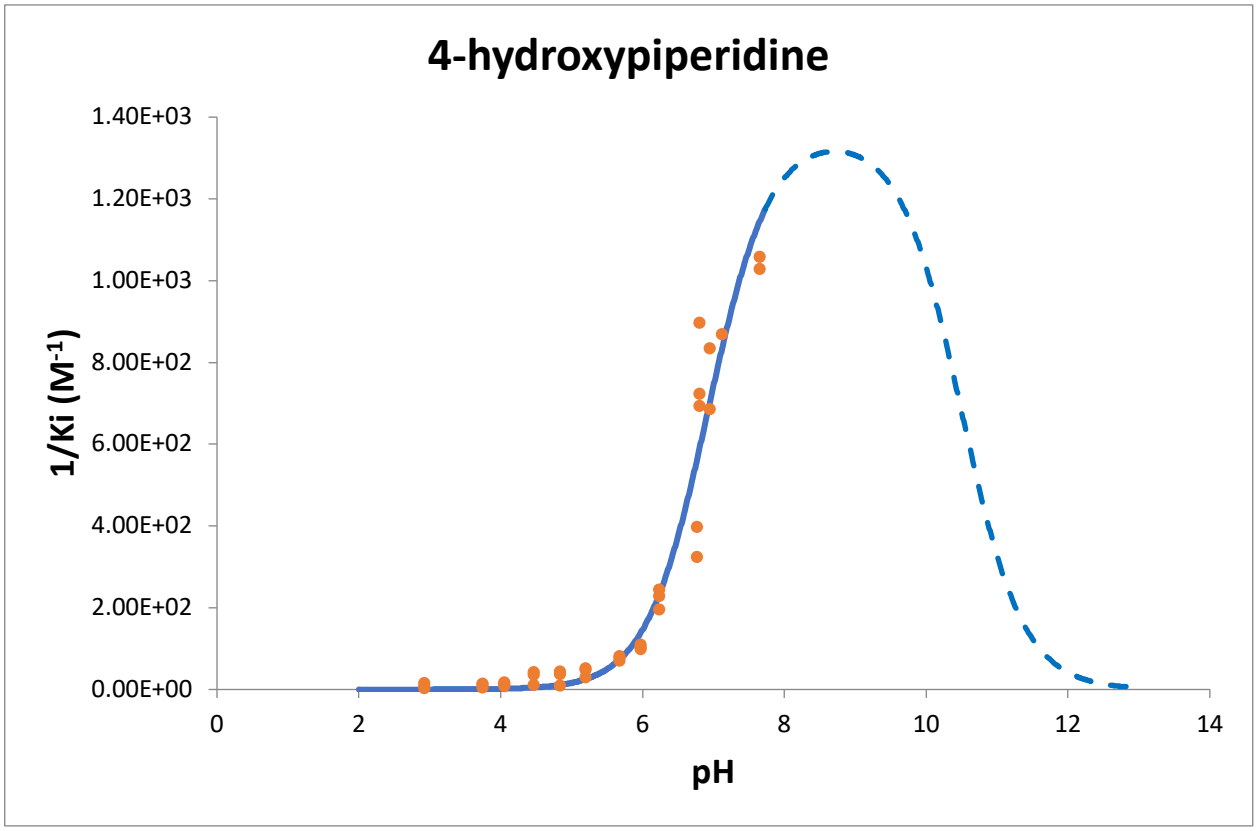


Figure 10: pH profile for 4-hydroxypiperidine (EH+I or E+IH)

The binding of 4-hydroxypiperidine is smooth and consistent. 4-hydroxypiperidine has a inhibition constant of $Ki_3 = 1.94(\pm 0.05)\times 10^{-7}$ M and has a pKa about 10.5 [14].

Discussion

Enzyme Inhibition

From table 1, when the hydroxyl group is on the 3rd position (#5) of the piperidine ring, it enhances the binding by the factor of 3 compared to when it is at the 4th position (#7). When the hydroxyl group is on the 3rd position, it may interact with the active site on enzyme more strongly by forming a hydrogen bond with groups on the active site. When the hydroxyl group is on the 4th position, the hydrogen bond may be weakened and the hydrogen bond may form between the 4' hydroxyl group on the inhibitor and the carbonyl oxygen on the group on the active site other than the one that form a hydrogen bond with the 3' hydroxyl group. In 1-deoxynojirimycin (#1), beside the 3'hydroxyl group, there are 3 more additional hydroxyl groups on the ring. The Ki for it is over a hundred fold lower than those compounds with only 1 hydroxyl group on the piperidines ring (#5, #6, #7), indicating that all of the hydroxyl groups on 1-deoxynojirimycin may involve in hydrogen bonds formation with different groups on the active sites, leading to very strong binding to the enzyme.

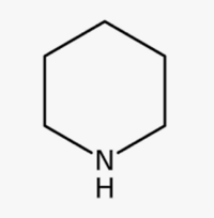


Figure 11: Structure of piperidine

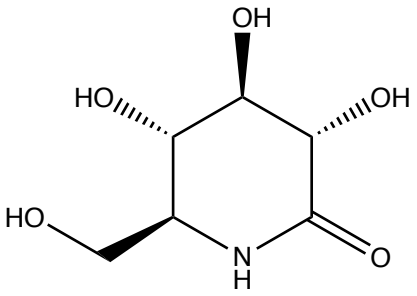


Figure 12: Structure of Gluconolactam

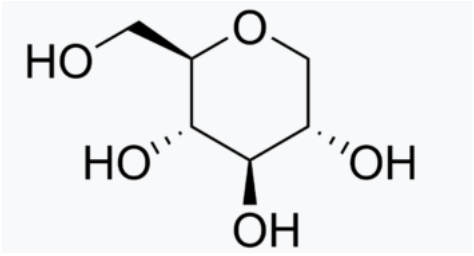


Figure 13: Structure of 1-deoxy-D-glucose

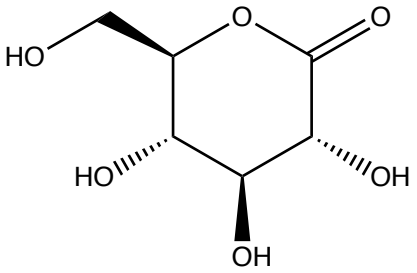


Figure 14: Structure of δ-gluconolactone

By comparing D-glucose (Ki=160mM) with 1-deoxy-D-glucose (Ki=60mM) and 1-deoxynojirimycin (Ki=0.01mM) with δ-gluconolactam (Ki=0.037mM) [14], it is clear that the extra functional groups on C-1 weaken the binding. With all other structures the same but no functional group on the C-1 carbon, 1-deoxy-D-glucose binds 2 to 3 times tighter than the D-glucose which has a hydroxyl group on the C-1 carbon. Similarly, 1-

deoxynojirimycin has no C-1 carbonyl oxygen compared with δ-gluconolactam which has a C-1 carbonyl oxygen presents. The difference in the C-1 carbonyl oxygen gives δ-gluconolactam a 3 to 4 times weaker binding than 1-deoxynojirimycin. These may serve as evidence that functional groups on the C-1 position hinder the binding sterically.

The greater binding promoting ability of the nitrogen in the ring than the oxygen in the ring can be illustrated by comparing 1-deoxynojirymycin (Ki=0.01mM) with 1-deoxy-D-glucose (Ki=60mM) and comparing δ-gluconolactam (Ki=0.037mM) with δ-gluconolactone (Ki=0.2mM). Both 1-deoxynojirymycin and δ-gluconolactam have nitrogen in the ring and both 1-deoxy-D-glucose and δ-gluconolactone have oxygen in the ring. The binding of the nitrogen containing compounds have 5 to 60 times greater binding affinity than the structure with oxygen containing ring. The difference may be caused by the interaction of the hydrogen on the nitrogen with hydrogen bond acceptors on the enzyme active site. The hydrogen on the nitrogen on 1-deoxynojirimycin may form a hydrogen bond with the hydrogen bond acceptor atom in the active site. However, the oxygen atom at the same position on glucose function also as a hydrogen bond acceptor which not only fails to form hydrogen bond with enzyme active site, but also may exert electron repelling effect with the active site.

The methyl group on the nitrogen also interferes with the binding. When there is a methyl group on the nitrogen (#8), the inhibitor binds to the enzyme 5 times less tightly than when there is a hydrogen connected to the nitrogen (#6), showing that the methyl group on the nitrogen may disturb the hydrogen bond forming between the hydrogen connected

on nitrogen and the hydrogen bond receptors on the active site of the enzyme. Alternatively, this may also be due to a steric effect.

In addition to the high affinity for the amine group, it is expected that beta-glucosidase interacts strongly with glucose in its half-chair conformation (Figure 15). Glucose naturally occurs in a chair conformation. During the reaction, the 1' alcohol or phenol group leaves the glucose and the empty p orbital of the oxocarbenium overlaps with the p orbital on oxygen to stabilize the structure. The 2' hydroxyl group will interact with the enzyme active site to adapt a structure that stabilizes the structure and push it into transition state. When the structure is destabilized, the 2' hydroxyl group is found in a position which distort the compound into a half-chair conformation. When the hydroxyl groups in glucose interact with the enzyme, those hydroxyl groups on glucose in a half-chair conformation bind more tightly than do the hydroxyl groups on glucose in the chair conformations. As a result, if a compound exists naturally in half-

chair conformation, the enzyme then does not need to use energy to distort it to induce tight binding, making it a strong inhibitor for the enzyme.

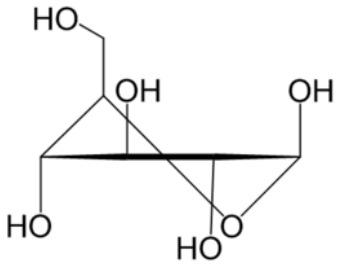


Figure 15: Structure of D-glucose in its half-chair conformation

δ-Gluconolactam (Ki=0.037mM) and δ-valerolactam (#9, Ki=67mM) both exist in half-chair conformation but differ in the presence of extra hydroxyl groups. δ-gluconolactam, with 3 extra hydroxyl groups and 1 extra hydroxymethyl group than the δ-valerolactam, has a Ki 2000 times smaller than the Ki of δ-valerolactam, indicating a 2000 times tighter binding than the δ-valerolactam. The result shows that additional hydroxyl groups in half-chair conformation also contribute to the binding.

pH profile for the binding

From figure 10 , the data of bisphenol S shows random distributions compared with those ionizable inhibitors. The overall binding increases as pH increases but the trend is does not have a uniform pattern. The non-pattern binding affinity change with pH change indicates the binding of bisphenol S may not be pH dependent.

For 1-deoxynojirimycin, with pH increase, the amount of unprotonated enzyme and unprotonated inhibitor exist increases. As a result, when the pH goes up, the contribution of single protonated enzyme with unprotonated inhibitor or unprotonated enzyme with the protonated inhibitor starts to predominate. At high pH, it is the binding of unprotonated enzyme to unprotonated inhibitor that predominates.

For 1-methyl-2-piperidinemethanol (figure 7), the binding occurs between the single protonated enzyme (EH form) and the unprotonated inhibitor (I form) or between the unprotonated enzyme (E form) with the protonated inhibitor (IH form).

For the agmatine (Figure 8), the binding affinity increases with higher pH and will presumably reaches a plateau at pH higher than 8, predicted by the best-fit curve derived from experimental data.

3-Hydroxypiperidine (Figure 9) has a binding pattern similar to that of 1-methyl-2-piperidinemethanol. The binding affinity starts to increase dramatically at around pH 6 when there are more unprotonated inhibitor and unprotonated enzyme exist; and will presumably start to decrease dramatically around pH 8 due to a decrease in single protonated enzyme and protonated inhibitor species in the reaction solution.

In the case of 4-hydroxypiperidine (Figure 10), the overall binding is contributed by the binding of single protonated enzyme to unprotonated inhibitor or unprotonated enzyme to protonated inhibitor.

For the agmatine, which acts as a non-ionizable inhibitor during the binding process, it is sure that the IH_2^{2+} binds to the unprotonated enzyme (E form).

For 1-methyl-2-piperidinemethanol, 3-hydroxypiperidine and 4-hydroxypiperidine whose pKa is much larger than the pK_{AE2} of the enzyme, zero order kinetics were measured for the purpose of narrowing down the possibilities of different binding forms. By comparing the diffusion limit with the calculated reaction rate of certain binding species, it may give suggestion to the real binding forms. If the calculated reaction rate of certain species, say, the double protonated enzyme with unprotonated inhibitor, is greater than the diffusion limit of reaction $10^9 M^{-1} s^{-1}$, then the binding species given by term 2 must be the single

protonated enzyme to the unprotonated inhibitor because the any reaction cannot be faster than the diffusion limit.

This method was used with inhibitors 1-methyl-2-piperidinemethanol, 3-hydroxypiperidine and 4-hydroxypiperidine. However, after comparing the reaction rate of certain species with diffusion limit rate, none of the possibilities can be excluded.

CONCLUSION

In this project, enzyme kinetics were tested and several important parameters were determined for sweet almond beta-glucosidase. The topology of the enzyme active site was further explored by comparing the binding affinity of different inhibitors, mainly nitrogen-containing cyclic compounds. The binding research is important in that inhibitors of enzyme give an insight into drug research as enzymes can be over-expressed in some diseases. With the understanding of the topology of the enzyme active site, drugs that target a certain enzyme can be devised to cure diseases.

By comparing the inhibitor constant (Ki) of these inhibitors and their structure difference, the contribution of additional hydroxyl groups and carbonyl groups on the ring structure to the binding to the enzyme active site was inferred. It is known by the comparing that the functional group on C-1 can hinder the binding while hydroxyl groups on other carbon promote the binding not only in compounds that exist in chair conformation but also in compound that exist in half-chair conformation. In addition, the nitrogen in the 6-member ring renders better binding than the oxygen in the ring. Also, methyl group on the nitrogen in the ring also hinders the binding to the enzyme active site.

The pH profiles of inhibitor binding were conducted to figure out the species of inhibitors and enzyme that are involved in the binding process. The binding pattern of 1-deoxynojirimycin, 1-methyl-2-piperidinemethanol, 3-hydroxypiperidine and 4-hydroxypiperidine are similar in that either the binding occurs between the single protonated enzyme (EH form) and the unprotonated inhibitor (I form) (or between the unprotonated enzyme (E form) with the protonated inhibitor (IH form)). In the case of

agmatine, the binding species is assured to be unprotonated enzyme (E form) and the inhibitor.

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APPENDIX

Derivation of Equation 1:

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

From the scheme, the reaction rate of the forward reaction and reverse reaction of ES formation should be the same, thus

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

At steady state, the rate of enzyme-substrate complex formation should be equal to the rate of complex dissociation. The rate of enzyme-substrate complex formation, v_f, can be expressed as

$$v_f = k_1[E][S]$$

The rate of complex dissociation,v_d, is measured by the rate of individual enzyme, substrate re-formation and the formation of product P.

$$v_d = k_{-1}[ES] + k_2[ES]$$

Since

$$[E_T] = [E] + [ES]$$

At steady state,

$$v_f = v_d$$

$$k_1[E][S] = k_{-1}[ES] + k_2[ES]$$

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

Rearrange the equation

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

$$[\mathrm{ES}] = \frac{[E_T] \cdot [S]}{K_M + [S]}$$

The reaction rate of an reaction is defined as the rate of product formation, thus

$$v = k_2[ES]$$

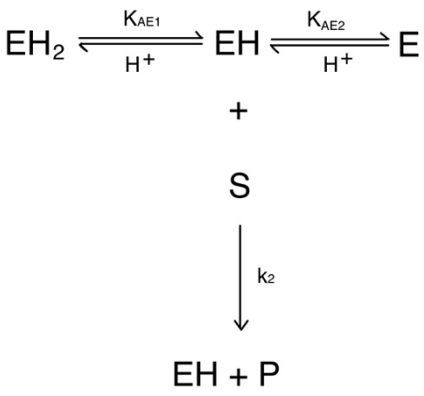
$$v = \frac{k_2 \cdot [E_T] \cdot [S]}{K_M + [S]}$$

Because $k_2 \cdot [E_T]$ is the maximum reaction rate,

$$v = \frac{V_{max} \cdot [S]}{K_M + [S]}$$

Derivation of Equation 4:

pH profile for the enzyme provides information for the 2 pKa value of the enzyme. The equation used to fit in the data is deduced by following steps.



From the scheme above:

$$K_{AE1} = \frac{EH \cdot H}{EH_2}$$

$$K_{AE2} = \frac{E \cdot H}{EH}$$

Since that the total enzyme E_T equals the sum of each form of the enzyme presents in the solution, $E_T = E + EH + EH_2$.

By substitution,

$$E_T = \frac{K_{AE2}}{H} \cdot EH + EH + \frac{H}{K_{AE1}} \cdot EH$$

$$E_T = (\frac{K_{AE2}}{H} + 1 + \frac{H}{K_{AE1}}) \cdot EH$$

The rate for product P to form is expressed as

$$\frac{dP}{dt} = k_2 \cdot EH \cdot S$$

By substitution,

$$\frac{dP}{dt} = \frac{k_2 \cdot E_T \cdot S}{1 + \frac{H}{K_{AE1}} + \frac{K_{AE2}}{H}} = \frac{V_{max}}{K_M} \cdot S$$

The Henderson-Hasselbalch Equation is used:

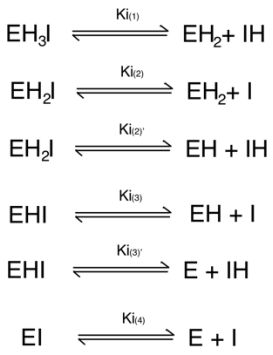
$$\mathrm{pH} = -\log_{10} H^+$$

$$\mathrm{pKa} = -\log_{10} Ka$$

Then, to substitute H with exponentials:

$$k^{obs} = \frac{k^{lim}}{1 + 10^{(pK_{AE1} - pH)} + 10^{(pH - pK_{AE2})}}$$

Derivation of Equation 5:



From the above scheme, Ki can be expressed by E, EH, EH_2, EH_3, I and IH.

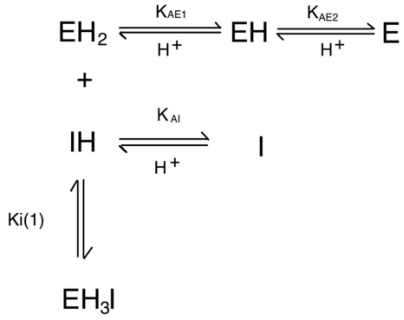
$$Ki_{(1)} = \frac{EH_2 \cdot IH}{EH_3 I}$$

$$Ki_{(2)} = \frac{EH_2 \cdot I}{EH_2 I}$$

$$Ki_{(3)} = \frac{EH \cdot I}{EHI}$$

$$Ki_{(4)} = \frac{E \cdot I}{EI}$$

For protonated ionizable inhibitor in IH form to bind to the EH_2 form of the enzyme:



By definition

$$K_i^{obs} = \frac{E_T \cdot I_T}{EI_T} = \frac{(EH_2 + EH + E) \cdot (I + IH)}{EH_3I}$$

Then take the reciprocal value of K_i^{obs}:

$$\frac{1}{K_i^{obs}} = \frac{EH_3I}{EH_2 \cdot I + EH_2 \cdot IH + EH \cdot I + EH \cdot IH + E \cdot I + E \cdot IH}$$

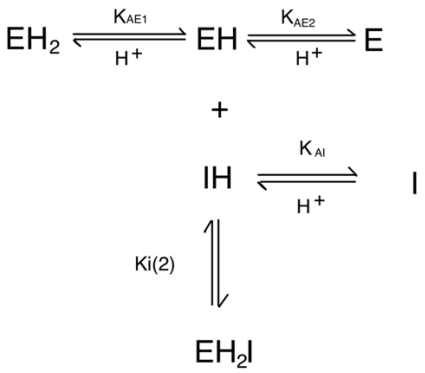
$$= \frac{EH_3I}{EH_2 \cdot \frac{K_{AI} \cdot IH}{H} + EH_2 \cdot IH + \frac{K_{AE1} \cdot EH_2}{H} \cdot \frac{K_{AI} \cdot IH}{H} + \frac{K_{AE1} \cdot EH_2}{H} \cdot IH + \frac{K_{AE2} \cdot K_{AE1} \cdot EH_2}{H^2} \cdot \frac{K_{AI} \cdot IH}{H} + \frac{K_{AE2} \cdot K_{AE1} \cdot EH_2}{H^2} \cdot IH}$$

$$= \frac{EH_3I}{(EH_2 \cdot IH) \cdot (\frac{K_{AI}}{H} + 1 + \frac{K_{AE1}K_{AI}}{H^2} + \frac{K_{AE1}}{H} + \frac{K_{AE1} \cdot K_{AE2} \cdot K_{AI}}{H^3} + \frac{K_{AE2} \cdot K_{AE1}}{H^2})}$$

$$= \frac{1}{Ki_{(1)} \cdot (\frac{K_{AI}}{H} + 1 + \frac{K_{AE1}K_{AI}}{H^2} + \frac{K_{AE1}}{H} + \frac{K_{AE1} \cdot K_{AE2} \cdot K_{AI}}{H^3} + \frac{K_{AE2} \cdot K_{AE1}}{H^2})}$$

Derivation of Equation 6 and Equation 7

For protonated ionizable inhibitor in IH form to bind to the EH form of the enzyme:



By definition,

$$K_i^{obs} = \frac{E_T \cdot I_T}{EI_T} = \frac{(EH_2 + EH + E) \cdot (I + IH)}{EH_2I}$$

Then take the reciprocal value of K_i^{obs}

$$\frac{1}{K_i^{obs}} = \frac{EH_2I}{EH_2 \cdot I + EH_2 \cdot IH + EH \cdot I + EH \cdot IH + E \cdot I + E \cdot IH}$$

$$= \frac{EH_2I}{\left(\frac{EH \cdot H}{K_{AE1}} \cdot \frac{K_{AI} \cdot IH}{H}\right) + \left(\frac{EH \cdot H}{K_{AE1}} \cdot IH\right) + \left(EH \cdot \frac{K_{AI} \cdot IH}{H}\right) + (EH \cdot IH) + \left(\frac{K_{AE2} \cdot EH}{H} \cdot \frac{K_{AI} \cdot IH}{H}\right) + (\frac{K_{AE2} \cdot EH}{H} \cdot IH)}$$

$$= \frac{EH_2I}{(EH \cdot IH) \cdot (\frac{K_{AI}}{K_{AE1}} + \frac{H}{K_{AE1}} + \frac{K_{AI}}{H} + 1 + \frac{K_{AE2} \cdot K_{AI}}{H^2} + \frac{K_{AE2}}{H})}$$

$$= \frac{1}{Ki_{(2)} \cdot (\frac{K_{AI}}{K_{AE1}} + \frac{H}{K_{AE1}} + \frac{K_{AI}}{H} + 1 + \frac{K_{AE2} \cdot K_{AI}}{H^2} + \frac{K_{AE2}}{H})}$$

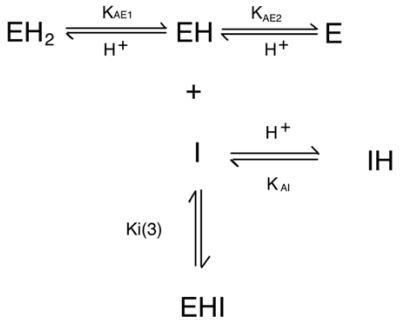
If the inhibitor is nonionizable, the equation will be transformed to

$$K_i^{obs} = \frac{E_T \cdot I_T}{EI_T} = \frac{(EH_2 + EH + E) \cdot I}{EH_2 I}$$

$$\frac{1}{K_i^{obs}} = \frac{1}{Ki_{(2)} \cdot (1 + \frac{K_{AE1}}{H} + \frac{K_{AE2} \cdot K_{AE1}}{H^2})}$$

Derivation of Equation 8 and Equation 9

For unprotonated ionizable inhibitor in I form to bind to the EH form of the enzyme:



By definition,

$$K_i^{obs} = \frac{E_T \cdot I_T}{EI_T} = \frac{(EH_2 + EH + E) \cdot (I + IH)}{EHI}$$

Then take the reciprocal value of Ki^{obs}

$$\frac{1}{K_i^{obs}} = \frac{EHI}{E \cdot I + E \cdot IH + EH \cdot I + EH \cdot IH + EH_2 \cdot I + EH_2 \cdot IH}$$

$$= \frac{EHI}{\left(\frac{K_{AE2} \cdot EH}{H} \cdot I\right) + \left(\frac{K_{AE2} \cdot EH}{H} \cdot \frac{I \cdot H}{K_{AI}}\right) + (EH \cdot I) + \left(EH \cdot \frac{I \cdot H}{K_{AI}}\right) + \left(\frac{EH \cdot H}{K_{AE1}} \cdot I\right) + (\frac{EH \cdot H}{K_{AE1}} \cdot \frac{I \cdot H}{K_{AI}})}$$

$$= \frac{EHI}{(EH \cdot I) \cdot (\frac{K_{AE2}}{H} + \frac{K_{AE2}}{K_{AI}} + 1 + \frac{H}{K_{AI}} + \frac{H}{K_{AE1}} + \frac{H^2}{K_{AE1} \cdot K_{Ai}})}$$

$$= \frac{1}{Ki_{(3)} \cdot (\frac{K_{AE2}}{H} + \frac{K_{AE2}}{K_{AI}} + 1 + \frac{H}{K_{AI}} + \frac{H}{K_{AE1}} + \frac{H^2}{K_{AE1} \cdot K_{Ai}})}$$

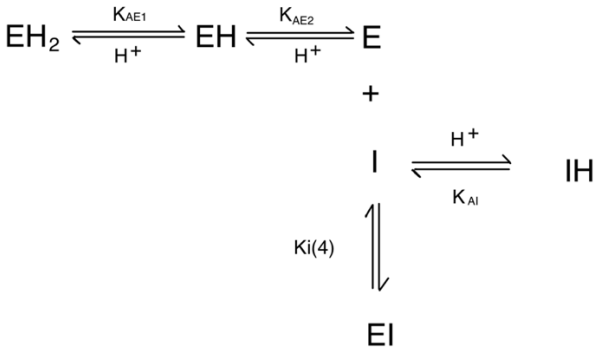
If the inhibitor is nonionizable, the equation will be transformed to

$$K_i^{obs} = \frac{E_T \cdot I_T}{EI_T} = \frac{(EH_2 + EH + E) \cdot I}{EHI}$$

$$\frac{1}{K_i^{obs}} = \frac{1}{Ki_{(3)} \cdot (\frac{H}{K_{AE1}} + \frac{K_{AE2}}{H} + 1)}$$

Derivation of Equation 10 and Equation 11

For unprotonated ionizable inhibitor in I form to bind to E form of the enzyme:



From the definition of K^{obs}, we get

$$K_i{}^{obs} = \frac{E_T \cdot I_T}{EI_T} = \frac{(EH_2 + EH + E) \cdot (I + IH)}{EI}$$

Then take the reciprocal value of K_i^{obs}

$$\frac{1}{K_i^{obs}} = \frac{EI}{EH_2 \cdot IH + EH_2 \cdot I + EH \cdot IH + EH \cdot I + E \cdot IH + E \cdot I}$$

$$= \frac{EI}{\left(\frac{EH \cdot H}{K_{AE1}} \cdot \frac{I \cdot H}{K_{AI}}\right) + \left(\frac{EH \cdot H}{K_{AE1}} \cdot I\right) + \left(\frac{E \cdot H}{K_{AE2}} \cdot \frac{I \cdot H}{K_{AI}}\right) + \left(\frac{E \cdot H}{K_{AE2}} \cdot I\right) + \left(E \cdot \frac{I \cdot H}{K_{AI}}\right) + (E \cdot I)}$$

$$= \frac{EI}{\left(\frac{E \cdot H^2}{K_{AE1} \cdot K_{AE2}} \cdot \frac{I \cdot H}{K_{AI}}\right) + \left(\frac{E \cdot H^2}{K_{AE1} \cdot K_{AE2}} \cdot I\right) + \left(\frac{E \cdot H}{K_{AE2}} \cdot \frac{I \cdot H}{K_{AI}}\right) + \left(\frac{E \cdot H}{K_{AE2}} \cdot I\right) + \left(E \cdot \frac{I \cdot H}{K_{AI}}\right) + (E \cdot I)}$$

$$= \frac{EI}{(E \cdot I) \cdot (\frac{H^3}{K_{AE1} \cdot K_{AE2} \cdot K_{AI}} + \frac{H^2}{K_{AE1} \cdot K_{AE2}} + \frac{H^2}{K_{AI} \cdot K_{AE2}} + \frac{H}{K_{AE2}} + \frac{H}{K_{AI}} + 1)}$$

$$= \frac{1}{Ki_{(4)} \cdot (\frac{H^3}{K_{AE1} \cdot K_{AE2} \cdot K_{AI}} + \frac{H^2}{K_{AE1} \cdot K_{AE2}} + \frac{H^2}{K_{AI} \cdot K_{AE2}} + \frac{H}{K_{AE2}} + \frac{H}{K_{AI}} + 1)}$$

If the inhibitor is nonionizable, the equation will be transformed to

$$K_i^{obs} = \frac{E_T \cdot I_T}{EI_T} = \frac{(EH_2 + EH + E) \cdot I}{EI}$$

$$\frac{1}{K_i^{obs}} = \frac{1}{Ki_{(4)} \cdot (\frac{H^2}{K_{AE1} \cdot K_{AE2}} + \frac{H}{K_{AE2}} + 1)}$$