Experiment 6 & 7
The Enzyme Kinetic Activity of Chymotrypsin: Temperature and pH Effects, Determination of $K_m$, $V_{max}$, and Inhibitor Effects

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Introduction

Enzymes are biological catalysts, which means that they change the rate of chemical reactions without being used up in the reaction. Enzymes are a special class of proteins that have active binding sites for specific substrates. Enzymes increase reaction rates by creating weak forces or binding interactions and positive interactions between the enzyme and the transition state of the substrate (Cok, 2011). The reaction state is the reaction intermediate, which equals the activation barrier. Biological enzymes are usually most active at mild temperatures and physiological pH (Holmes, 2004). Enzyme kinetics is the study of enzyme reactions and mechanisms (Holmes, 2004). Advances in enzyme kinetics have allowed researchers to develop treatments for diseases (Holmes, 2004). The activity of an enzyme is controlled by many factors such as pH and temperature of the environment as well as enzyme inhibitors (Cok, 2011).

Enzymes have an optimum pH and temperature in which they are most active. The pH of the environment affects the ionization state of catalytic groups in the active site as well as the ionization of the substrate and because of this electrostatic interactions are thus controlled by pH (Holmes, 2004). Control by pH of the conformation of an enzyme through ionizable amino acids is critical for the three-dimensional shape of the molecule (Holmes, 2004). Temperature is associated with entropy the amount of disorder. As temperature increases disorder increases resulting in increases molecular collisions and interactions. These molecular forces are associated with increased enzymatic activity. Enzyme activity increases with temperature until it reaches an optimal temperature. At high temperatures proteins become denatured, this holds true for enzymes being that they
are made of protein. Thus, temperature increases enzymatic activity until it affects the functional structure of the protein at which time activity ceases.

An Inhibitor can be thought of as a negative catalyst. Inhibitors decrease the activity of an enzyme by changing the enzyme substrate relationship at the binding site. They may be large or small organic molecules or an ion. Inhibition may be reversible or irreversible. In irreversible inhibition the inhibitor bonds very tightly or covalently to the enzyme causing it to dissociate and become inactive (Lehninger, 2008). Reversible inhibition causes rapid dissociation from the enzyme, hindering enzymatic activity but not becoming part of the enzyme (Lehninger, 2008). Competitive, noncompetitive and mixed-inhibition are the three main classes of reversible enzyme inhibitions. They differ by how they bind to enzymes as well as how they respond to substrate concentration (Holmes, 2004). Competitive inhibition the substrate and inhibitor compete for the same binding site, uncompetitive inhibition the inhibitor binds to the enzyme substrate complex preventing formation of product, and finally with mixed inhibition the inhibitor can bind to the substrate or the enzyme substrate complex (Lehninger, 2008). Enzyme inhibition is studied by close examination of reaction rates and how they change in varying experimental conditions (Holmes, 2004).

The relationship between substrate concentration and reaction rate can be displayed graphically by the curve expressing the relationship between the two (Lehninger, 2008). For most enzymes, this curve is a hyperbola ( \( y = \frac{ax}{b + x} \) ) and can be expressed by the Michaelis Menten equation. The Michaelis Menten equation is based on the idea that the rate-limiting step in an enzymatic reaction is the breakdown of the enzyme substrate (ES) complex (Lehninger, 2009). The Michaelis Menten equation is
known as the rate equation: \( V_0 = V_{\text{max}} [S] / K_m + [S] \), where \( V_0 \) = initial velocity, \( V_{\text{max}} \) = maximum velocity, \( [S] \) = substrate concentration, and \( K_m \) = the Michaelis Menten constant, which is based on the rate of ES breakdown over ES formation (Lehninger, 2009). The place at which the line on the double reciprocal plot crosses the y axis is related to \( V_{\text{max}} \) (y-intercept = \( 1/V_{\text{max}} \)). \( K_m \) has a special relationship to \( V_{\text{max}} \); \( K_m \) is the substrate concentration at which \( V_0 = \frac{1}{2} V_{\text{max}} \). \( K_m \) can be understood by the [S] required for the reaction to occur. So, an enzyme with a high \( K_m \) requires a greater [S] to achieve a given reaction velocity than an enzyme with a low \( K_m \). Also, the x-intercept = -1/Km.

The Lineweaver-Burk plot, a double reciprocal plot of the Michaelis Menten equation, provides a more accurate determination of \( V_{\text{max}} \) and has been very useful in determining different types of reaction mechanisms (Lehninger, 2009). In the case of inhibition study of the graphical lines for a series of double reciprocal plots shows inhibition mechanisms. Parallel lines indicate a displacement mechanism or non-competitive mechanism where the enzyme and substrate do not compete for the same binding sites, thus the enzyme binds and prevents formation of a product (Lehninger, 2008). Lines that cross or intersect is indicative of the formation of a tertiary complex either from competitive inhibition or mixed inhibition. If the lines cross on the y-axis it is an indicator of competitive inhibition. \( K_m \) value changes while \( V_{\text{max}} \) is constant. Lines that cross off the y-axis represent mixed inhibition. Double reciprocal plots with mixed inhibition have increasing \( V_{\text{max}} \) values as inhibition increase and decreasing \( K_m \) values as inhibition increases (Lehninger, 2008).

From determination of \( V_{\text{max}} \) and knowledge of the total enzyme concentration \( K_{\text{cat}} \) can be determined. \( K_{\text{cat}} \) represents the efficiency or “turn-over” rate of catalysis for a
given enzyme. $K_{\text{cat}}$ is useful in comparison among enzymes. A high $k_{\text{cat}}$ would indicate a rapid turn over of one substrate. The $k_{\text{cat}}$ is a measurement of time measuring the number of substrate molecules turned over per enzyme mole of molecule per second (Holmes, 2004). This statement assumes that the enzyme is fully saturated with substrate so that the reaction is proceeding at a maximum rate (Campbell, 1991).

A spectrophotometer is used to gather absorbance values of enzymatic reactions over time. By the use of Beer’s Law ($A = \epsilon bc$) change in absorbance over time can be converted to change in concentration over time (Cok, 2011). When using a spectrophotometer, the absorbance of a sample depends on the concentration in moles per liter ($c$), light path length in centimeters ($b$), and the extinction coefficient ($\epsilon$) (Dean, J.A., Ed, 1975). The Beer-Lambert Law shows the correlation between the concentration ($c$) of an analyte and the length of the light path ($b$). At a given wavelength, light absorption is dependant on both concentration and path length of light (Cok, 2011). Absorption and concentration have a direct relationship. That is as the concentration of an analyte increases the amount of light absorption also increases. The slope of the absorbance measurements (change in absorbance over time) can be divided by the extinction coefficient ($\epsilon$) of the substrate multiplied by the pathlength ($b$) equal change in substrate concentration over time (velocity) (Cok, 2011).

Chymotrypsin is a proteinase found in pancreatic juice. It is a protyolitic enzyme that breaks down protein; it clots milk and hydrolyzes casein and gelatin (McGraw-Hill, 2011). Chymotrypsin is synthesized in the pancreas and released in an inactive form into the small intestine through the bile duct (Cok, 2011). Chymotrypsin is the most common reaction mechanism. Chymotrypsin uses an acid-base mechanism where it attacks...
aromatic bulky side chains (Lehninger, 2008). Chymotrypsin breaks peptide bonds through hydrolysis and decreases activation energy by stabilizing the reaction intermediate. Chymotrypsin depends on the strategic positioning of its three critical amino acid residues asparagine, histidine, and serine. Like all enzymes chymotrypsin works best under certain conditions and this lab is intended to determine the, pH, temperature, and inhibition conditions at which it functions the more efficiently. Experimental kinetic parameters in these categories will be used to understand the enzyme kinetic activity of Chymotrypsin and to determine it’s Km, Vmax, and Kcat values. Lastly, indole will be characterized for its inhibition effects on chymotrypsin.

**Materials and Methods**

**Part I: pH and Temperature**

Prepare twelve reaction cuvettes with 0.5 mL stock chymotrypsin enzyme. Add 4.0 mL buffer of increasing pH beginning with pH 5 to the first six test tubes (i.e. pH 5, pH 6, pH 7, etc.). Add 4.0 mL pH 7 to the remaining test tubes and place in ice baths increasing by 10°C beginning with 0°C for a minimum of 10 minutes. One by one starting with the lowest pH add 0.5 mL N-glutaryl-L-phenylalanine-p-nitroanilide (GPNA) substrate and measure its absorbance over 4 minutes at 410nm using each enzyme-buffer mixture without substrate as the blank in each scenario. Repeat for each of the first six test tubes, being sure to mix thoroughly throughout. Remove test tube seven from ice bath, dry the outside of the test tube, blank your spectrophotometer, add 0.5 mL GPNA substrate, and measure the absorbance over a 4 minute period. Repeat for each of the (5) remaining test tubes.

Plot absorbance versus time for each reaction tube combining test tubes 1-6,
effect of pH on one graph and 7-12, effects of temperature on a second graph. Determine the slope of each line. Convert the change in absorbance per minute into change in GPNA concentration per minute using Beer’s Law and a molar extinction coefficient of 8200 M-1cm-1 for GPNA. Plot velocity versus pH and Temperature.

**Part II: Inhibitor Effects**

Prepare 12 more test tubes with 4.0 mL pH 7.0 buffer. Add 0.1 mL GPNA to every fourth test tube beginning with test tube 1. Add 0.2 mL to every fourth test tube beginning with test tube 2, 0.3 mL beginning with test tube 3 and 0.4 mL with test tube 4. Then, add enough N,N-dimethylformamide (DMF) to each test tube so that GPNA + DMF equals 0.5 mL for test tubes 1 – 4 and equals 0.4 mL for the remaining test tubes. Use each mixture as a blank for absorbance readings at 410 nm before starting each reaction (Cok, 2011). Start the reactions one tube at a time, by adding 0.5 mL of chymotrypsin enzyme, mixing, and then recording absorbance measurements over 5 minutes. Construct Michaelis-Menten and Lineweaver-Burke plots where appropriate. Establish the $K_m$, $V_{max}$ and $k_{cat}$ values.

**Results**

A Spec – 200 was used for all measurements of absorbance. From the regression lines of absorbance over time for reactions of increasing pH and temperature (Figure 1 and Figure 2) slopes were calculated. Using Beer’s law with a molar extinction coefficient ($\varepsilon$) of 8,200 M-1cm-1 for p-nitroanilide (GPNA product) and a 1.0 cm path length, concentrations per second (velocities) were calculated. Results for the effects of pH and temperature on enzyme kinetics are displayed below in data table 1.

**Data Table 1.**

<table>
<thead>
<tr>
<th>Tube #</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Velocity (uM/sec)</th>
</tr>
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</table>
As seen in the bar graph of velocity versus pH (Figure 3), as pH increases, velocity increases until it peaks at pH 8 and then steadily decreases in velocity as pH continues to increase. All $r^2$ values for all trend lines for pH kinetics were greater than 98%. As seen in the bar graph of velocity versus temperature (Figure 4), as temperature increases, velocity increases until it reaches $40^\circ$C, and then drastically decreases in velocity at $50^\circ$C. All $r^2$ values for trend lines of temperature kinetics were greater than 99% with the exception of the trend line for $0^\circ$C displaying an $r^2$ value of 73% and $50^\circ$C, 97%.

From the trend lines of the Lineweaver-Burke plot (Figure 6), $V_{\text{max}}$ values for no inhibition, 0.1 M inhibition, and 0.2 M inhibition were calculated to be $4.16 \times 10^{-2}$ $\mu$M/sec, $2.07 \times 10^{-2}$ $\mu$M/sec, and $1.00 \times 10^{-3}$ $\mu$M/sec, respectively. $K_m$ values were calculated to be $9.97 \times 10^{-4}$ $\mu$M, $1.46 \times 10^{-4}$ $\mu$M, and $4.45 \times 10^{-6}$ $\mu$M, respectively. $K_{\text{cat}}$ ($V_{\text{max}}$/ total enzyme concentration) calculated using the $V_{\text{max}}$ of the enzyme without inhibitor and an enzyme total of 10 uM equates to $4.61 \times 10^{-3}$ sec$^{-1}$. The Lineweaver-Burk plot displays double reciprocal plots that cross very close to the y-axis.

**Discussion**

Since the pH at which the chymotrypsin displayed the greatest velocity was a pH of 8 (Figure 2) it can be concluded that chymotrypsin has its greatest activity
at a pH close to 8. As pH increases the activity of chymotrypsin also increases until it reaches its optimal pH.

This is also seen to hold true for temperature. As temperature rose, the enzymatic activity of chymotrypsin also increased until it peaked at 50°C (Figure 4). After 50°C the enzymatic activity decreased significantly suggesting that the chymotrypsin has underwent protein denaturation. Like most chemical reactions, the rate of the reaction increases as temperature increases. Enzymes are greatly affected by temperature. Even a one or two degree difference can increase or decrease the activity of most enzymes by 10 to 20% (Pfeiffer. 1954). This is however, complicated by the fact that proteins become denatured at high temperatures. The reaction rate for enzymes increases to a maximum level then abruptly decreases with further increase in temperature (Pfeiffer, 1954).

The constant $k_{cat}$ can be compared to literature for experiments under the same conditions. Experiments found used a pH near 8, since this is the optimum pH for chymotrypsin, note optimal enzyme pH is often given in a range.

Chymotrypsin-Catalyzed Hydrolysis of p-Nitrophenyl Trimethylacetate at pH 8.2: $k_{cat} = 1.3 \times 10^{-4}$ s$^{-1}$, with a $K_M = 5.6 \times 10^{-7}$ with a 0.01 M tris-HCL buffer (Bender, 1967). The pH used in this experiment was of 7 for analyzing the enzyme activity of chymotrypsin with indole inhibitor, so it cannot be compared to this particular literature. The value of $k_{cat}$, when used in comparison to other enzymes, will give insight to its relative efficiency (Cok, 2011). Knowledge of physiological conditions and study of enzyme kinetics gives scientists great knowledge of how enzymes work in vivo.
The Lineweaver-burk plot of chymotrypsin with varying concentrations of substrate and indole inhibitor depicts a graph that represents competitive inhibition. It virtually crosses on the y-axis represented by the nearly identical $V_{\text{max}}$ values and the varying $K_m$ values, both characteristic are indicative of a formation of a tertiary complex by competitive inhibition (Lehninger, 2008). Knowledge of enzyme inhibitors is very valuable in the medical world. Inhibitors are important because they can be useful for chemotherapeutic treatment of disease, and for providing experimental insights into the mechanism of enzyme action.

There are many practical uses for knowledge of enzyme activity. For example, chymotrypsin is excreted from the pancreas in an inactive form (Cok, 2011). If for any reason the chymotrypsin were to be activated within the pancreas it would begin to digest the pancreas itself, this is known as pancreatitis. It is critical for the proper functioning of chymotrypsin for it to not be activated until it leaves the pancreas through the bile duct and resides in the small intestine where it can continue its proper purpose for protein digestion. Being able to control the activity of enzymes in the body through knowledge of their kinetics and mechanism is a key to disease cure and prevention.
Literature Cited

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• Cok. Experiment 7: The Enzyme Activity of Chymotrypsin: Determination of $K_m$, $V_{max}$, and Inhibitor Effects. (2011).
• Pfeiffer, J. Enzymes, the Physics and Chemistry of Life. (1954). Simon and Schuster.