Enzymes: Potato Polyphenol Oxidase

A Quasi-Quantitative Study of Enzymatic Activity With Inhibitors

by

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for

BIOL 224 Spring 2018

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Introduction to Enzymes: Biological Catalysts

Enzymes have specific functions: Enzymes are categorized into one of 6 biological activities according to the Enzyme Commission:

- Oxidoreductases: catalyze redox reactions -- involve NAD and FAD (E.C. 1.X.X.X)
- Transferases: catalyze group transfers (E.C. 2.X.X.X)
- Hydrolases: use water to lyse bonds (E.C. 3.X.X.X)
- Lyases: nonhydrolytic and non-oxidative group removal (E.C. 4.X.X.X)
- Isomerases: catalyze isomerization reactions (E.C. 5.X.X.X)
- Ligases: catalyzes reactions requiring ATP hydrolysis (E.C. 6.X.X.X)

Enzyme Terminology

Cofactors are molecules or ions of a non-protein nature that are required by an enzyme for complete catalytic capacity, e.g., Mn^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+}, Mo^{2+}.

A coenzyme is a carbon-based molecule required by an enzyme for complete catalytic capacity, e.g., NAD^{+}, FAD, vitamins – and is bound loosely to the apoenzyme.

An apoenzyme is the active enzyme minus the cofactor; it's catalytically inactive.

A prosthetic group is a non-protein moiety tightly bound to the apoenzyme.

A holoenzyme is the apoenzyme plus a prosthetic group (the fully active enzyme).

Zymogens are immature enzymes that need "clipping" for activation – more later in the course during the lecture on the Digestive System or Enzymes.

Enzymes are globular proteins with the exception of ribozymes. Without enzymes, cellular reactions go too slowly to be conducive to life. All enzyme names end in "ase".

The active site of an enzyme is a 3-dimensional cleft in the enzyme caused by/coded by the primary structure of the protein; it's complimentary to the shape (geometry) of the substrate. Specificity characteristics are due to the active site; the crevice allows binding of 1) only one substrate or 2) 1 kind of R group.

Constitutive enzymes are always in the cell without regard to the availability of substrate.

Induced enzymes are present in the cell ONLY when the substrate activates gene mechanisms causing intracellular release of the active enzyme.

Practical Conditions To Study Enzymes:

The substrate (S) must be converted to product (P) by the enzyme (E) under the following conditions:

- The reaction is thermodynamically feasible
- S goes through and above the appropriate E_a for P to form

Example

Reaction	Catalyst	E_a (@ RT)
$2H_2O_2 \rightarrow 2H_2O + O_2$	None	319.2 kcal/mol
$2H_2O_2 \rightarrow 2H_2O + O_2$	I^-	239.4 kcal/mol
$2H_2O_2 \rightarrow 2H_2O + O_2$	Catalase	33.6 kcal/mol

Efficiency of Enzymes

Enzymes increase the rate of reaction without being consumed themselves. Enzymes lower the E_a (see table above), yet have no effect on K_{eq}.

Enzymes permit reactions to reach equilibrium quicker and have pH and temperature requirements.

Enzymes cause reactions to go within seconds as opposed to lab reactions that may take years; enzymes are a necessity to/for life:

E.g., $CO_2 + H_2O \leftrightarrow H_2CO_3$

The condensation of water with carbon dioxide to form carbonic acid, above, is catalyzed by carbonic anhydrase at a rate of $6*10^5$ molecules of CO_2 condensed per second.

Specificity of Enzymes

Enzymes are specific in the reaction types catalyzed. Enzymes are specific in the substance (substrate) involved in the reaction (S). There are three specific kinds of specificity for enzymes:

- Absolute specificity ≡ catalyzes reaction with only one S
- Relative specificity ≡ catalyzes reaction of substrates with similar structures
- Stereochemical specificity ≡ D vs L – this can be thought of as "handedness" – the enzyme being left or right "handed".

Enzyme Regulation

The cell regulates which enzymes function and when, i.e., not ALL enzymes are working at the same time. Some enzymes catalyze uni-directionally (either catabolic or anabolic); some catalyze bi-directionally (amphibolic).

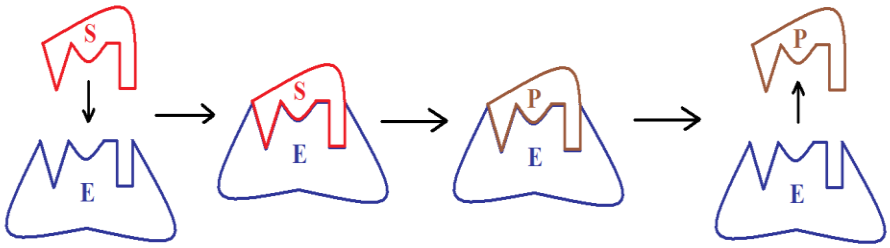
Enzyme Activity

The activity of an enzyme is defined the catalytic capacity of the enzyme to increase the reaction rate. The turnover number is the # of S molecules acted upon by ONE enzyme molecule per minute. Enzyme assays measure enzyme activity in "Units (U's)". One unit (**U**) is the amount of **enzyme** consuming 1 µmol substrate or forming 1 µmol product per minute under standard conditions (at a given pH, T and [S]).

Enzyme Models

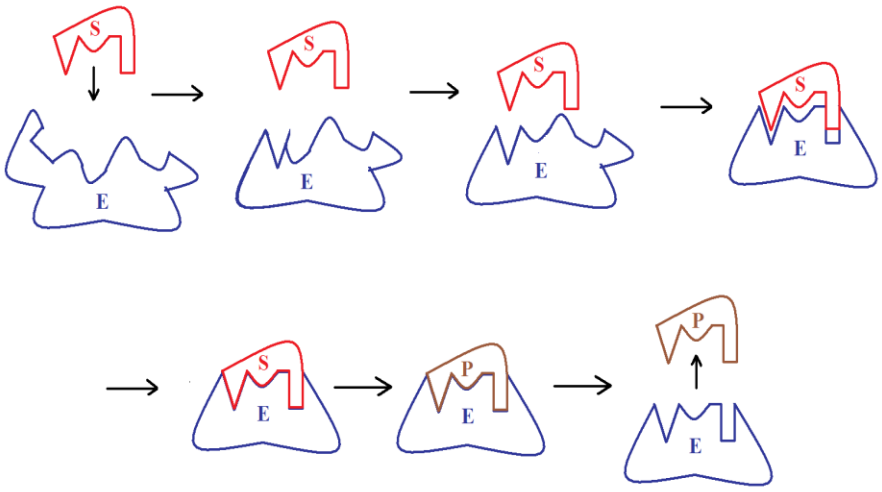
Of significance, of course, is the fact that the shape of the enzyme gives it its function (the structure of a protein gives it its function). Enzymes speed up the reaction rate in biological systems 100,000 - 1,000,000 fold! Some are known to increase the reaction rate > 10^{20}-fold! Enzymes have specific substrates (chemical group upon which the enzyme works), but can work on limited kinds of substrates. There are two generally accepted models for the functioning of enzymes: the lock and key model and the induced fit model.

Model #1: The Lock and Key Model



In this model, see graphic, right, the substrate (S) is complimentary to the binding/active site in the enzyme (E). This is likened to the lock and key, where the lock is complimentary to the key. As the E and S bind, they form the Enzyme-Substrate complex (ES). This is an intermediate in the reaction that will cause S to be changed into a product (P). The enzyme acts as a sort of scaffold, holding the substrate so that one specific reaction may occur. In this case, a bond (or bonds) is (are) broken as the enzyme changes its shape ever so slightly, causing the substrate to break exactly where it's supposed to, releasing the new products and the enzyme for use, again. Remember that the active sites (a, b, c) of the enzyme are complimentary to the SHAPE of the substrate.

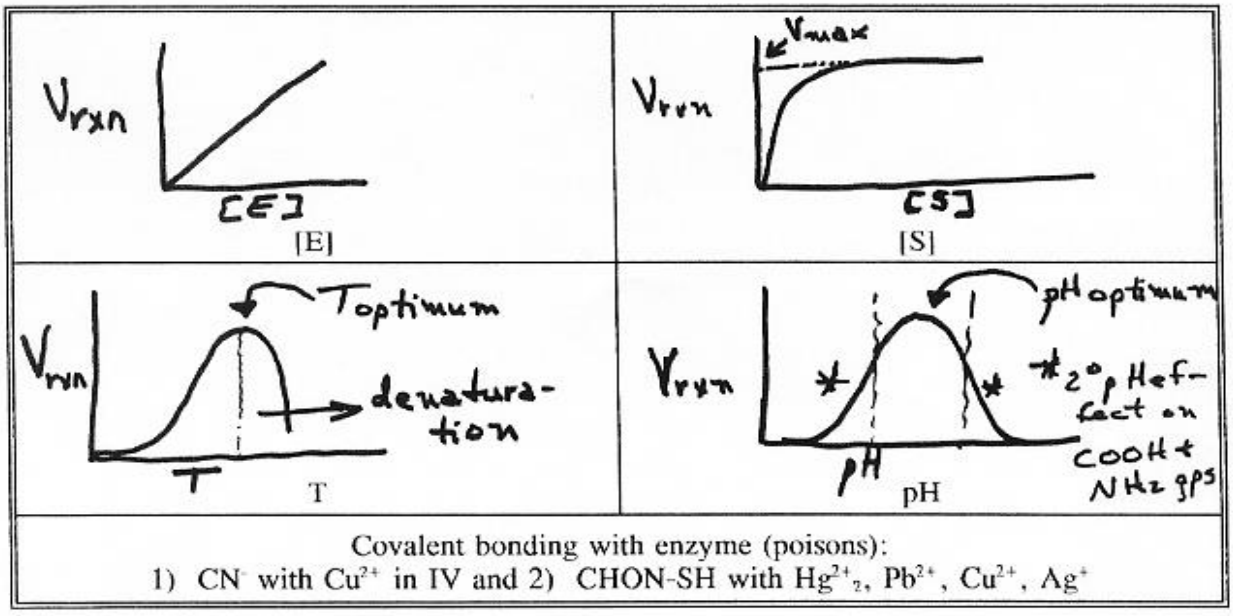
Model #2: Induced Fit



The second model is called the induced fit model (image, right). This means that as the S gets closer to the E, the E actually undergoes a conformational change (shape change) to fit the S, i.e., its shape is INDUCED to change by the presence of the substrate. Note that as S gets closer to E, the active site "a" (left site) changes shape to match the complimentary site on S. As S continues to get even closer, site "b" (middle site) shifts its shape, as does site "c" (right site) when S is all but bound to the enzyme. Once ES is formed, this model conforms to the remainder of the lock and key theory of enzyme-substrate binding.

What Effects Enzyme Activity?

The easy answer to this question is all of the following: [E], [S], T, pH and covalent bonding:



Covalent bonding with enzyme (poisons):
1) CN^- with Cu^{2+} in IV and 2) CHON-SH with $Hg^{2+}{}_2$, Pb^{2+}, Cu^{2+}, Ag^+

The longer answers start in the upper left of the above graphic. In general terms, the velocity of the reaction increases with increasing enzyme, e.g., 3 [E] = 3 V_{rxn} .

With increasing [S] (upper right), this causes S to bind at the activation site causing conformational changes so that the active site binds S. At some [S], E is sat'd with S and will not work any faster. This rate is called the V_{max}.

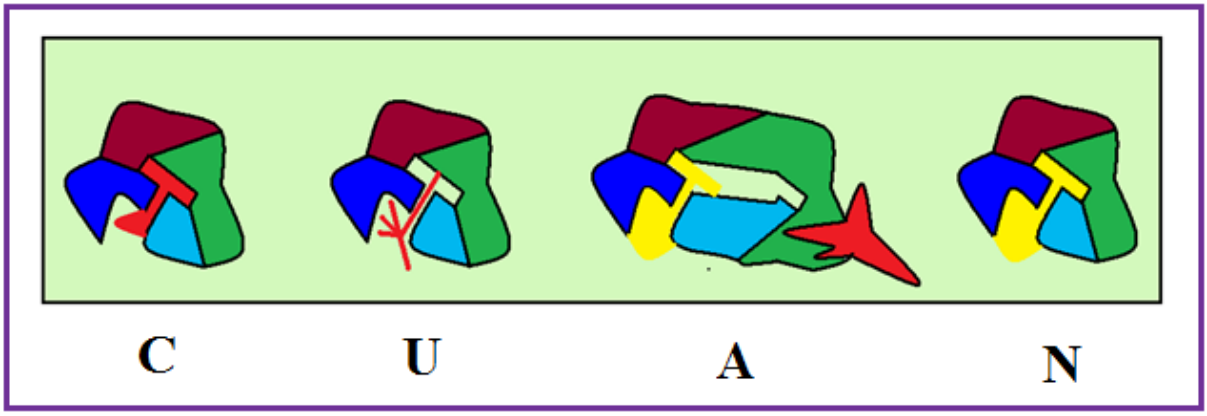
At lower left, temperature increases the rate of the reaction (V_{rxn}) to a point where activity drops off (denaturation).

The pH optimum is where enzyme has greatest activity (bottom right); at pH's above and below this pH, the enzyme still has some activity until pH extremes are reached. This causes enzyme denaturation at either end of extremes.

Lastly, covalent bonding with the enzyme by poisons impacts the activity of the enzyme. This may include the effect of cyanide on complex IV in ETOP or the impact of the mercurous ion, plumbous ion, cupric ion and silver (I) ion binding to the thiol group on cysteine in proteins.

Is there anything else that impacts enzymatic activity? YES!!!! Effectors. Effectors are non-substrate[s] that turn[s] on the E, e.g., calmodulin (in most cells) and troponin (in muscle cells {skeletal and cardiac}). To activate E's, both must bind Ca^{2+}.

Enzyme Inhibition: Descriptive Introduction



Normal ES Complex

The far right graphic represents the normal (N) ES complex. Any inhibition pattern MUST be compared to the "normal" so that the pattern may be identified.

Competitive Inhibition

The farthest left graphic represents competitive (C) inhibition of an enzyme, i.e., an inhibitor specific to this enzyme COMPETES with the substrate for the active site of this enzyme. It is reversible; will block S from binding. One example of this sort of inhibition is carbamoyl choline that competitively inhibits acetylcholinesterase.

Uncompetitive Inhibition

The second graphic from left graphic represents uncompetitive (U) inhibition. This sort of inhibition involves covalently bound inhibitor and inactivates the enzyme irreversibly. Two examples of this sort of inhibitor are nerve gas and organophosphates that inhibit acetylcholinesterase. Organophosphate poisoning may be reversed by injecting a drug called 2-PAM. Valium and atropine are useful to treat muscle spasms and breathing difficulties, as well.

Non-Competitive Inhibition

The second from right graphic represents noncompetitive (A) inhibition. Note that the inhibitor does NOT bind to the active site of the enzyme, rather it has its own unique binding site. When a noncompetitive inhibitor binds to an enzyme, it causes the enzyme to change shape and shuts off its activity reversibly by not allowing S to bind completely. This sort of inhibition is also referred to as allosteric inhibition and plays major roles in metabolic regulation. An example of a noncompetitive inhibitor is aspirin. Aspirin inhibits cyclo-oxygenase (COX) which is the main enzyme in prostaglandin biosynthesis. Prostaglandins mediate pain, Inflammation, blood pressure, gastric mucous secretion, blood clotting, labor and delivery, to name a few.

Mixed Inhibitor Types

An example of mixed inhibitor types is aspirin (ASA) and Ibuprofen (IBU). ASA is an **UN**competitive inhibitor of COX-1 (CycloOXygenase type 1). ASA and IBU inhibit cyclo-oxygenase variants which is the main enzyme in prostaglandin biosynthesis. Prostaglandins mediate pain, Inflammation, blood pressure, gastric mucous secretion, blood clotting, labor and delivery, dysmenorrhea, to name a few. This inhibition is IR-reversible – unlike other NSAID's (Non-Steroidal Anti-Inflammatory Drug's).

ASA acetylates COX-1 to inhibit it. The half life ($t_{½}$) of ASA varies by dosage: 250 mg dose $t_{½}$ = 2-4.5 hrs; 1 g dose $t_{½}$ = 5 hrs; 2 g dose $t_{½}$ = 9 hours; > 4 g $t_{½}$ = 15-30 hrs. ASA CHANGES COX-2 activity to produce anti-inflammatory lipoxins ("LX's"; derived from ω3 fatty acids (EPA) as well as ω6 fatty acids such as $20{:}4^{\Delta 5,8,11,14}$), see image at right. ASA acetylates COX-1 to inhibit it.

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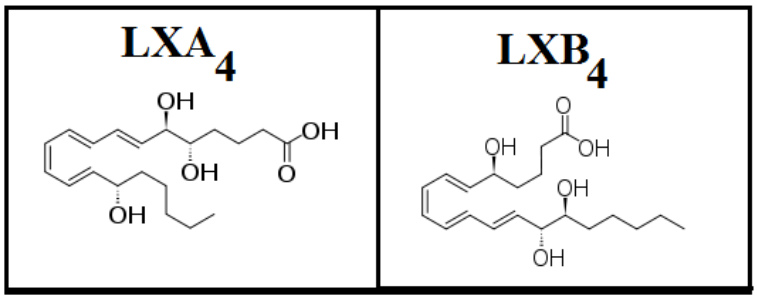
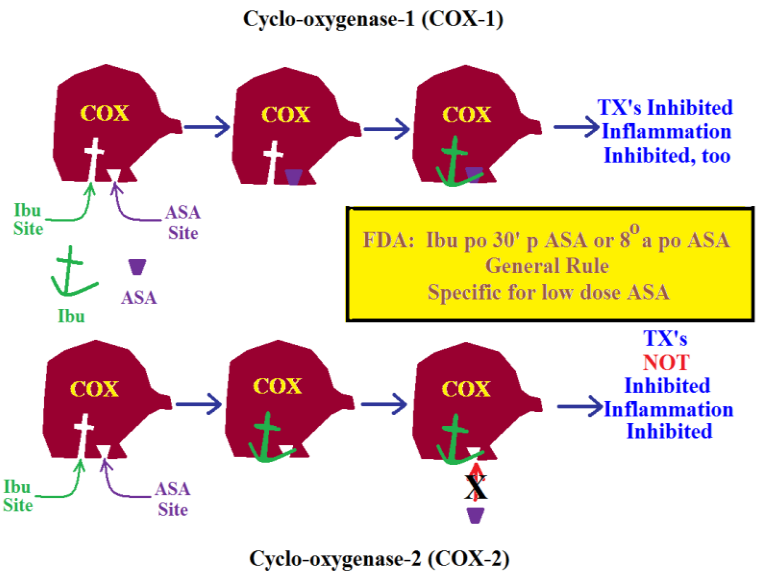


Lipoxins
anti-inflammatory

IBU is a **NON**competitive inhibitor of COX-2. It is reversible in its inhibition. IBU works primarily through COX-2 (Like Vioxx and Celebrex by reducing PGI_2. This permits "normal" TX(A_2 or B_2) production which increases the incidence of blood clots [IBU has lowest incidence of GI/Hematological Sx of the NSAID's, by the way].

The half life for IBU is unique in that it's about 1.8-2 hrs while its duration of action is about 2-4X the $t_{½}$.

The problem with these two medications is that IBU binds to COX-2 inhibiting the production of PGI_2 – the natural titrant of TX's. What does this mean? Blood clots, potentially. If one is a cardiac patient and is taking low dose oral ASA on a daily basis to prevent blood clot formation, yet needs IBU for pain control, what is one to do?



Per

http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm

125222.htm, the patient needs to take their ASA first and wait 30 minutes to take their IBU (top of graphic at bottom of previous page) or take their IBU 8 hours before their ASA dose. If IBU is taken first or is not taken long enough before the ASA dose, IBU not only binds, it also blocks the binding of ASA, to COX-1 and COX-2 (graphic, bottom right, previous page). Should this occur, the potential for a fatal MI due to thrombosis of [a] coronary arter[y]ies is elevated.

Reaction Kinetics: A Brief Introduction

A condition of equilibrium is reached in a system when 2 opposing changes occur simultaneously at the same rate. The rate of a chemical reaction may be defined as the # of mols of a substance which **disappear** or **are formed** by the reaction per unit volume in a unit of time.

Example

For the reaction I $\rightarrow$ C, the forward rate equation is as illustrated in the formula, below:

$$I \rightarrow C$$

$$Rate_{forward} = \frac{[I]_2 - [I]_1}{t_2 - t_1} = \frac{\Delta [I]}{\Delta t}$$

The previous rate is for the DISAPPEARANCE of I, therefore:

$$rate = -\frac{\Delta [I]}{\Delta t}$$

$$where\ the\ negative\ sign\ means\ disappearing\ or\ "loss\ of"$$

Backwards example (rate of formation of I) using the same simple reaction:

$$Rate_{bkward} = \frac{[C]_2 - [C]_1}{t_2 - t_1} = \frac{\Delta [C]}{\Delta t} = +\frac{\Delta [C]}{\Delta t}$$

$$where\ the\ positive\ sign\ means\ for\ \min g$$

$$when\ both\ reaction\ rates\ study\ I$$

For more complex reactions of the following type:

$$X \rightarrow 3\ Z$$

$$Rate = -\frac{\Delta\ X}{\Delta\ t} \neq \frac{\Delta\ Z}{\Delta\ t}$$

$$this\ \ is\ \ due\ \ to\ \ Z\ \ appearing\ \ 3X\ \ as\ \ fast\ \ as\ \ X$$

$$is\ \ disappeari\ ng$$

$$\therefore$$

$$Rate = -\frac{\Delta\ X}{\Delta\ t} = \frac{1}{3}\frac{\Delta\ Z}{\Delta\ t}$$

In general, for the reaction:

pP + qQ $\rightarrow$ rR + sS , the rate may be expressed as follows:

$$Rate = -\frac{1}{p}\frac{\Delta\ P}{\Delta\ t} = -\frac{1}{q}\frac{\Delta\ Q}{\Delta\ t} = \frac{1}{r}\frac{\Delta\ R}{\Delta\ t} = \frac{1}{s}\frac{\Delta\ S}{\Delta\ t}$$

Reaction Order

For the Reaction:

pP + qQ $\rightarrow$ rR + sS

$$Rate = -\frac{1}{p}\frac{\Delta\ P}{\Delta\ t} = -\frac{1}{q}\frac{\Delta\ Q}{\Delta\ t} = \frac{1}{r}\frac{\Delta\ R}{\Delta\ t} = \frac{1}{s}\frac{\Delta\ S}{\Delta\ t}$$

$$and\ \ is\ \ proportion\ al\ \ to$$

$$[P]^n\ [Q]^m$$

$$or$$

$$Rate = k\ [P]^n\ [Q]^m$$

$$where$$

$$k = proportion\ ality\ \ cons\tan t\ \ or\ \ rate\ \ cons\tan t$$

k is a rate constant that is unique for every reaction. A reaction with an incredibly large rate constant is faster than a reaction with an incredibly small rate constant.

For the Reaction pP + qQ $\rightarrow$ rR + sS, the reaction is "n" order in [P] and "m" order in [Q] OR Is OVERALL "(n + m)" order. The KEY!!!!!! is that "n" and "m" DO NOT necessarily equal "p", "q", "r" or "s" (the stoichiometry – see below), although they can.

Example

$$2\,N_2O_5\ (g) \rightarrow 4\,NO_2\ (g) + O_2\ (g)$$

$$Rate = -\frac{1}{2}\frac{\Delta[N_2O_5]}{\Delta t} = \frac{1}{4}\frac{\Delta[NO_2]}{\Delta t} = \frac{\Delta[O_2]}{\Delta t}$$

$$or$$

$$= k\,[N_2O_5]$$

This reaction is FIRST order in N_2O_5, NOT SECOND order as one might intuit **<u>from the stoichiometry</u>**.

The order of the reaction is the specification of the empirical (experimentally-determined) dependence of the rate of the reaction on CONCENTRATIONS of **<u>reactants</u>**.

The order may = 0, a whole number or a non-whole number, e.g., 0, 1, 1½, or 2. We'll focus on whole numbers and 0 (zero) for reactions of the type: fF + gG $\rightarrow$ Products

AND! The order of the reaction is defined in terms of REACTANTS not the products, therefore, products do not need to be specified

First Order Reaction Example

Assume reaction is 1st order in F and zero order in G:

$$Rate = -\frac{1}{f}\frac{\Delta F}{\Delta t} = k'[F]^1[G]^0$$

$$f\,k' = k \;\therefore\; \frac{\Delta F}{\Delta t} = k\,[F]$$

$$\text{Re}\,arrange:\ \frac{\Delta F}{F} = k\,\Delta t\ \ and\ \ \text{int}\,egrate:$$

$$\ln\frac{[F]}{[F]_o} = -\,k\,t$$

$$OR$$

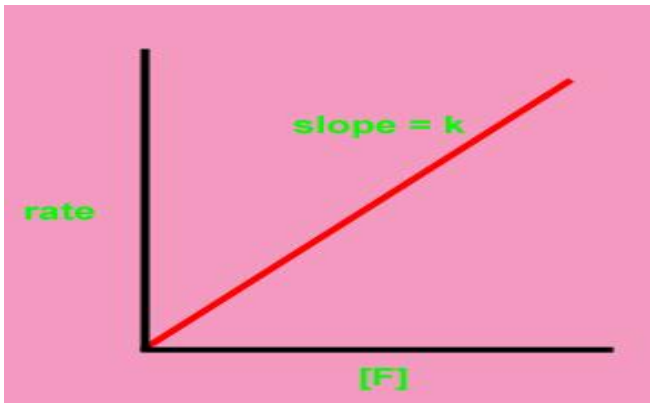
$$[F] = [F]_o\,e^{-k\,t}$$

Many radioactive decays fit 1st order reactions:

- $^{226}Ra_{88} \rightarrow {}^{222}Rn_{86} + {}^{4}He_{2}$
- $^{238}U_{92} \rightarrow {}^{234}Th_{90} + {}^{4}He_{2}$

The rate of the example reaction is proportional to [F] (table below and image at right, below):

Double [F]	$k\,[F]^1$	$k\,[F]^2$	$k\,[F]^3$
Rate Change	⇑ X 2	⇑ X 4	⇑ X 8
Arithmetic	$2^1 = 2$	$2^2 = 4$	$2^3 = 8$
Reaction Order in [F]	First	Second	Third



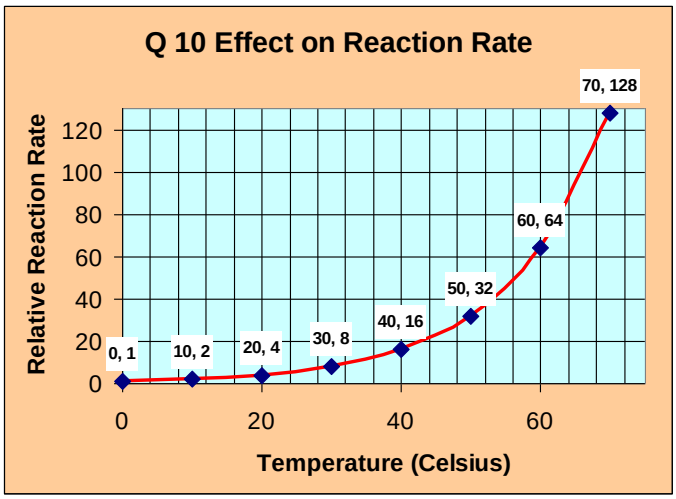
Q_{10} Effect

Example: If the rate of a chemical reaction doubles for every 10°C rise in temperature, how much faster would the reaction proceed at 55°C than at 25°C?

Solution

The temperature increased 30°C, therefore, the reaction rate increases 8-fold (there are three 10's, hence $2^3 = 8$; image at right).

What if the temperature was increased to 105°C from 25°C?



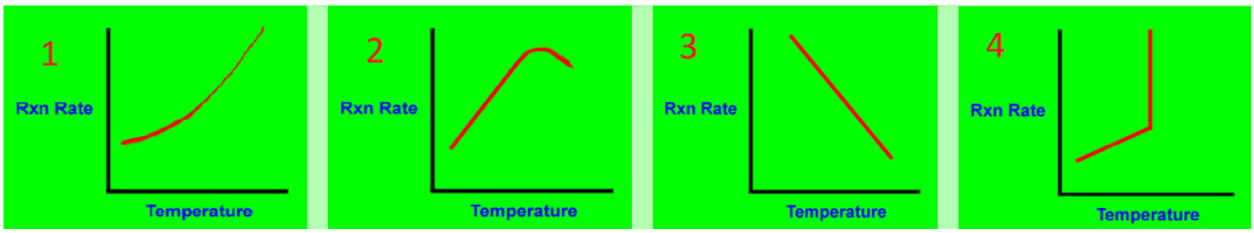
The temperature increased 80°C, therefore reaction rate increases 256-fold (there are eight 10's, hence $2^8 = 256$).

How much faster would a reaction go at 100°C than at 25°C?

For every 10°C increase in temperature, the reaction rate doubles. The change in temperature is 75°C. This is 7.5 (seven and a half) 10°C increases. Hence $2^{7.5} = 181$ times faster

In an experiment, a sample of NaOCl was 85% decomposed in 64 minutes. How long would it have taken if the temperature was 50°C higher?

For every 10°C increase, the reaction rate doubles. 50°C increase is 5 10°C increases. Hence: $2^5 = 32$ times faster. So: (64 minutes)/(32 times faster) = 2 minutes.



There are 4 types of temperature dependence for reaction rates (labeled images above):

– 1 The reaction rate increases with increasing temperature: NORMAL.

– 2 The reaction rate increases to a point, then reduces with increasing temperature, e.g., enzymes being denatured.

– 3 The reaction rate decreases with increasing temperature: VERY RARE. This sort of reaction is known only for a few reactions that are multi-step reactions:

- $A \rightarrow B$ Fast step
- $B \rightarrow C$ Rate limiting step

– 4 The reaction rate increases with increasing temperature: odd behavior. The result is an explosive reaction when the temperature shoots up. The gradual rise in temperature is due to chain reactions.

Apply Kinetics to Enzymes

Enzymes are, with a couple of exceptions, proteins. Enzymes are biological catalysts. Enzymes speed up biological reactions incredibly. For this discussion: E = enzyme, S = substrate, ES = enzyme-substrate complex, EP = enzyme-product complex and P = product; in addition, I = inhibitor. The derivation of the basic enzyme-related equation follows (left panel, then, right panel):

$$E + S \xrightarrow{k_1} ES \qquad FAST$$

$$ES \xrightarrow{k_{-1}} E + S \qquad FAST$$

$$ES \xrightarrow{k_2} E + P \qquad SLOW$$

$$AND$$

$$\frac{d\,[ES]}{d\,t} = -\frac{d\,[ES]}{d\,t}$$

$$Short \quad Method$$

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

$$Solve \quad for \ [ES]:$$

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

$$Stop \quad temporaril \ y$$

Lest we forget,

- Rate limiting step is step 3
- Rate equation is: k_2 [ES]
- Substitute as before (image at right):

$$\left(\frac{k_1 k_2}{k_{-1}}\right)[E][S] = k[E][S]$$

$$\textit{Write the overall reaction:}$$

$$E + S \Leftrightarrow ES \rightarrow E + P$$

Enzyme Inhibition: An Introduction to Quantitative Enzymology

From this point on, the arithmetic manipulations to study enzymes are all based on the following reaction sequence:

$$E + S \leftrightarrow ES \rightarrow EP \rightarrow E + P$$

Inherent to this sort of study are 3 assumptions:

1. [E] << [S]
2. P ≠ S
3. ES forms either
    1. E + S OR
    2. E + P

Fundamental Reaction and Constants

$$E + S \underset{k_2}{\overset{k_1}{\Leftrightarrow}} ES \xrightarrow{k_3} E + P$$

We can use the above sequence to write a statement about the rate of the reaction (V) which is proportional to [E], [S], and k_1, k_2 and k_3 (these latter three rate constants are "fudge factors" – they can also be used to determine K_{eq} as we'll see in a bit). Note that we're "ignoring" EP, now.

Catalytic Velocity

The catalytic velocity (speed) is the product of [ES] and k_3 (rate limiting step is the rate of P formation and, hence, ES loss) or $V = k_3$ [ES]

Can we write equation[s] to tell us about [ES] in terms of measurable [E], [S] and [P]? YES!!!!!!

$V_{formation\ [ES]} = k_1$ [E] [S] $\qquad\qquad$ $V_{loss\ of\ [ES]} = (k_2 + k_3)$ [ES]

!!! ES = INTERMEDIATE in reaction sequence!!!

When $V_{formation} = V_{loss}$, [ES] is a constant as [S] decreases and [P] increases.

We may write that arithmetically as

$k_1 [E] [S] = (k_2 + k_3) [ES]$

Steady State Approximation

The steady state approximation is so called because the [intermediate] does not change.

Let's solve for [ES] since it DOES remain constant:

$$[ES] = \frac{k_1 [E][S]}{(k_2 + k_3)} \Leftrightarrow \frac{\frac{[E][S]}{(k_2 + k_3)}}{(k_1)}$$

Remember that equilibrium constants express what is going on in a reaction in terms of products and reactants. They can also be expressed in terms of rate constants. In this case, the equilibrium constant is given a special name and icon: K_M – the Michaelis-Menton constant.

K_M Equals

$$K_M = \frac{k_2 + k_3}{k_1}$$

$$\therefore$$

with substituti on

$$[ES] = \frac{[E][S]}{K_M}$$

Is there a way to determine [E]? YES!!!!!! [E] = $[E_T]$ – [ES] which is the UNCOMBINED E. By substituting $[E_T]$ – [ES] for [E], we can obtain the following equation:

$$[ES] = \frac{([E_T] - [ES])[S]}{K_M} = \frac{[E_T][S]}{K_M} - \frac{[ES][S]}{K_M} = \frac{[E_T][S] - [ES][S]}{K_M}$$

By rearranging, we get

K_M [ES] = [E_T] [S] – [ES] [S]

[ES] K_M + [ES] [S] = [E_T] [S]

[ES] (K_M + [S]) = [E_T] [S]

And

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

Remember, now, the rate limiting step equation is:

V = k_3 [ES]

So let's substitute for [ES]

$$V = \frac{k_3[E_T][S]}{K_M + [S]}$$

$$When\ [S] >>>> K_M,$$

$$\frac{[S]}{K_M + [S]} \rightarrow 1$$

This means E is saturated with S at AND causing V_{max} and that V_{max} = k_3 [E_T]. When we substitute below, we get:

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

which is the Michaelis-Menton Equation.

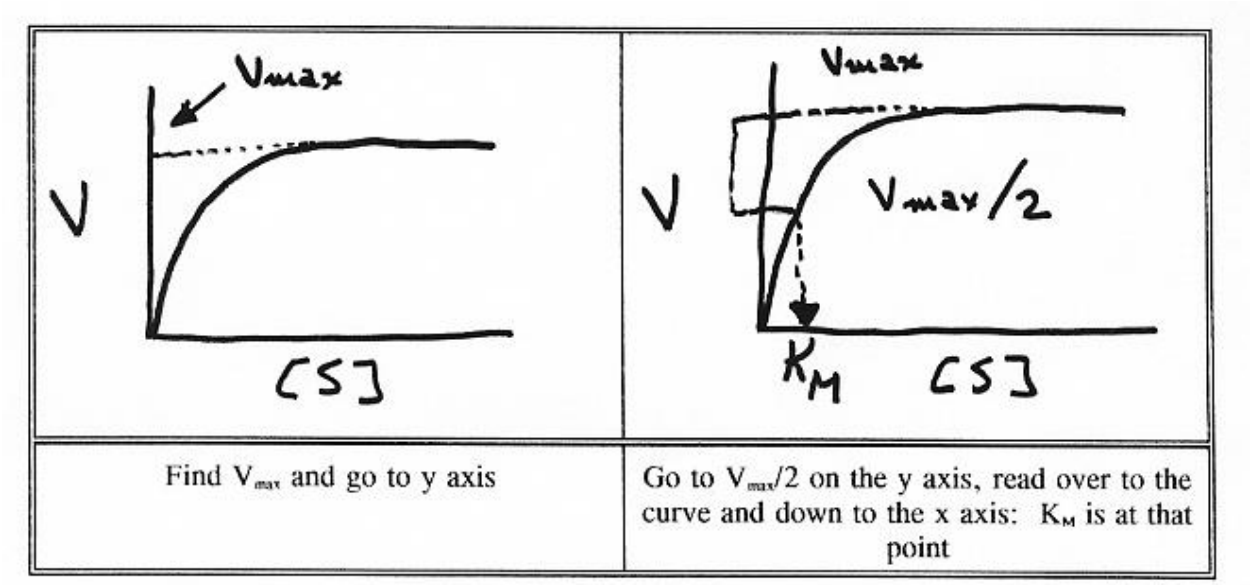
Application #1 – when [S] = K_M

$$V = \frac{V_{max}\,[S]}{K_M + [S]} = \frac{V_{max}\,K_M}{2\,K_M} = \frac{V_{max}}{2}$$

hence

$$K_M = [S] \;@\; \frac{V_{max}}{2}$$

This application can be illustrated as follows:



Find V_{max} and go to y axis	Go to $V_{max}/2$ on the y axis, read over to the curve and down to the x axis: K_M is at that point

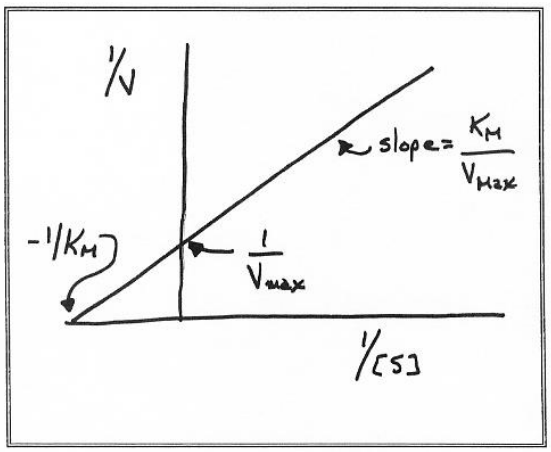
This is in the form of a rectangular hyperbola -- not very useful to the enzymologist.

Lineweaver & Burke Modification

Lineweaver and Burke took double reciprocals (the other name of this method) of the Michaelis-Menton equation and obtained the following equation:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}\,[S]}$$

Graphically, this is what Lineweaver-Burke looks like in general (image at right):



This is very useful! -- more to come later.

Application #2: When k_2 >>> k_1 (for most – not all – enzymes, image immediately below):

$$K_M = \frac{k_2}{k_1}$$

When K_M is high, k_2 is increased and/or k_1 is decreased and favors ES → E + S, hence, a high K_M = weak bonding of S with E to form ES.

When K_M is low, k_2 is reduced and/or k_1 is increased and favors ES → E + P, hence, a low K_M = strong bonding of S with E to form ES.

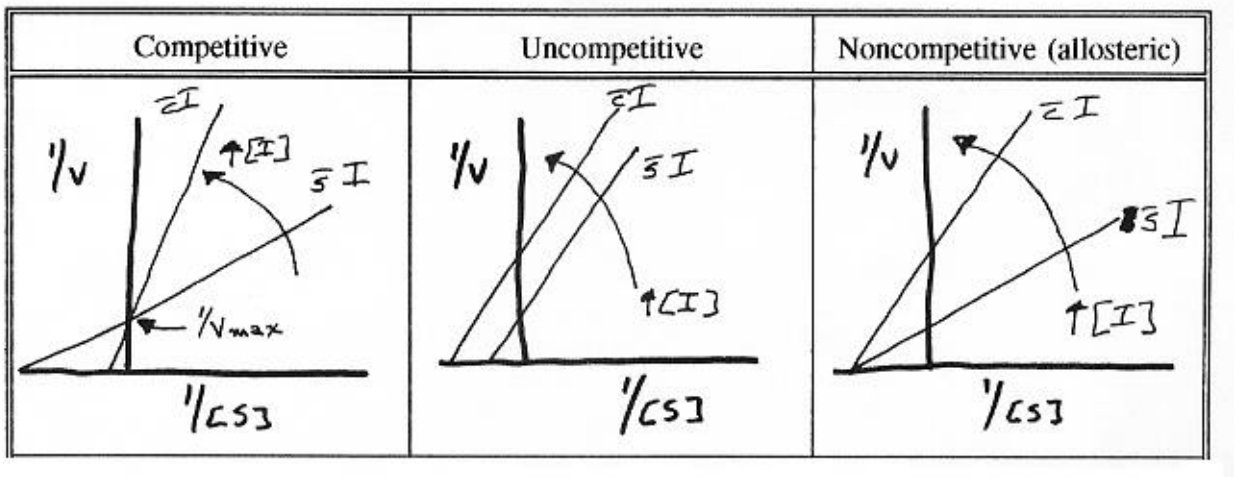
$K_M = 1*10^{-9}$ M ← low K_M

vs

$K_M = 1*10^{-3}$ M ← high K_M

E binds with S 1,000,000-fold tighter with the E that has the low K_M than with the E with the high K_M.

Lineweaver-Burke and Inhibitors



Graphically, the three inhibition styles, when illustrated using Lineweaver-Burke, appear as immediately above.

Competitive Inhibitors

The inhibitor drives no change in V_{max}. K_M changes with inhibitor. Inhibition can be overcome by very high [S], i.e., "swamping out" the inhibitor. The inhibition is reversible; "temporary". The competitive inhibitor is completely or partially identical to S molecular shape. If the inhibitor effects slope and K_M = competitive inhibitor. There's NO effect on y-intercept.

Un-Competitive Inhibitors

This inhibitor drives V_{max} changes, which decreases with inhibitor. The K_M changes. Inhibition can not be overcome with high [S] since inhibition is covalent at active site. Inhibition is overcome only 1) with proper drug therapy or 2) with synthesis of new enzyme. As a general rule, this inhibition is irreversible; "permanent". If the inhibitor effects x and y intercepts = un-competitive inhibition. There is no effect on the slope.

Non-Competitive Inhibitors

This inhibitor drives V_{max} changes (decreases with inhibitor). There is no change in K_M. Inhibition can not be overcome by high [S] since inhibition does not occur at active site or E, but at a separate surface site. The inhibition is reversible; "temporary". Non-competitive inhibition is aka feedback inhibition and/or allosteric inhibition. If the inhibitor effects slope and y intercept = non-competitive inhibition. There is no effect on the x-intercept.

Potato Polyphenol Oxidase (PPO) Assay and Data Analysis

Introduction

Potato polyphenol oxidase (PPO) is an enzyme that is activated upon injury to the potato, e.g., sliced with a knife, cut with a spade, poked with a pitch-fork **[1]**. It is this enzyme that causes the potato to turn brown where it shows damage **[2,3]**. The same enzyme (polyphenol oxidase -- PPO) is also found in apples and bananas and grapes **[4]**.

PPO catalyzes the following reaction (note that **Figure 1** shows the benzoquinone as the product of catechol oxidase catalysis and that **Figure 2** illustrates the o-quinone as the product of polyphenoloxidase catalysis – same things, different names):

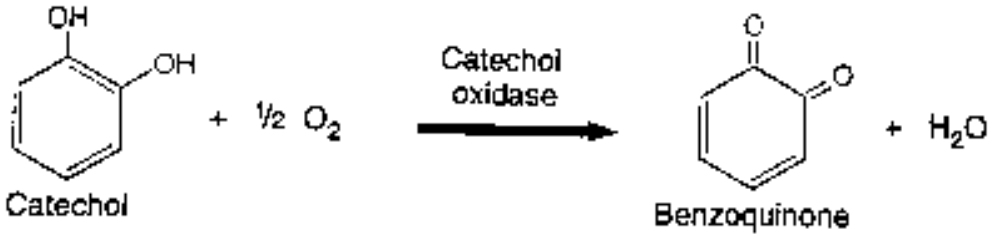


Figure 1. Catechol oxidase (PPO) catalyzes the oxidation of catechol to benzoquinone. The oxidation product is used to synthesize melanins.

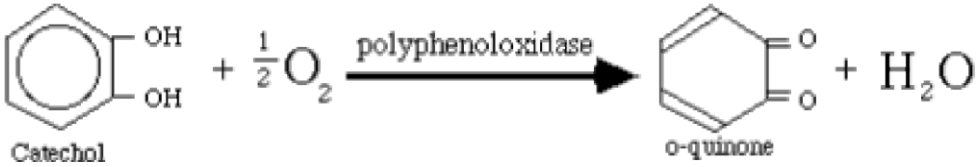


Figure 2. Polyphenoloxidase (PPO) catalyzes the oxidation of catechol to o-quinone. The oxidation product is used to synthesize melanins.

The benzoquinone/o-quinone product is then utilized to synthesize melanins – pigments that cause the damaged tissues to turn reddish-brown. It is these pigments that will be measured in the laboratory to determine the KM and Vmax of this enzyme. In addition, the effects of three proposed inhibitors of PPO will be studied, as well: Tyrosine, *trans*-Cinnamic Acid and Phenylthiourea (PTU).

Classic Michaelis-Menten enzyme kinetics have been discussed above and will not be discussed as part of this lab write-up. The applications of Lineweaver-Burke analysis discussed above will be applied and discussed in the "Results" and "Discussion" sections.

Materials

The materials required for this experiment are tabulated, below:

Blender (Food Processor)	Distilled water	A potato
24 -- Disposable test tubes	Test tube rack	Pencil
Spectrophotometer at 480 nm	24 -- Disposable cuvets	Parafilm
Disposable pipets	Scissors	Waste container
Clock and Parafilm™	Ice bath	Catechol (0.03M)[A]
Tyrosine (0.01M)[A]	*trans*-Cinnamic Acid (0.01M)[A]	Phenylthiourea (0.01M)[A]
Glass stirring rods	Beakers	Pan balances
Buchner funnel	Filter paper	Filter Flask with Tubing
Large disposable pipets	Pipet bulb	Lab cart

Methods

The method for this experiment is a conglomeration of several methods, optimized for this experiment **[5-7]**. To prepare the reagents[A], refer to a previously performed experiment in BIOL 190L and/or CHEM 121 lab: An Experimental Approach to the Practicality of Making One's Own Solutions in Biology and Chemistry.[A]

Set up 4 rows of test tubes – 6 test tubes per row (refer to **Table 1**, below).

Tube #	**1**	**2**	**3**	**4**	**5**	**6**
mL Distilled Water	3	3	3	3	3	3
gtts Distilled Water	5	4	3	2	1	0
gtts catechol	0	1	2	3	4	5
Tube #	**1-tyr**	**2-tyr**	**3-tyr**	**4-tyr**	**5-tyr**	**6-tyr**
mL Distilled Water	3	3	3	3	3	3
gtts Distilled Water	5	4	3	2	1	0
gtts catechol	0	1	2	3	4	5
Tube #	**1-t-CA**	**2-t-CA**	**3-t-CA**	**4-t-CA**	**5-t-CA**	**6-t-CA**
mL Distilled Water	3	3	3	3	3	3
gtts Distilled Water	5	4	3	2	1	0
gtts catechol	0	1	2	3	4	5
Tube #	**1-PTU**	**2-PTU**	**3-PTU**	**4-PTU**	**5-PTU**	**6-PTU**
mL Distilled Water	3	3	3	3	3	3
gtts Distilled Water	5	4	3	2	1	0
gtts catechol	0	1	2	3	4	5

Table 1. Tabular summary of test tube organization for the experiment from initial study through the three potential inhibitors.

Label the tubes in Row 1 as 1 through 6. Label the tubes in Row 2 as 1-tyr through 6-tyr. Label the tubes in Row 3 as 1-t-CA through 6-t-CA. Label the tubes in Row 4 as 1-PTU through 6-PTU.

Into each test tube in each row, pipet 3 mL distilled water. Into the first tube (#1) of each row add 5 drops of distilled water. Into the second tube (#2) of each row add 4 drops of distilled water. Into the third tube (#3) of each row add 3 drops of distilled water. Into the fourth tube (#4) of each row add 2 drops of distilled water. Into the fifth tube (#5) of each row add 1 drop of distilled water. Into the sixth tube (#6) of each row add no additional water.

Now, into the first tube (#1) of each row add no (0) drops of catechol. Into the second tube (#2) of each row add 1 drop of catechol. Into the third tube (#3) of each row add 2 drops of catechol. Into the fourth tube (#4) of each row add 3 drops of catechol. Into the fifth tube (#5) of each row add 4 drops of catechol. Into the sixth tube (#6) of each row add 5 drops of catechol.

By row, each #1 tube is now 0M catechol; each #2 tube is 0.006M catechol; each #3 tube is 0.012M catechol; each #4 tube is 0.018M catechol; each #5 tube is 0.024M catechol; each #6 tube is 0.03M catechol – this is the concentration of substrate ([S]) for your kinetic studies.

Next, into each of the "#-tyr" tubes, add 5 gtts of 0.01M tyrosine. Into each of the "#-t-CA" tubes, add 5 gtts of 0.01M *trans*-cinnamic acid. Into each of the "#-PTU" tubes, add 5 gtts of 0.01M PTU. Each row of tubes now contains its potential PPO inhibitor.

> **NOTE: PTU and catechol are poisonous and are to be handled with care. Wash your hands after using these chemicals and do not mouth pipet them. If you choose to wear gloves for this experiment, you must comply with the glove use policy, Section VI (A), Lines 37-50. In addition, with the exception of catechol, these chemicals don't "like" to dissolve in water. Ask your instructor for assistance with this.**

Chop the potato into small cubes and place in the blender. Add 300 mL distilled water and pulse the blender until the potato is blended in the water. Set up the Buchner funnel with filter paper and attach to the aspirator on the sink. Turn the water on as high as it will go and filter the potato extract. You want to save the filtrate (the liquid in the filter flask) for your assay. The potato pulp and filter paper may be discarded in the trash.

The PPO extract and all tubes are now ready to be taken to the spectrophotometer for the assay.

Make sure the visible light source is on and that the spectrophotometer is set on absorbance. Set the spectrophotometer at 480 nm. Place a cuvet with water in it in the cuvet holder. Close the lid. Press the "Calb" button and wait until the LED reads "0.000".

Use the following protocol for Tube #1 for every tube – one at a time.

Using a disposable pipet, place 8 drops of your PPO extract in the tube. Fairly quickly, cover the tube with a piece of Parafilm ™ and invert to mix without shaking (shaking allows air to mix with the protein and denature it). Pour enough out of the test tube into a cuvet to fill the cuvet about two-thirds full. Place in the spectrophotometer and read as soon as the LED stabilizes the time zero value of the absorbance at 480 nm. Time the sample for 30 more seconds and read the Absorbance at 480 nm. Record both readings in the following data table. Remove the sample, place in an appropriate container, and repeat with each tube until done.

Your instructor/professor will assist you in disposal of reagents and mixed cuvets and test tubes.

Data Collection Table

				5 gtts 0.01M inhibitor		5 gtts 0.01M inhibitor		5 gtts 0.01M inhibitor	
		WITHOUT Inhibitor		WITH Tyr		WITH t-Cinnamic Acid		WITH PTU	
		A_{480} readings		A_{480} readings		A_{480} readings		A_{480} readings	
	[Catechol] M	30 sec	0 sec	30 sec	0 sec	30 sec	0 sec	30 sec	0 sec
Tube 1	0.000								
Tube 2	0.006								
Tube 3	0.012								
Tube 4	0.018								
Tube 5	0.024								
Tube 6	0.030								

Results and Data Manipulation

Remember that the calculation of the velocity of the reaction is as follows:

$$\boldsymbol{V = \frac{A_{480}^{30} - A_{480}^{0}}{0.5\ \text{min}} = \frac{\text{Change in Absorbance}}{\text{minute}} = \frac{\Delta A_{480}}{\text{min}}}$$

Once you have your date recorded, set up an Excel spreadsheet so that you can set up tables for $\frac{1}{V}$ and $\frac{1}{S}$ with and without inhibitors (remember to identify the inhibitor). Once you have those tables set up, use your data to construct the following curves/graphs/plots in Lineweaver-Burke format, i.e., for $\frac{1}{V}$ vs $\frac{1}{S}$: 1) enzyme only, 2) enzyme with tyr (you'll have 2 lines: one for unhibited enzyme and one for inhibited enzyme), 3) enzyme with PTU (you'll have 2 lines: one for unhibited enzyme and one for inhibited enzyme), 4) enzyme with trans-cinnamic acid (you'll have 2 lines: one for unhibited enzyme and one for inhibited enzyme). Using the curves (and remember, **no dot-to-dot** – linear trend line), determine the

kind of inhibition pattern, the K_M and the V_{max} for the four conditions. Using Excel, label each plot with the K_M and the V_{max}, as well as the equation for the line and the R^2 value for each line. Your plots will be uploaded into Canvas for grading.

Discussion

Potato polyphenol oxidase was chosen for this experiment because of the ease of acquisition of potatoes. The enzyme is also easily studied in the lab (with appropriate safety precautions) without requiring any expensive equipment or sophisticated solutions, e.g., buffers set at specific pH's. PPO can be easily prepared simply in water and stored on ice just before use. One might note that as soon as the potato is cut, PPO is activated and the reaction begins in the blender and continues after filtration. It is this activation that storing on ice slows down.

Classically, V or V_{max} is not measured in "absorbance units per minute". For the purposes of this experiment, however, those units were chosen to simplify the experiment.

The function of catecholase (another name for PPO) is to provide quinone compounds that are used to produce melanins. Tyrosine is used to synthesize melanins in humans. Based upon both its structure and its human functions, one would expect that tyrosine might inhibit PPO. Based upon the results of previous experiments, it seems that PPO "sees" tyrosine as a noncompetitive inhibitor. Since non-competitive inhibitors inhibit in an allosteric manner, it seems reasonable that tyrosine regulates PPO activity by a negative feedback mechanism, i.e., to shut down the enzyme at a binding site not identical to the active site of the enzyme.

Trans-cinnamic acid tends to not strike one as a metabolite, i.e., tyrosine is something that PPO is continuously exposed to as a result of biological activity and the need for tyrosine in protein synthesis. Trans-cinnamic acid doesn't serve the same purpose, hence, from previous experiments that it is also a non-competitive inhibitor of PPO is somewhat surprising.

That the data from previous experiments doesn't conform as closely to Excel's linear regression as did the analyses of enzyme and enzyme-plus-tyrosine, suggests that technique may be a bit of an issue, i.e., consistency between sample preparation and analysis might could be rendered improved.

Knowing that all samples (short of adding enzyme) were prepared by the same person and that the only variables impacting analysis are enzyme addition, sample inversion and reading the spectrophotometer tend to suggest the latter contributing to the deviation away from the regression equation.

That PTU is a competitive inhibitor (based on these results) of PPO comes as a surprise. PPO contains copper(II) ions as an inorganic cofactor. The copper perpetuates a redox cycle between the Cu(II) and Cu(I) ions much as during collagen synthesis and catecholamine synthesis. PTU is well known to react with and bind to (chelate) the copper ion to inactivate PPO.

Typically, competitive inhibition occurs as a result of the inhibitor interacting with the enzyme's active site, competing with the substrate, blocking it out of binding in the pocket. Competitive inhibitor may be "swamped out" by the addition of substrate and the enzyme reactivated. This effect was not studied in

this experiment. It would be helpful to study the effect of PTU on the enzyme in varying concentrations of PTU to more clearly understand this inhibitive effect.

For further details and clarification of this experiment, including previous data (Excel) applications, see Potato Polyphenol Oxidase (PPO) Assay and Data Analysis.

Conclusions

Per previous experiments, trans-cinnamic acid and tyrosine are both non-competitive inhibitors of PPO. PTU is a competitive inhibitor of PPO – per previous experiments. Student results may not necessarily reflect previous results.

In the future, it would be of interest to explore the inhibitive properties of phenol and DOPA given their similar structures to either catechol or catechol derivatives.

This experiment is easily performed in the laboratory setting and consistent results may be obtained with minimal laboratory experience.

Acknowledgements

Without the good-natured participation of Erin, Marlo, Travis and Alvin (CHEM 220 Students, Spring 2005), this experiment would not have been completed or accomplished as efficiently or as effectively.

As a result of personal e-communications with JR Frey (28 Feb 2018, 1 Mar 2018 and 2 Mar 2018), Chemistry Education graduate student (Dr. Marcy Towns' lab at Purdue University; regarding this experiment being implemented at Purdue for their general chemistry course for agriculture and health science majors), recent research appears to suggest that one issue with PTU and PPO may very well be that *"… for the diphenolase activity[,] substrate inhibition was observed, which was apparently abolished by adding PTU. These observations lead to the hypothesis that a secondary, allosteric binding site exists, which binds dopamine and PTU and reduces the catalytic activity."* **[8]**. While this hypothesis remains to be tested, it, however, seems reasonable in light of the reported uncharacteristically inconsistent inhibition effects of PTU on PPO.

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9. References 1-5 accessed in early Spring 2005; links may or may not still work (3 March 2018).

This experiment designed/re-written 2 and 3 March 2018; final 15 March 2018, 0948 hours PST.
Updated 23 January 2021, 1440 hrs, PST.