# Iso-Orotate Decarboxylase:

# Purification, Isolation and the rate of the Enzymatic and Non-enzymatic reaction

by

Vincient R. Barnes Sr.

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In the

Chemistry

Program

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Iso-Orotate Decarboxylase:

Purification and Isolation and the Rte of the Enzymatic and Non-enzymatic reaction

Vincient R. Barnes Sr.

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## Thesis Abstract

*N.crassa* is known to utilize a unique salvage pathway for the production of uracil. This is known as the pyrimidine salvage pathway. This pathway consists of four enzymatic steps to convert thymidine to uracil. The final enzyme is isoorotate decarboxylase (IDCase), which forms uracil from isoorotate through a decarboxylation reaction. An in vitro assay for IDCase activity has been developed, which allowed the determination of specific activity of the enzyme in various strains of Neurospora. In addition attempts at isolating the gene as well as measuring the kinetic properties will be performed.

**Acknowledgements**

First and foremost I would like to acknowledge God and my mother Shurlie, the underlying source of my faith and strength. Secondly, my wife for her unwavering compassion and support as I pursued my academic dreams. My four children who are the key to my eternal youth, I thank you for understanding my dream. The professionals of YSU, I owe you my undying gratitude for all your help, support and understanding. The ongoing bonds that have formed will never be broken. To the professors who saw in an undergraduate the potential to go further, I thank you. To the professors who acted as a shield in times of despair a special thanks to Drs. Peter Norris and John Jackson for sharing their knowledge. I would also like to express my undying gratitude and appreciation to Dr. Jeffrey Smiley for the guidance, patience, faith and support he has provided me with throughout my tenure at YSU. Finally, I would like to express gratitude to everyone I have worked with in the lab. The JAS group 2001-2004 all my love and respect. Finally, to the Department of Chemistry and the School of Graduate Studies at YSU, thank you for allowing me this opportunity.

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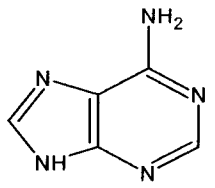
## List of Symbols and Abbreviations

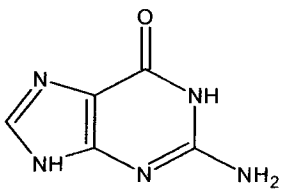
| | |
|---|---|
| α | alpha |
| β | beta |
| G | gram |
| L | liter |
| LCMS | liquid chromatography mass spectrometry |
| M | molarity |
| μ | micro- |
| MHz | megahertz |
| Min | minute |
| Mol | mole |
| N | nano- |
| Nm | nanometer |
| NMR | nuclear magnetic resonance |
| °C | degrees Centigrade |
| Ppm | parts per million |
| Rpm | revolutions per minute |
| TLC | thin layer chromatography |
| UV | ultraviolet |
| v/v | volume by volume |
| w/v | weight by volume |

## Chapter I: Introduction

Nucleotides are involved in almost every type of biochemical process and exist in two distinct forms: purines and pyrimidines. Adenine and guanine are classified as purines, heterocyclic compounds with fused rings; whereas cytosine, thymine and uracil are classified as pyrimidines a single-ringed nitrogen-containing molecule. In both DNA and RNA, cytosine consistently pairs with guanine; however this consistency does not apply to the pairing of adenine with thymine in DNA and adenine and uracil RNA (Figure 1-1, and 1-2). Specifically, in DNA adenine pairs with thymine, while in RNA adenine pairs with uracil. The purines and pyrimidines adenine, guanine, cytosine, thymine and uracil are the monomeric units of nucleic acids. Nucleotide triphosphates (NTPs), most commonly ATP, store energy via phosphodiester bonds, the release of which is used to propel thermodynamically unfavorable reactions. NTPs regulate many degradative and biosynthetic metabolic pathways. Finally, nucleotides are part of coenzymes, such as $NAD^+$, FAD, and others.

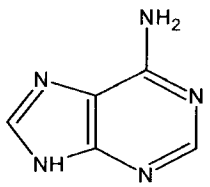



Adenine

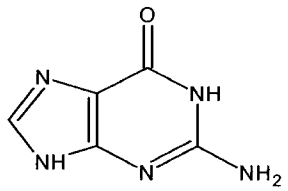



Guanine

Figure 1a. DNA bases

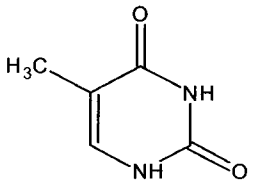



Adenine

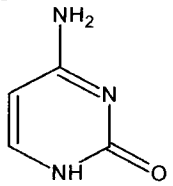



Guanine

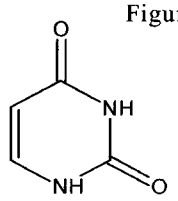
Figure 1b. RNA bases



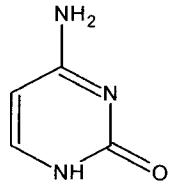

Thymine




Cytosine




Uracil




Cytosine

Figure1-1/1-2. DNA, RNA base pairing depicting the difference in base pairing.

The *de novo pyrimidine* biosynthetic pathway, through which pyrimidine ribonucleotide biosynthesis occurs, was first discovered in *Neourospora crassa* by Mary Ellen Jones.[1] She determined that strains of *N. crassa* which normally require cytosine and uracil grew normally with orotic acid as the sole pyrimidine source. The *de novo* pyrimidine nucleotide biosynthesis is a six step process (Figure 1-3). This is a channeled series of reactions, meaning intermediates are not released into the medium, but instead move directly from one enzyme to the next as a result of enzyme associations.[2] It starts with the cytosolic enzyme carbamoyl phosphate synthase II combining glutamine, water, and bicarbonate anion to produce carbamoyl phosphate. Two molecules of adenine triphosphate (ATP) are required for this reaction, one to provide a phosphate group, and the other to energize the reaction. Aspartate transcarbamoylase (ATCase) then catalyzes the addition of aspartate to the carbamoyl group, with loss of a phosphate. Next is a ring formation by the enzyme dihydroorotase, producing dihydroorotate, and loss of water. Oxidation by dihydroorotate dehydrogenase gives orotate, with quinone being reduced. Orotate phosphoribosyl transferase adds a ribose monophosphate group in the β configuration to form orotidine-5'-monophosphate (OMP). The final step is a decarboxylation by orotidine-5'-monophosphate decarboxylase (ODCase) to produce uridine-5'-monophosphate (UMP).

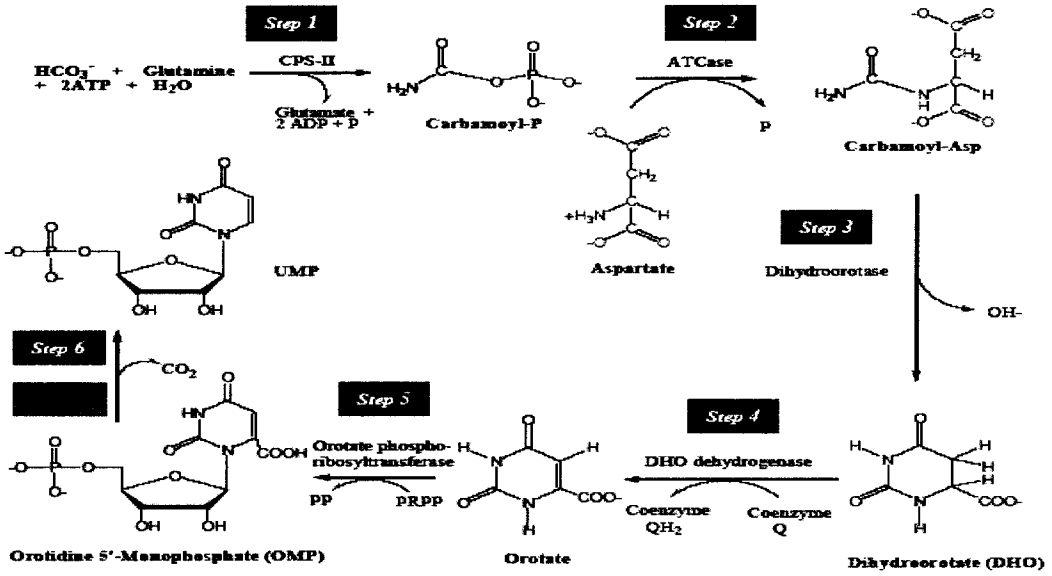



Figure 1-3. The *de novo pyrimidine* biosynthesis pathway

Regulation of the *de novo pyrimidine* pathway in bacteria normally occurs at ATCase with activation by ATP and inhibition by CTP. In animals, regulation occurs by activation of carbamoyl phosphate synthase II with ATP or PRPP, and inhibition with UDP or UTP.

Once UMP has been synthesized, it is converted to UDP. From there, it is converted to dNDP by a ribonucleotide reductase. There are three classes of ribonucleotide reductases, dependent on the type of organism. In keeping with the characteristics of the class, most reductases operate on diphosphate nucleotides. Types I and II are found in prokaryotic organisms. All eukaryotic organisms utilize the type I ribonucleotide reductase. Type III is found in anaerobic organisms living in $O_2$ depleted environments. All three classes follow the same basic mechanism: a free radical replacement of OH at the 2` position by an H Atom.

For incorporation into DNA, Uridine nucleotides must be converted to dTTP (Figure 1-4).

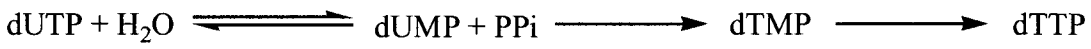



Figure 1-4. Conversion of deoxyuridine tripohsphate to deoxythymidine triphosphate.

The reaction starts with didephosphorylation to dUMP by dUTP diphosphohydrolase. This seemingly wasteful process occurs to minimize the amount of dUTP, so that uracil is not incorporated into DNA. Methylation of dUMP to dTMP is catalyzed by thymidylate synthase, with $N^5$, $N^{10}$ methylenetetrahydrofolate as a methylene donor (Figure 1-5). The reaction starts with an enzymatic nucleophilic attack at C6 of dUMP, followed by C5 attack of the methylene group. Loss of the proton at C5, then hydride transfer to the methylene group displaces the enzymatic nucleophile, terminating the reaction. This reaction is unique in that it is a reversible reaction. The reactants together can go from dUMP to dTMP, but they can not go in reverse as seen with many reactions.

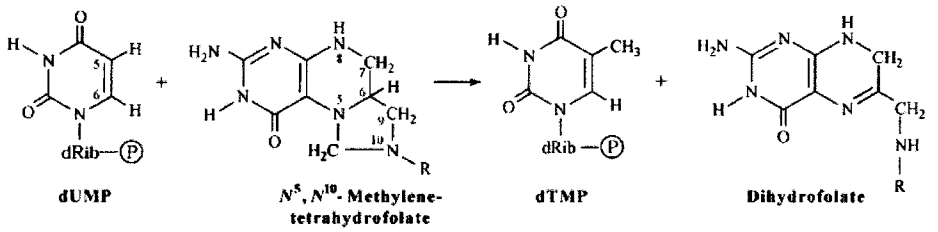



Figure 1-5. Uridine first reacts with ATP to become Uridine mono-phosphate. After this conversion, methylation occurs via a transfer from N5, N10-Methylen-tetrahydrfolate orchestrated by thymidylate synthase. The resulting product is dTMP.

The pyrimidine metabolic pathway is normally unidirectional. Thymidine ribonucleotides and ribonucleosides cannot be reused in most organisms. However, a pathway discovered by Palmatier *et al.* named the thymidine salvage pathway, converts thymidine to uracil in a four-step process.[3] The uracil can then be converted to UMP and reincorporated into the total pyrimidine pool. Their group discovered that, when labeled thymidine is incorporated into the system, the label appears in both RNA, and DNA, whereas the normal incorporation of thymidine is only in DNA.

The thymidine salvage pathway is found in only a few organisms: *Neurospora crassa, Aspergillus nidulans,* and *Rhodotorula glutinis*. The pathway, as described first by Smiley, begins with an oxidation of thymidine to thymine ribonucleoside[4] (Figure 1-6). Next is cleavage of the ribose, producing thymine and ribose. Oxidation of the thymine to give uracil-5-carboxylate (iso-orotate, IOA) is next, and finally decarboxylation at the 5 position to produce uracil.

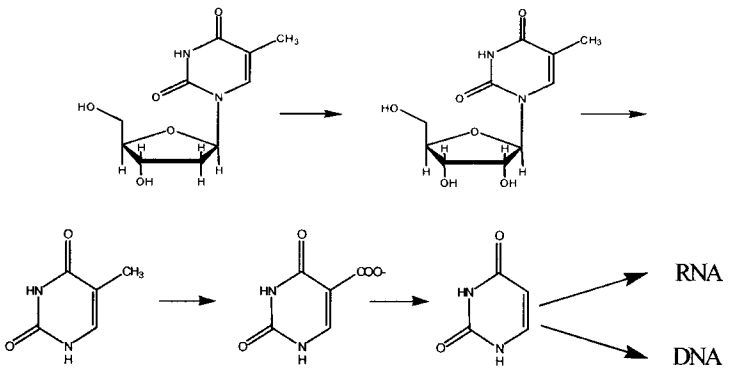



Figure 1- 6. The pathway as proposed by the Smiley Group.

Iso-orotate decarboxylase (IDCase) completes the conversion of thymidine to uracil in the unusual thymidine salvage pathway identified to date only in certain fungi.[5] This enzyme has been studied very little in the past, and therefore little is known about

it.[3, 4] A possible mechanism for the enzymatic decarboxylation of iso-orotate has been offered by Smiley *et al.*[4] (Figure 1-7). The proposed mechanism involves an attack by a nucleophilic residue in the enzyme at the 6 position of the iso-orotate, followed by the loss of carboxylate, leaving the enolate of uracil bound to the active site residue. Finally, the enzyme is removed from the substrate, leaving uracil.

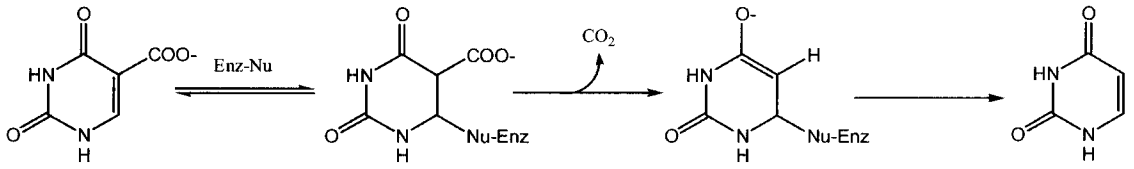



Figure 1-7. Proposed IDCase mechanism.

There are four main types of decarboxylation reactions: α-keto acid, β-keto acid, amino acids, and oxidative decarboxylations.[6] α-keto acid decarboxylation occurs in the presence of a metal ion. One example is pyruvate decarboxylase (Figure 1-8). This reaction requires both thiamine pyrophosphate (TPP) and a divalent metal ion. Anion formation occurs by addition to C2 of pyruvate, forming a covalent adduct, then decarboxylation occurs, where the TPP is protonated. The key of this reaction is the heterocyclic compound as an electron sink, to provide intermediate stabilization.

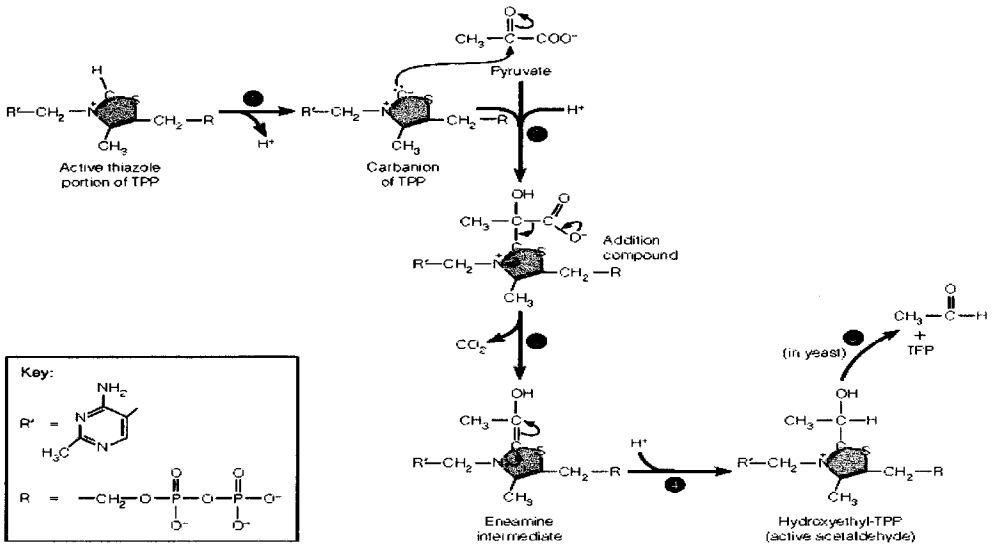



Figure 1-8. A depiction of the pyruvate decarboxylase reaction leading to the formation of Hydroxymethy-TPP and finally acetaldehyde and TPP. The key to this reaction is the thiamine compound used as an electron sink.

As with α-keto acid decarboxylases, β-keto acid decarboxylation reactions require a metal ion for catalysis. However, no other cofactors are needed. An α carboxyl and keto carbonyl are formed, with the metal stabilizing the enolate. Related to this reaction is oxidative decarboxylation. Two distinct steps occur in oxidative decarboxylation: oxidation to the ketone, followed by the decarboxylation of the β $CO_2$. Schiff base dependent β-keto acid decarboxylations rely on nitrogen stabilization of the negative charge formed after loss of $CO_2$ (Figure 1-9). The reaction requires no metals or other cofactors. It begins by covalent attachment of an amine group, then loss of water to form an imine. Loss of $CO_2$ occurs, then the loss of the amine to form the carbonyl product.

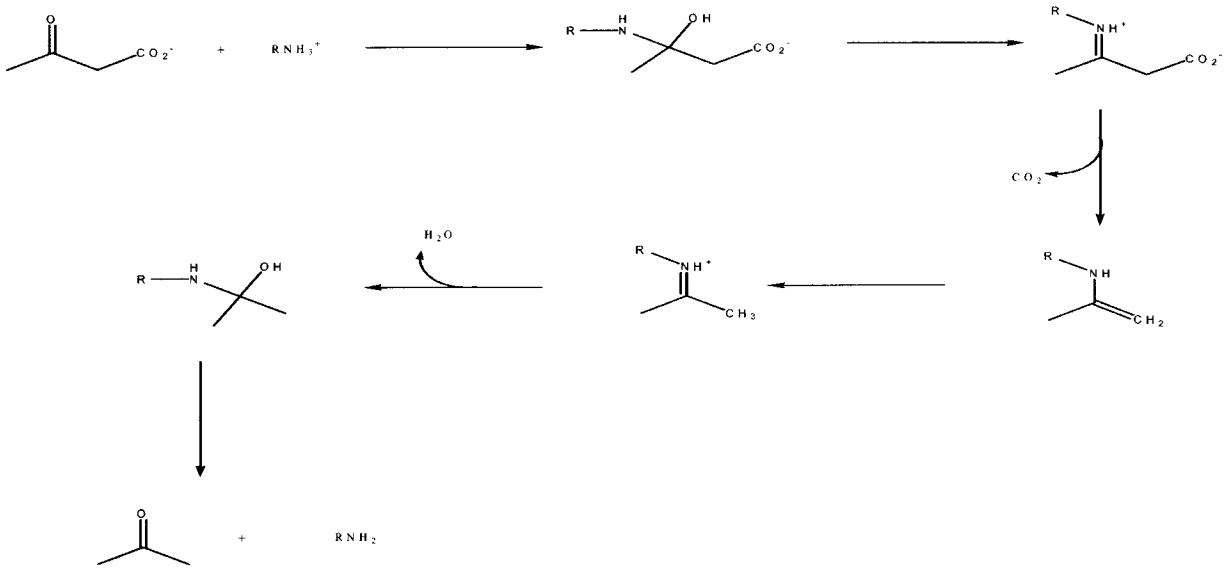


Figure 1-9. A depiction of the decarboxylation of a β-keto acid using a Schiff base as a form of stabilization.

Pyridoxyl-5`-phosphate is used in the decarboxylation of amino acids (Figure 1-10). As with β-keto acid decarboxylations, a Schiff base promotes the decarboxylation. The reaction begins with PLP attachment to the enzyme; binding the substrate with the Schiff base followed by loss of $CO_2$ and charge neutralization by the pyridinium nitrogen to form a stabile quinonoid intermediate. Reprotonation occurs, and the amine product is released.

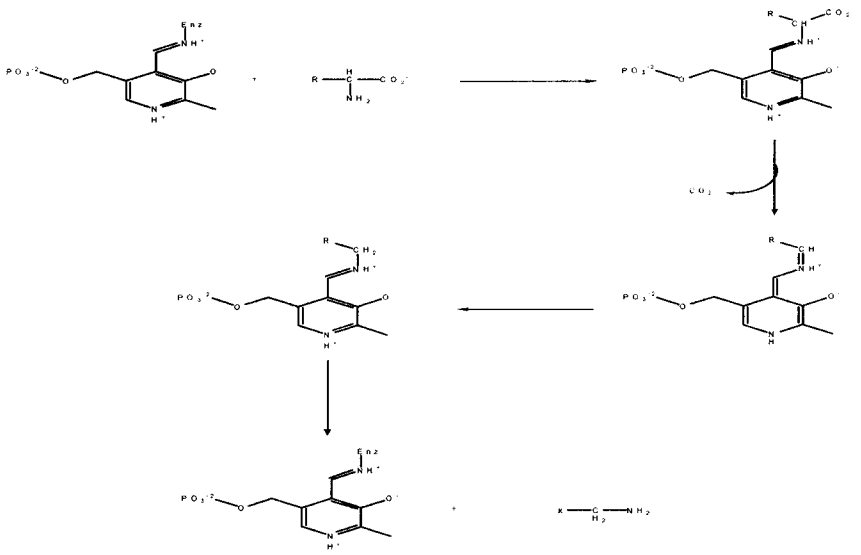


Figure 1-10. PLP/amino acid complex showing the decarboxylation, in the next step reprotonation occurs releasing the amine product.

Though it is not one of the types of decarboxylases discussed above, a possible mechanism for IDCase resembles a β-keto acid decarboxylation after a proposed conjugate addition. Several pyrimidine-metabolizing enzymes, such as thymidylate synthase, make this type of nucleophilic addition. (Figure 1-11) Therefore, this provides a unique and interesting enzyme to study.

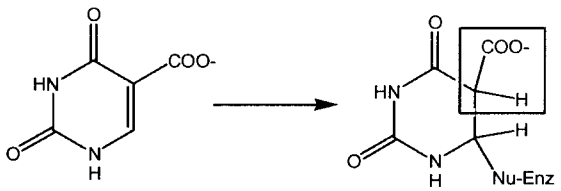



Figure 1-11. β-keto acid like mechanism of IDCase, after a conjugate addition.

The first published research of the IDCase enzyme was done by Palmatier *et al.*[3] The pathway in *N.crassa* was shown by [$^{14}$C]-labeled thymidine being incorporated into RNA, but not DNA. This led them to believe that thymidine, normally a precursor of DNA thymine nucleotides, undergoes a demethylation to uracil, a precursor to RNA nucleotides. They also described the procedures they used to show that IDCase is present in the cells, and determined from the results that an enzyme or pathway which converts thymidine to RNA pyrimidines is present in *N. crassa.*

In the research published by Smiley *et al.*, they describe two different assaying methods to determine the amount of IDCase activity, and also propose a mechanism for the thymidine salvage pathway.[4] One technique discussed involves the use of radioactive labeled [carboxy-$^{14}$C] IOA to assay for IDCase activity. The other technique spectrophotometrically records the enzymatic conversion of 2-thioIOA to 2-thiouracil.

The final research to be mentioned, done by Brody and Westheimer, deals with orotidine-5'-monophosphate decarboxylase (ODCase).[7] Their interest resided in the isolation of pure, intact, and active enzyme. To achieve their goal, they used a custom affinity chromatography column to purify the ODCase enzyme. To construct a custom affinity chromatography column, a spacer arm terminated by an amino group was attached to 6-azaUMP, a known inhibitor of the ODCase enzyme. This amino group could then form an amide bond with carboxymethyl agarose, thus creating an agarose chromatography resin decorated with covalently bound 6-azaUMP. The total protein slurry collected from yeast cells was allowed to pass over the column, which bound only ODCase. All other proteins flowed through the column unhindered. Utilization of this

method afforded the authors a 3200-fold purification after one pass over the column, and multiple columns yielded up to a 6700-fold purification of ODCase.

We will try an approach similar to that of Brody and Westheimer's for the purification of IDCase. 5-nitrouracil (5Nu) is known to inhibit IDCase[8], and may be a mechanism based inhibitor. We will attach 5Nu to activated agarose via a spacer arm, and a protein slurry containing IDCase will be passed over the agarose chromatography resin in an effort to separate the IDCase from unwanted proteins.

Another way to isolate IDCase is to isolate the gene responsible for encoding the enzyme. One way to effectively isolate the gene is by gene screening. By transforming a gene library of an organism which contains IDCase into an organism which does not contain the enzyme, and screening the transformed cells on a limiting media plate, individual colonies can be isolated and tested for insertion of the gene.

This screening process was earlier performed by Theisen, Kelln, and Neuhard.[9] Their goal was to isolate and characterize the *pyrF* gene, which is responsible for the encoding of ODCase in *Salmonella typhimcrium*. By transforming an ODCase deficient bacteria with plasmid containing the *pyrF* gene and plating the transformed bacteria on a selective media, only those bacterium which contained the *pyrF* gene were able to grow. They then determined that addition of the *pyrF* gene in plasmid form to the cells yielded a 15-20 fold increase in ODCase activity. Analysis of the plasmid harvested from the deficient bacteria grown on the selective media confirmed the plasmid contained the *pyrF* gene.

We intend to isolate the gene from *R. glutinis/N. crassa*. The sequence can then be converted to the plasmid form, and transformed into the desired vector of a bacterial

strain which is deficient in both IDCase and ODCase. Allowing the transformed cells to incubate on minimal media, with IOA as the sole pyrimidine source, only the bacteria which incorporate the plasmid containing the gene responsible for IDCase will be able to grow. We will then be able to isolate and gene sequence the plasmid. We will also be able to use the plasmid to overexpress IDCase, so that the protein can be isolated and further studied. With the purified protein studies into the mechanism and the enzyme's kinetic properties can be studied.

## Chapter II: Kinetics: Enzymatic and Non-enzymatic Reactions

### Introduction

Enzymes are extraordinarily efficient and selective biological catalysts that accelerate a chemical reaction towards the achievement of equilibrium. Chemical reactions in many metabolic pathways necessary for sustaining life would not proceed at sufficient rates without the presence of enzymes. Enzymatic reactions increase in rate $10^3$ to $10^{17}$ times faster than their corresponding uncatalyzed reactions. Enzymes are and have the function characteristics of globular proteins. This allows for the binding one or more substrate molecules. Substrates are bound in a hydrophobic cleft know as the active site. Enzymes whose activity is regulated generally have a more complex structure than unregulated enzymes. Most regulated enzymes have two binding sites, one for the substrate and the other for the regulator or inhibitor.

For many enzyme reactions the rate (V or d[P]/dt) varies with the substrate concentration [S] a shown by the studies of Lineweaver and Burke and then again by Michaelis and Menten. The rate (V or d[P]/dt) is defined as the number of moles converted to product per second which is measure of enzyme activity. At low substrate concentration the rate or velocity (V) is almost proportional to substrate [S] concentration. At high substrate [S] concentrations, the velocity is not linear with the [S] concentration and rate approaches a maximum velocity called $V_{max}$.

The model proposed by Michaelis and Menten is also used to explain the kinetics of an enzyme reaction. One of first great advances in biochemistry was the discovery that an enzyme transiently binds to a substrate to form an enzyme-substrate complex [ES].

The substrate binds non-covalently to the active site of the enzyme; this could be followed by subsequent covalent binding. The rate of an enzymatic reaction depends on the concentrations of both the substrate and the catalyst (enzyme). In the case where the amount of the enzyme is much less than the amount of the substrate in the reaction vessel, the reaction is considered to be pseudo first order. The plot of this type of reaction yields a straight line that illustrates the effect of enzyme concentration on reaction velocity in a pseudo first-order reaction (Figure 2-1). The more enzyme present, the faster the reaction. Pseudo first-order conditions are used in analyses that determine the concentration of an enzyme in a sample. The concentration of enzyme can be easily determined by comparing its activity to a pre determined reference curve.

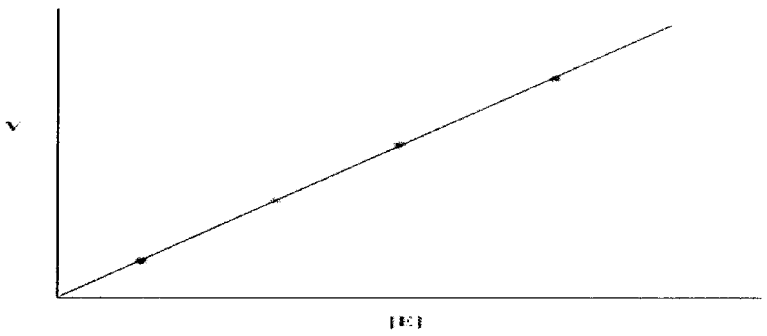


Figure 2-1. The plot is the reaction velocity v verses the concentration of the enzyme.

At low [S] the velocity is directly proportional to the substrate concentration. At high substrate concentration, when [S] is much greater than $K_m$; The velocity (v) is the maximal rate ($V_{max}$) and independent of the substrate concentration which depends on the total enzyme concentration, [E], and rate constant $k_3$. The meaning of $K_m$ is evident from the Michaelis-Menten equation. When [S] = $K_m$, then $v=V_{max}/2$. Thus $K_m$ is equal to the substrate concentration at which the reaction rate is half of its maximal value. The Michaelis constant, $K_m$, and the maximal rate, $V_{max}$, can be derived from the rates of catalysis measured at different substrate concentrations if an enzyme operates according to the simple scheme give in the Michaelis Menten equation. It is convenient to transform the Michaelis-Menten equation into one that gives straight line plot. This can be done by taking the reciprocal of both sides of the equation to give a plot of 1/[S] versus 1/V.

$$\mathbf{V = (V_{max} * [S]) / (K_m + [S] \text{ and } 1/v = (K_m + [S] / V_{max} * [S]}$$

$$\mathbf{= (K_m/V_{max}) * 1/[S] + 1/V_{max}}$$

This plot, dubbed the Lineweaver-Burk plot, yields a straight line with an intercept of $1/V_{max}$ and slope of $K_m/V_{max}$, the intercept on the vertical axis is $1/V_{max}$, and the intercept on the horizontal axis is $-1/K_m$.

$K_m$ and $V_{max}$ for an enzyme-catalyzed reaction can be measured in several ways. Both values can be obtained by analysis of initial velocities at a series of substrate concentrations and a fixed concentration for the enzyme. In order to obtain reliable values for the kinetic constants, the [S] point must be spread out both below and above $K_m$ to produce a hyperbola. It is difficult to determine either $K_m$ or $V_{max}$ directly from a graph of

initial velocity versus concentration because the curve approaches $V_{max.}$ However, using a suitable computer program, accurate values can be determined by fitting the experimental results to the equation for the hyperbola. The Michaelis-Menten equation can be rewritten in order to obtain values for $V_{max}$ and $K_m$ from straight lines on graphs. The most commonly used transformation is the double-reciprocal Lineweaver-Burk plot. Values of $K_m$ can be determined even when enzymes have not been purified, provided that only one enzyme in the impure preparation can catalyze the observed reaction. At high substrate concentration, when [S] is much greater than $K_m$ than $V = V_{max}$ that is the rate is maximal, independent of substrate concentration. When $V=V_{max}$ then [S]/ ([S] + $K_m$) approaches 1. When $[S] = K_m$ then $V = V_{max}/2$. Thus $K_m$ is equal to the substrate concentration at which the reaction rate is half of its maximal value. When the [S] is less than $K_m$ then the $V=V_{max}$ $[S]/K_m$ and the substrate concentration is proportional to the velocity.

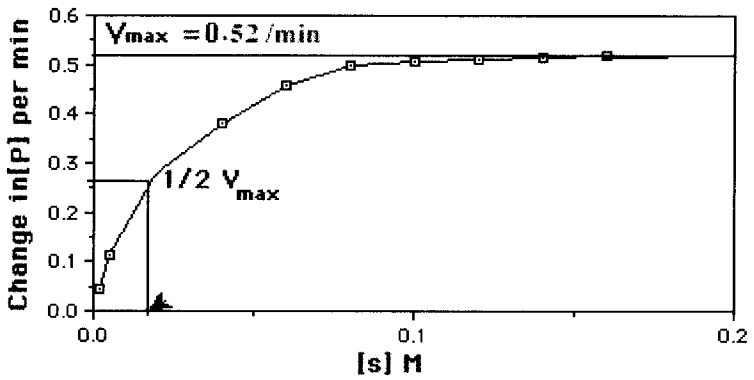



Figure 2-2. This figure is a representation of the saturation plot used to attain values for [S] and V.

The significance of $K_m$ and $V_{max}$ values:

The $K_m$ values of enzymes range widely (0.4 μM for Arginine-tRNA synthetase to 5,000 μM for chymotrypsin). For most enzymes $K_m$ lies between $10^{-1}$ and $10^{-7}$ M. The $K_m$ value for am enzyme depends on the particular substrate and also on environmental conditions such as pH, temperature, and ionic strength. The Michaelis constant, $K_m$ has two meanings. First, $K_m$ is the concentration of substrate at which half the active sites are filled. Once the $K_m$ is known, the fraction of sites filled at any substrate concentration can be calculated from the following equation:

$$f_{ES} = \frac{V}{V_{max}} = \frac{[S]}{[S] + K_m}$$

$f_{ES}$ **Fraction of site filled at any substrate concentration**

Kinetic perfection in Enzymatic Catalysis (The $k_{cat}$ /$K_m$) When the substrate concentration is much greater than $K_m$, the rate of catalysis is equal to $k_{cat}$, the turnover number. However, most enzymes are not normally saturated with substrate. Under physiological conditions, the $[S]/K_m$ ratio is typically between 0.01 and 1.0 or when $[S] << K_m$, the enzymatic rate is much less than $k_{cat}$ because most of the active sites are unoccupied. When $[S] << K_m$, the concentration of free enzyme, [E], is nearly equal to the total concentration of enzyme $[E_T]$, and so: $V=k_{cat}/K_m([S][E_T]$

Thus the ultimate limit on the value of $k_{cat}/K_m$ is set by $k_1$, the rate of formation of the ES complex. This rate cannot be faster than the diffusion-controlled encounter of an enzyme and its substrate. Diffusion limits the value of $k_1$ so that it cannot be higher than between $10^8$ and $10^9$ $M^{-1}S^{-1}$. Hence, the upper limit on $k_{cat}/K_m$ is between $10^8$ and $10^9$ $M^{-1}S^{-1}$.

This restriction also pertains to enzymes having more complex reaction pathways. Their maximal catalytic rate when substrate is saturating, denoted by $k_{cat}$ depends on several rate constants rather than on $k_{cat}$ alone. The pertinent parameter for these enzymes is $k_{cat}/K_m$. In fact, the $k_{cat}/K_m$ ratios of the enzymes acetycholinesterase, carbonic anhydrase, and trisephophate isomerase are between $10^8$ and $10^9$ $M^{-1}$ $S^{-1}$, which shows that they have attained kinetic perfection. Their catalytic velocity is restricted only by the rate at which they encounter substrate in the solution. Any further gain in catalytic rate can come only by decreasing the time for diffusion. Indeed, some series of enzymes are associated into organized assemblies so that the product of one enzyme is very rapidly found by the next enzyme. In effect, products are channeled from one enzyme to the next, much as in an assembly line.

The constants $k_{cat}$ and $K_m$ are useful for comparing the activities of different enzymes. It is also possible to assess the efficiency of an enzyme by measuring the rate acceleration that it provides. This value is the ratio of the rate constant for a reaction in the presence of the enzyme ($k_{cat}$) divided by the rate constant for the same reaction in the absence of enzyme ($k_{non}$). Surprisingly few rate acceleration values are known because most cellular reactions occur extremely slowly in the absence of enzymes.

For full understanding of the decarboxylation of IDCase, it is important to understand both aspects of the decarboxylation reaction being studied. Knowledge of the kinetics of both the non-enzymatic decarboxylation of iso-orotate to uracil as well as the enzyme assisted reaction will aid in the understanding of the total process and lend to the eventual understanding of the mechanism. In addition the studies involving the use of

isotope labeled substrates may also aid in uncovering this unique mechanism. The mechanism is believed to be dependant upon the pH of the environment. (Figure 2-3) If the nature of the environment is highly acidic then the $CO_2$ occupying the fifth position of the 5-carboxy-uracil will be protonated and therefore not a good leaving group. Likewise if the conditions are too basic the nitrogen at position 1 is in an ionic state and the molecule is not suitable for nucleophilic attack at C-6. When the conditions are right and the p OH is in the proper range, then nucleophilic attack could begin at C-6 and start the decarboxylation reaction. The start of the reaction is thought to proceed through the formation of an enolate double bond between C4-C5 of the molecule. Protonation at C5 would occur next, followed by first the loss of $CO_2$ (decarboxylation) and then the nucleophile at the C-6 position.

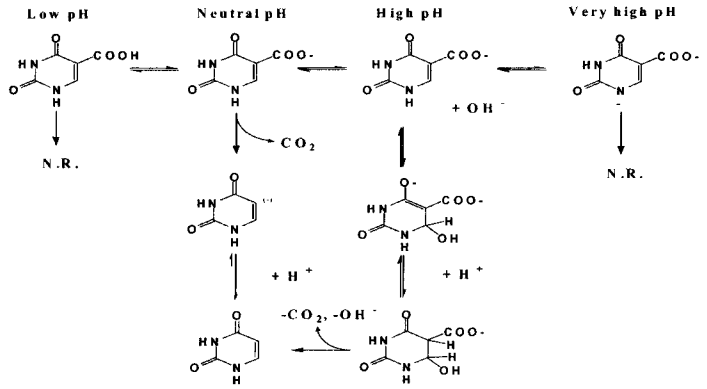



Figure 2-3. The proposed conditions for the mechanism of the non-enzymatic decarboxylation of IOA.

To test the kinetic efficiency and the proposed mechanism described above various experiments have been planned. Due to the slow spontaneous conversion rate of the non-enzymatic reaction all sample reactions were run at 100°C.

**Materials/Methods**

A stock solution of 50 mM Iso-orotic acid was prepared with unpurified iso-orotic acid from Sigma. The 50 mM stock solution then was diluted 10-fold to yield 5 mM reaction solutions. The reactions were run in Ace Glass pressure tubes at 100 °C under varying pH conditions. IOA (0.390 g, 0.05 mol) is dissolved in 50 ml of 500 mM $KH_2PO_4$ / $Na_3BO_3$ and adjusted to desired pH's of 4.0, 5.4, 6.0, 7.2, 8.2, 9.0, 10.2, 10.6 11.0 and 13.2. 3.0 mL solution was placed in a pressure tube and heated at 100°C. Periodic checks are made removing small aliquots for testing. Each time the reaction mix was removed from the oil bath and placed in cold water and methanol the time was stopped for that point. The total reaction time was calculated by adding the time trials together excluding the amount of time subtracted due to the cooling process. The 5.0 mM reaction solution was buffered with either 50 mM potassium phosphate or sodium borate buffer to minimize pH changes through the reaction's progression. The pH of the buffered iso-orotic acid solution was then adjusted to select values with concentrated NaOH. The initial aqueous reactions were run at varying pH's while the temperature was held constant. The preparation of the standard curve was achieved via the use of UV spectroscopy and the HPLC. A sample of 78 mg of uracil is dissolved in water and NaOH and the absorbance is measure at 260 nm. Using the extinction coefficient and Beer's law the absorbance of the sample is then converted to a corresponding concentration. With a known concentration of the sample it is then run on the HPLC to check retention time and the area of the peak. This peak area is directly correlated to the amount of the uracil injected. The injection size ranges from a 5 µl to 50 µl injection. The values for the peaks area are then plotted on a graph and a linear relationship is taken (Graph 2-1). The equation for

the slope of the line is then used in subsequent steps to give the remaining molar amount of IOA in the reaction vessels as well as the molar amount that has converted to uracil by way of C5 decarboxylation.

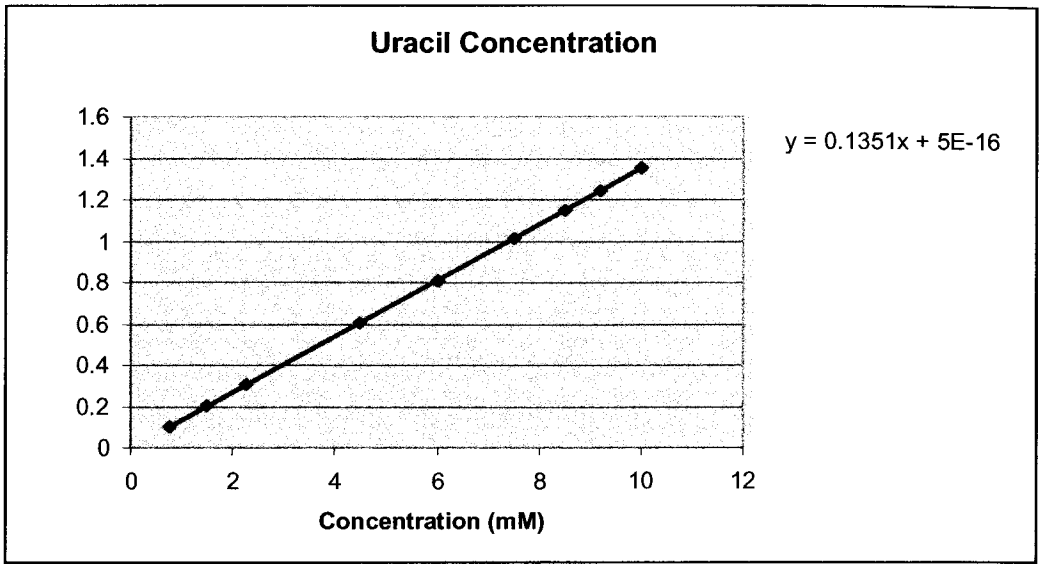
**Results/Discussion**

Progress of the reactions was checked using the Waters HPLC system and the Breeze software. Using the Isocratic Pumping method, a flow rate of 1 ml/minute and a 10 µl injection the peaks with in a certain range for IOA and Uracil were used. Generally the first peak with a retention time of 1.5-1.8 minutes indicated the IOA in the injection. The second peak with a retention time of about 2.5-2.8 minutes indicated the uracil formed during the reaction. Comparing the area of the peaks of the uracil against the values from the previously determined concentration curve the concentration of uracil formed from the heated reaction can be determined. The concentration of the uracil formed gives a remaining molar concentration for IOA, and this subtracted from the starting concentration give the total amount of:

1. Uracil formed in the reaction and
2. The amount of IOA remaining in the original reaction vial.

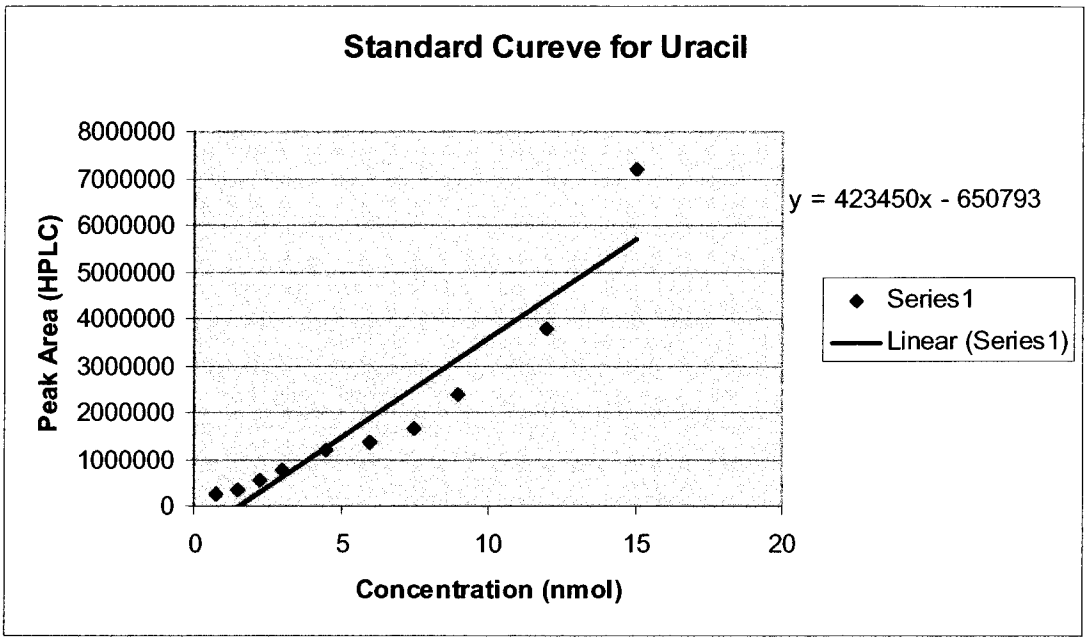
By plotting the natural log of the remaining IOA concentration against the reaction time in seconds yields the rate constant of a reaction *(k)*.

Abs (nm)

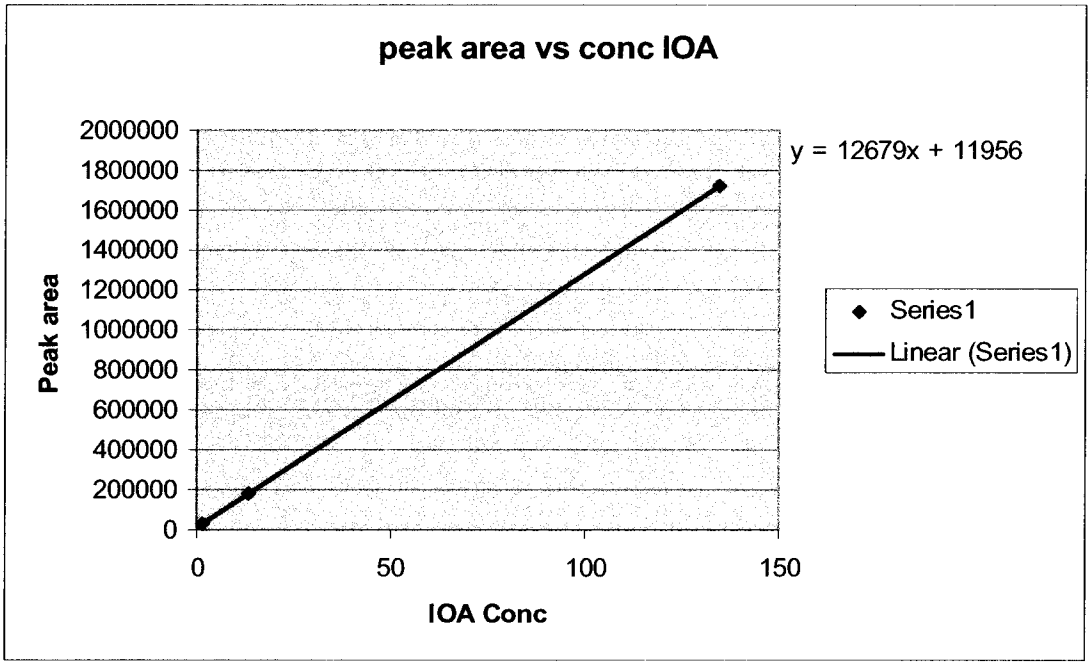



Graph 2-1, 2-2 and 2-3. The graphs constructed to convert the absorbance to concentration for uracil conversion. This is accomplished using Beer's law and the corresponding extinction coefficients for the two products.

Using the data above, the concentration curves for known amounts of uracil and IOA where constructed using the HPLC peak area that corresponds to the concentration injected and then by using Excel to yield the equation of the line we were able to measure the amount of protein converted in each reaction vessel.




Graph 2-2. Standard curve for concentration of uracil injected. The equation is used throughout the non-enzymatic reaction to calculate the number of nmols of uracil converted for each time point.

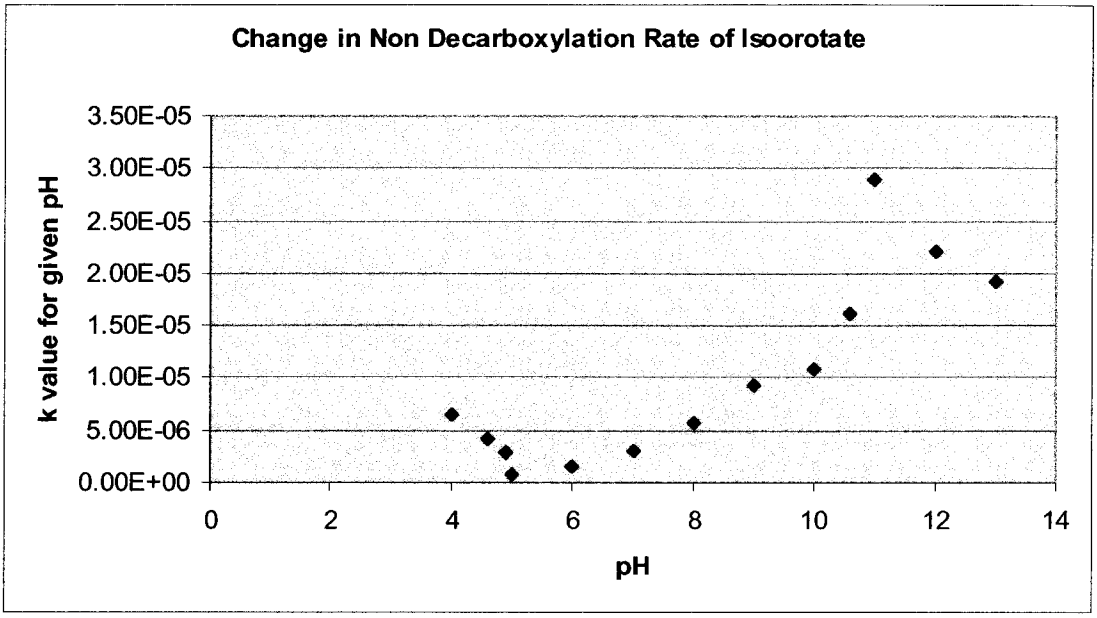



Graph 2-3 Standard curve for IOA.

With the concentration curves in place the data gathered from the various experiments were plotted to yield points in the scope of the proposed mechanism. Each point on the graph is the corresponding *k* value for that particular pH tested. In keeping to the proposed mechanism we find that between the range of 5 and 13 the mechanism appears to hold true. The rate of the reaction increases at a steady rate from pH 5 up to pH 11. Past the pH of 11 the rate starts to decline. The sample tested at pH 4 however gave unexpected results that require further study. Graph 2-4. Graph of *k* values from the non-enzymatic reaction.

Table 2-1. The data in this table was used to compile and compare the different k values obtained from the non-enzymatic reaction. It is evident from the graph and the table that at low pHs below a pH of 5 a different mechanism may be at work.

| pH of the Sample | Calculated k value |
|---|---|
| 4 | 6.5500E-06 |
| 4.5 | 4.1300E-06 |
| 4.8 | 2.8400E-06 |
| 5 | 8.0700E-07 |
| 6 | 1.4900E-06 |
| 7 | 3.1000E-06 |
| 8 | 5.7100E-06 |
| 9 | 9.2500E-06 |
| 10 | 1.0800E-05 |
| 10.6 | 1.6200E-05 |
| 11 | 2.8900E-05 |
| 12 | 2.2000E-05 |
| 13 | 1.9300E-05 |




Graph 2-4. Graph of the combined k values from the non-enzymatic reaction. (For the full table containing the data necessary to yield this plot see Appendix A.)

From the graph above it is evident that the reaction occurs with the aid of a nucleophile. As the concentration of this nucleophile (OH) increases so then does the value of k or the rate of conversion from IOA to uracil. The points, representative of the pH's above 11.02, show a decrease in rate and this can be attributed to the change in the state of the IOA molecule. As proposed the presence of excessive OH ion causes the deprotonated specie of IOA and this form does not favor decarboxylation.

## Synthesis of the Deuterated Iso-Orotate Substrate

### Introduction

The effects of isotopes can play a role in determining the mechanistic aspects of a reaction. There are three types of isotope effects commonly employed in the study of enzymes. These are primary, secondary and solvent. A primary isotope effect results from the cleavage of a bond to the substituted atom. Secondary isotope effects result from cleavage of bonds adjacent to the isotope substituted atom. Solvent KIE's or *Kinetic Isotope Effects* are obtained from the comparison of the reaction rate in $H_2O$ with that in $D_2O$.

Commonly used isotopes are D, T, $^{15}N$, $^{18}O$. The various isotopes of an element have slightly different chemical and physical properties because of their mass differences. Under the proper circumstances, such differences can manifest themselves as a *mass-dependent* isotope fractionation effect. Nuclear interactions, on the other hand, lead to a *non-mass-dependent* effect in the sense that they depend on the nuclear structure, rather than on the weight difference per se. In the first case, for example, the properties of molecules with $^{17}O$ will be intermediate between those of molecules with $^{16}O$ and $^{18}O$; this is not necessarily the case for the non-mass-dependent effects. For elements of low atomic numbers, these mass differences are large enough for many physical, chemical, and biological processes or reactions to *fractionate* or change the relative proportions of different isotopes of the same element in various compounds. As a result of fractionation

processes, waters and solutes often develop unique isotopic compositions (ratios of heavy to light isotopes) that may be indicative of their source or the process that formed them.

Two main types of phenomena produce isotopic fractionations: isotopic exchange and kinetic process. It is this kinetic process and its effects that we intend to study. The effects of isotopes on the kinetics of a reaction depend on the size of the isotope and the type of reaction it is involved in. If the reaction proceeds through nuclear interactions the binding effects are changed and it is this change that can be measured.

The use of isotope labeling for enzyme kinetics is widely used and simple to employ. The use of the isotope labeling method will in part assist in the determination of the mechanism used by the protein. The typical effect of the deuterium isotope is about 1.5. This means that by adding the deuterium in such a way as to ensure that the proposed mechanism has to operate through the labeled bond and there by producing a change in the rate of the reaction. If the nucleophile proceeds as proposed then the deuterium should slow down the rate of the reaction. If the rate is unchanged by the isotope addition, then the deuterium affect would not aid in the determination of the mechanism.

**Materials and Methods**

Uracil was suspended in a solution of $D_2O$, NaOD, DMSO, and held at room temperature for two days. The uracil is then dried and redisolved in $D_20$ and DMSO and allowed to react for one week in an oil bath at 58°C. The purified product of this reaction was deuterated uracil and was confirmed through proton and carbon NMR. The second step of the synthesis was the addition of a hydroxy-methyl group to position five of the pyrimidine ring. This reaction was carried out in 27% w/v formaldehyde, deionized water

and 2 M KOH. The mixture is allowed to react for six days (*Smiley)*[4]. The transformation of the 5-hydroxymethyluracil to iso-orotic acid was carried out using Pt-catalyzed oxidation. To begin the reaction 2.5 g of the $PtO_4$ catalyst is added to 5ml of water and under an atmosphere of hydrogen gas at 3.0atm the mixture is shaken using a Parr apparatus. After the one hour activation time the mixture is removed from the apparatus and molecular oxygen is slowly bubbled through for thirty minutes [4]. Then the previously synthesized HMU is added. Using the Parr apparatus the resulting solution is allowed to mix and react further under atmospheric $O_2$ for 24 hours. To complete the reaction the pH of the solution is raised and gravity filtered to separate the IOA from the Pt catalyst. The pH, after separation is again lowered, and the resulting precipitation is collected.

**Results:**

The conversion of uracil to deuterated uracil at positions five and six yields nearly a 100% conversion of reactants to products. TLC attempts show both starting material and product with the same $R_f$, therefore it is necessary to exploit the differences in the product and starting material by mass spectrometry and NMR. The distinguishing factor on the $^{13}$CNMR is the presence of triplets appearing at positions five and six. The need for this step was eliminated by the purchase of uracil 4-d from CDN Isotopes. Step two of the conversion to hydroxyl-methyluracil goes to completion in six days with a yield of about 77%. Step three in the synthesis of iso-orotic acid-6-d is the oxidation of the hydroxyl-methyl group now attached to the C5 position of the molecule. This is achieved

at about an 87% percent yield and verified by the use of TLC, NMR and mass spectrometry. The conversion from HMU to IOA reaches competition after 36 hours, the need to maintain 100% deuterium labeling at C6 has been the elusive portion of the synthesis. Further studies and attempts may include the use of $D_2O$ in place of $dH_2O$ during the platinum catalysis step of the reaction to ensure the deuterium label is not lost.

## Enzymatic Reaction: $K_m$ and $V_{max}$

### Materials and Methods

The method for assaying this protein was set to follow the design as illustrated by the enzyme kinetics standards and the equations of Michaelis-Menten and Lineweaver-Burke. The first of these assays, all of which are set at 1ml volumes, were used to determine the amount of substrate needed. The substrate used would have to be at a concentration that would yield cpms sufficient for measurements, without the substrate being totally consumed by the enzyme. With the [S] value determined the next series of assays were employed to test for $K_m$ and $V_{max}$.

The reaction parameters used to determine $K_m$ and $V_{max}$ were set at concentrations of 100, 50, 25, 16.7, 12.5 and 10 mM, using the same buffering system with a pH range from 5 to 10. (The composition of the assays are listed in appendix C.) With the conditions in place the procedure follows that of the earlier mentioned $^{14}C$ assaying standards [4]. The difference in the two assays would be total volume of the reaction mix. In the assay where crude protein from the expression system is used the volume is adjusted to 1ml for the purpose of easy substrate concentration adjustments.

**Results and Discussion**

In unpublished work from this lab, the IDCase gene was identified and transferred to an *E. coli* expression system. The protein taken from the *E. coli* containing the IDCase gene was used for the assays of IDCase activity. The protein concentration was calculated by Bradford assays and found to be at a concentration of 16.844 μg/ml. The protein although in a crude state could be used as purified protein is not necessary to determine the $K_m$ and $V_{max.}$ The procedure used to assay the protein and measure the activity is similar to the technique described by Smiley et al. using $^{14}C$ labeled IOA. In order to determine the amount of protein needed in the assay, various concentrations were tested to verify activity and ensure the assays would yield measurable counts with out complete consumption of the substrate. With the concentration determined the assays were performed in order to determine the Km and Vmax. For each 1ml assay a phosphate buffer of the desired pH was used in a concentration of 10:1 buffer to protein. The protein was diluted 1:100 in dialysis buffer containing 50mM Tris, 10% Glycerol and 5uM BME. Added to reaction mix containing the substrate in concentrations of 100, 50, 25, 16.7, 12.5, and 10 mM were the phosphate buffer of the desired pH for that particular assay, and the corresponding amount of water to ensure volume. (For complete assay conditions see the appendices) The assay time of five minutes was used and at intervals of: 15 seconds the protein is added to the various preassembled reaction mixture and capped using a stopper with a wick moistened with KOH. The reaction vials are allowed to mix in a counter top shaker for five minutes. At the end of the five minutes the reactions are

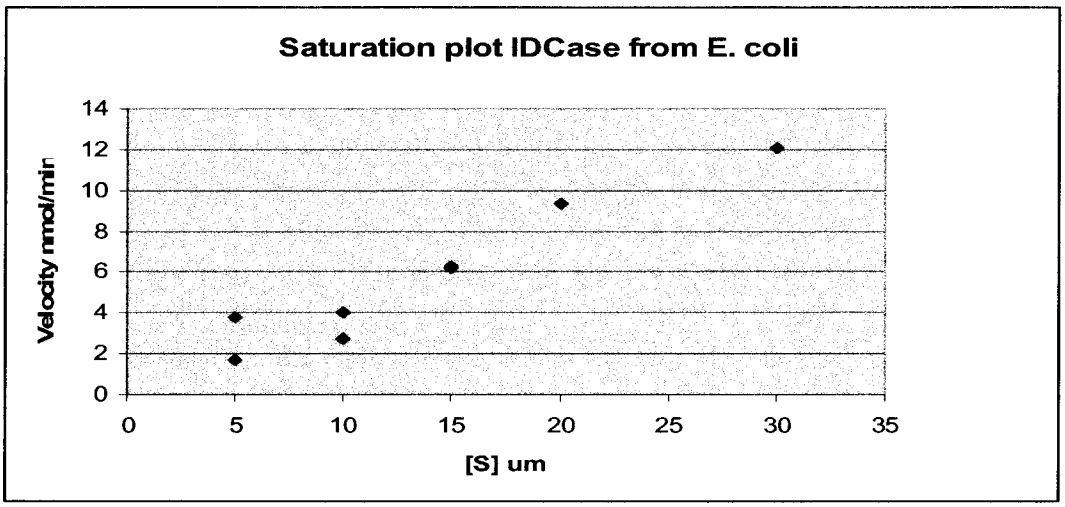
quenched with 0.2 mL of HCl. The addition of the acid denatures the protein causing the release of the $^{14}C$ labeled $CO_2$ that is then trapped on

the wick. After about an hour the wicks are placed on a glass pan and dried in an oven. After drying the wicks were placed in vials containing Scintisafe liquid. These vials are then placed in the scintillating counter and counted for 10 minutes each. The background from the fluid and the counter are added together, averaged out and the final number represents the counts per minute of the $^{14}C$ labeled $CO_2$ being released from the wicks. It is these numbers that are used to measure the activity.

The data gathered from the testing of the IDCase protein were similar to those published by Smiley *et al.* The $K_m$ measured in their study was 32 +/- 7 μM. This data was comparable to the $K_m$ of 40 μM data attained from the testing of the IDCase from the *E. coli* at pH 7.5. The data from the determination of the proper [S] showed that 10 μL addition of a 1:10 dilution of the crude protein sample was sufficient to yield counts that were measurable and would not be too much protein to the point of maxing out or totally consuming the substrate. The saturation plot is an indicator of the number and time needed for a portion of the active sites of an enzyme to be filled or come in contact with the substrate. With this data it is possible to deduce the number of active sites occupied by substrate at a given time point. As the active sites are filled, the graph takes on a tapered appearance near the level were the most amounts of substrate have been added. This tapering has a lot to do with the diffusion principles and how fast the [ES] complex can form. These limits are set and can not be overcome with the addition of more substrate.

| [S] | V (cpm/nml |
|---|---|
| 5 | 3.8 |
| 5 | 1.7 |
| 10 | 2.7 |
| 10 | 4.0 |
| 15 | 6.2 |
| 15 | 6.3 |
| 20 | 0 |
| 20 | 9.3 |
| 30 | 12.1 |
| 30 | 12.1 |
| | |

Table 2-2. The data from the determination of the proper [S] showed that a 10µl addition of a 1:10 dilution of the crude protein sample was efficient enough to give counts that were measurable and would not be overly efficient to the point of maxing out or totally consuming the substrate.

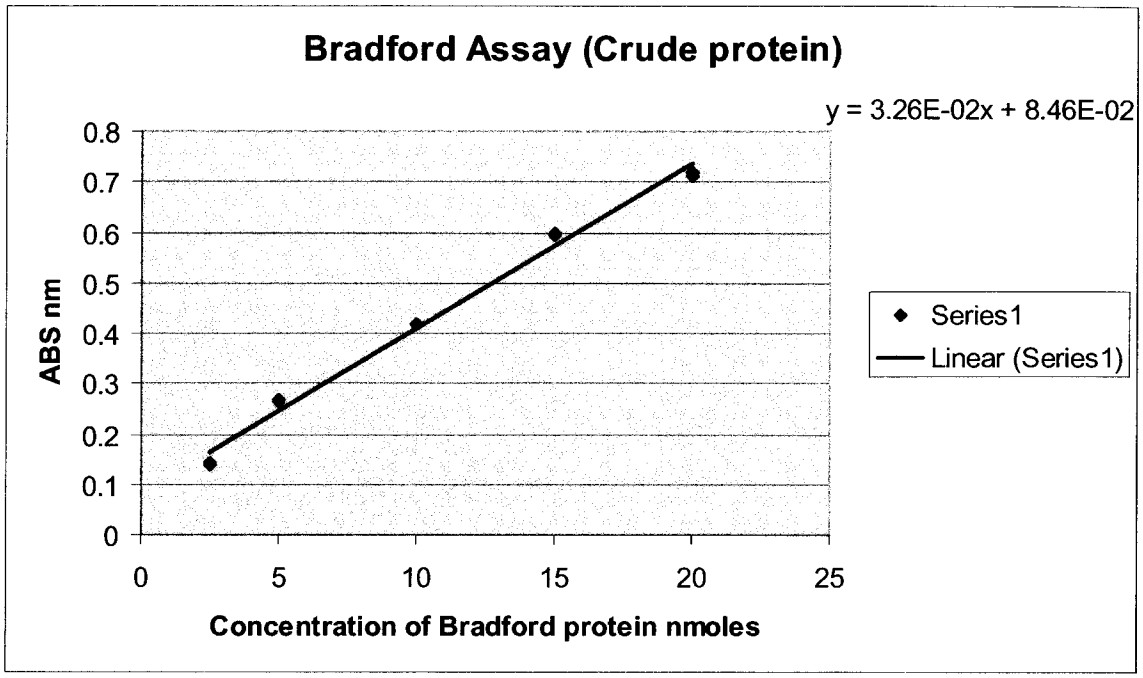



Graph 2-5. The graph of the saturation plot formulated from the table 2-1. The data plots represent the velocity of the conversion of IOA to uracil by the IDCase enzyme. Each point refers to a set concentration of enzyme and substrate. From this graph the number

of active sites occupied at a given time can be measured. The slight tapering of the end points are indicative of the enzyme reaching its maximal velocity.

With this data at hand the next step in the process was to run the series of assays at the different pH levels to test and compare the enzymatic efficiency and the optimal pH range at which the enzyme would function. From the data presented in Graph 2-6 we find the optimal range to be between the ph of 5.5 and 9.5.The values calculated for the $K_m$. Under the conditions of the assay the resulting Km is in the range calculated by Smiley *et al.* The experiment itself was geared to find the upper and lower limits of the enzyme and for the most part it fit the predicted scheme, but at ph points above 10 and below 5.5 the data lacked the plotting consistency to be used. This barrier could have been overcome by the modification of the $^{14}C$ IOA concentration in the substrate mix to make the assay "Hotter" and the shortening of the reaction time for example, instead of 5 minute assays only allow 1 minute reaction times. As time restraints are a big issue it was safe to say that using the same assay method and procedures, the enzyme is inactive at pH's below 5.5 and above 10.

The IDCase protein from the *E. coli* expression system, used in the assays had a Bradford assay absorbance of 0.6336 and this corresponds to a concentration of 16.84 µg/mL. The crude protein used for the assays is then diluted 1:10 giving each reaction a total concentration of 16.84 µg of protein. From the assays we first find the $K_m$ of the protein.

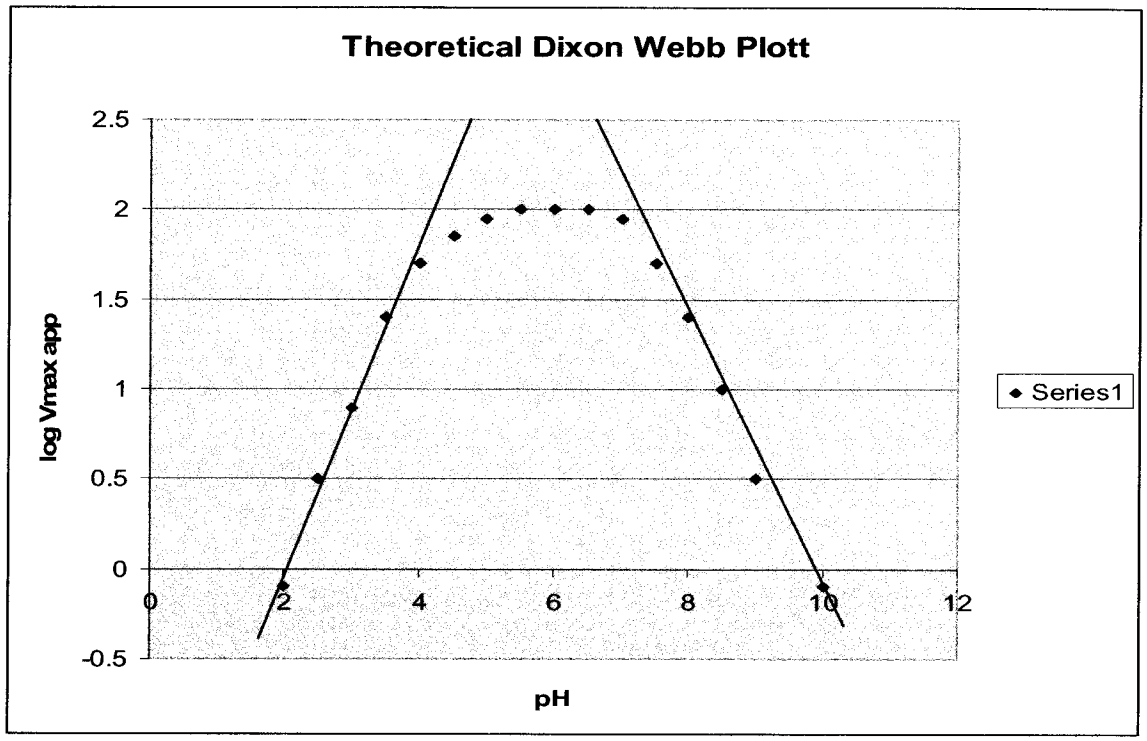



Graph 2-6. Graph of the Bradford assay used for determining the concentration of the crude protein from the *E. coli* expression system.

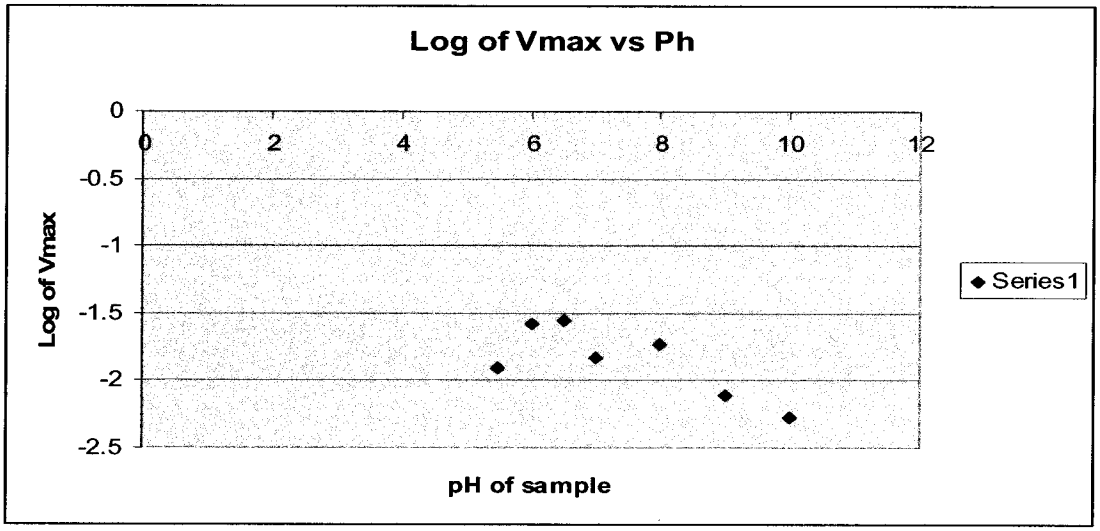
| pH | $K_m$ |
|---|---|
| 5.5 | 46 |
| 6 | 45 |
| 6.5 | 15 |
| 7 | 51 |
| 8 | 36 |
| 9 | 121 |
| 10 | 16 |

Table 2-3. $K_m$ values for the corresponding pH used for the assay.

The plot of the above data would theoretically yield a graph with a parabolic line, with the intersection being the pka of the corresponding point in the reaction. The graph labeled? Is an example of the perfect experimental conditions and results of the experiment, the graph that follows is the graph generated from the data of the actual experiment.




Graph 2-6 Dixon-Webb Plot




Graph 2-7. Data collected from actual experiments plotted using the Dixon Webb Method to determine the $pk_{es}$ complex at given points. The slope of the lines are 1 and -1, indicating the titration of our titratable groups at high and low pH. From this plot we can

determine the $_{p}k_{a}$ of the enzyme substrate complex at a given pH. (A complete listing of the kinetic data and the individual graphs are in Appendix B.)

The data from the assays of the protein hold true to many of the studies performed using the same method. Enzymes have a set range in which they maintain functionality, outside that range the activity of the enzyme becomes erratic and unstable. This can be attributed to the process of denaturization. A barrier such as this could be eliminated with a change in the assay conditions. By increasing the amount of $^{14}C$ IOA in the reaction mix (making the reaction hotter) and reducing the time of the reaction these barriers or opportunities could be eliminated. As these are the preliminary studies that portion of the experiment will come at a later date.

## Chapter III: Inhibitor Synthesis and Affinity Chromatography

### Introduction

The use of a known inhibitor attached to the stationary phase of a chromatography column to isolate specific proteins has previously worked on other proteins,[7,8] and it is believed that it can be successful in the isolation attempts of IDCase. 5-nitrouracil (5-Nu) is a strong inhibitor of IDCase. Previous inhibition studies of N1 alkylated 5Nu by Dr. Smiley (unpublished) showed little to no inhibition of IDCase. However, inhibition was retained when N3 alkylated 5Nu was used. Therefore, the protein still binds to the N3 linked 5Nu. This product can be conjugated to an activated agarose column to produce the custom affinity resin for chromatography and separation of the IDCase.

Chains of 5 and 9 atoms in length will be attached to N3 of 5Nu. Similar molecules have been previously synthesized.[8] Analysis of products were obtained by thin layer chromatography (TLC), $^{1}$H NMR (Varian Gemini 2000, 400MHz spectrometer), UV spectroscopy (HP 8452 diode array spectrophotometer), and mass spectrometry (Bruker Data Analysis Esquire LC).

### Methods/Materials

The following synthesis was originally described by Yun.[8] In a clean, dry, 50 mL roundbottom flask, 0.5 g 5-nitrouracil (3.2 mmol), 6.4 mL 25% tetrabutylammonium hydroxide in methanol (6.4 mmol), and 5 mL N,N-dimethylformamide (DMF) were mixed at room temperature, until all the 5-nitrouracil was dissolved. The resultant mixture was evaporated until a syrupy solution was obtained. This solution was then redissolved in 458 μL ethyl-4-bromobutyrate (3.2 mmol) and 5 mL DMF, mixed at room

temperature for 24 hours, then quenched with 4 mL glacial acetic acid. The product was extracted with 30 mL water and 3x 20 mL ethyl acetate. The organic layer was dried over $MgSO_4$, and then evaporated to dryness. Starting material and unwanted side products were removed from the desired product by flash chromatography, using a 19:1 methylene chloride:methanol solvent system. Column fractions were checked by thin layer chromatography (TLC) using the same $MeCl_2$:MeOH solvent system. Those fractions containing only the desired product were pooled and evaporated to dryness (Figure 3-1 and 3-2).

800 mg [product 1] (2.95 mmol) and 3 mL ethylenediamine were mixed in a clean, dry, roundbottom flask, and allowed to mix under reflux for four days. The resultant mixture was separated by flash chromatography, using a 4:1 isopropanol:ammonium hydroxide solvent system.

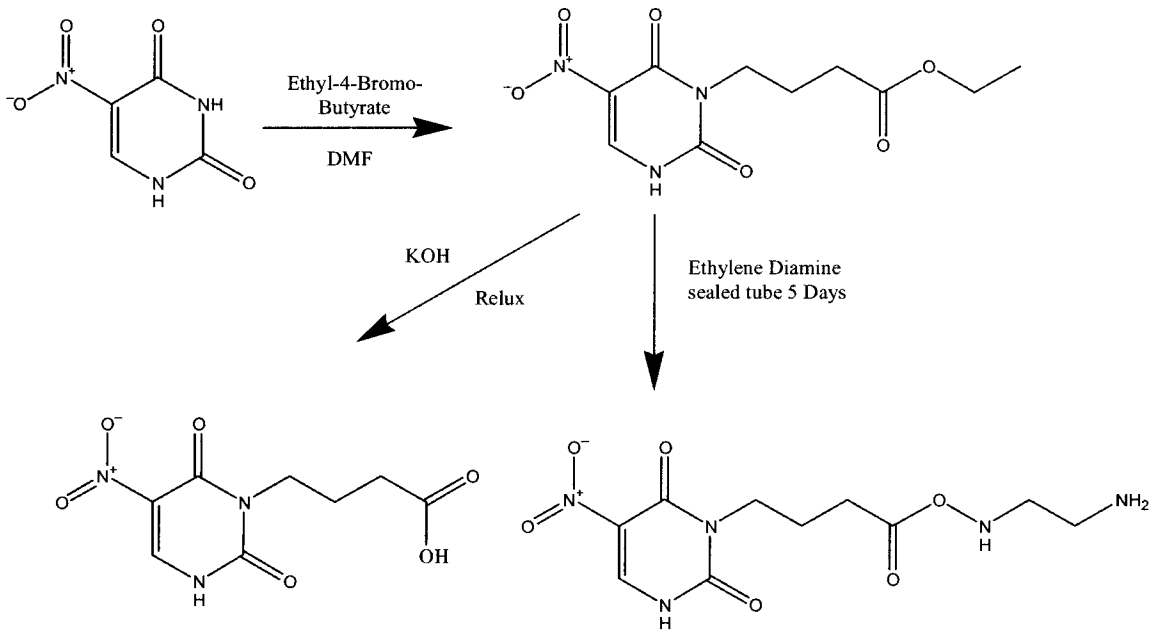



Figure 3-1

Figure 3-2.

Figure 3-1 and 3-2 Inhibitor synthesis.

Analysis of products were done by 10 μL injection into a high performance liquid chromatography (HPLC, Exterra $RP_{18}$ column) using a solvent system of 500 mM $KH_2PO_4$, and 0.01 mM Acetonitrile ($CH_3CN$). Further analysis was also done by mass spectrometry using electrospray ionization (ESI) in both positive and negative modes. The progression of the synthesis was monitored closely using the HPLC. The mass of each product could first be calculated by the atoms contained in the molecule. Using this number the major peak from the HPLC data not only confirmed the existence of the product with the proper weight but also the purity of the product which is evident by the number of unexpected peaks.

**Results and Discussion**

Compound 1 was synthesized with a 26.4% yield. A TLC chromatogram provided 3 peaks, and after flash column purification only the desired would be used to synthesize compound 1b. Product 1b is the hydrolyzed form of compound 1, and was synthesized with a near 100% yield. The hydrolysis of product 1 was carried out by dissolving the compound in 2M KOH and refluxing at 60 $^0$C for two hours. The hydrolysis yielded 100% conversion. The process for the conversion of Product1 to Product 2 was similar to that of the hydrolysis of the original. The compound was dissolved in 5 ml of ethylene-diamine and allowed to react in a closed 125 ml round bottomed flask. HPLC of compound 2 after 5 days showed new peaks when compared to the compound 1 chromatogram 87% of the reactants converted to product 2. The TLC of product 2 yielded two spots, which were isolated by flash chromatography and checked by mass spectrometry. The mass spectrometer was also used to check the progression of the synthetic process. All spectra were taken in negative mode and the resulting spectrum

represents the weight of the sample minus a proton. Product 1 in negative mode showed 1 peak at 270.0 (Figure 3-3), product 1 hydrolyzed showed a peak of 243.3 (Figure 3-4) and product 2 showed a peak at 284.0 (Figure 3-5) all consistent with the expected values for the product.

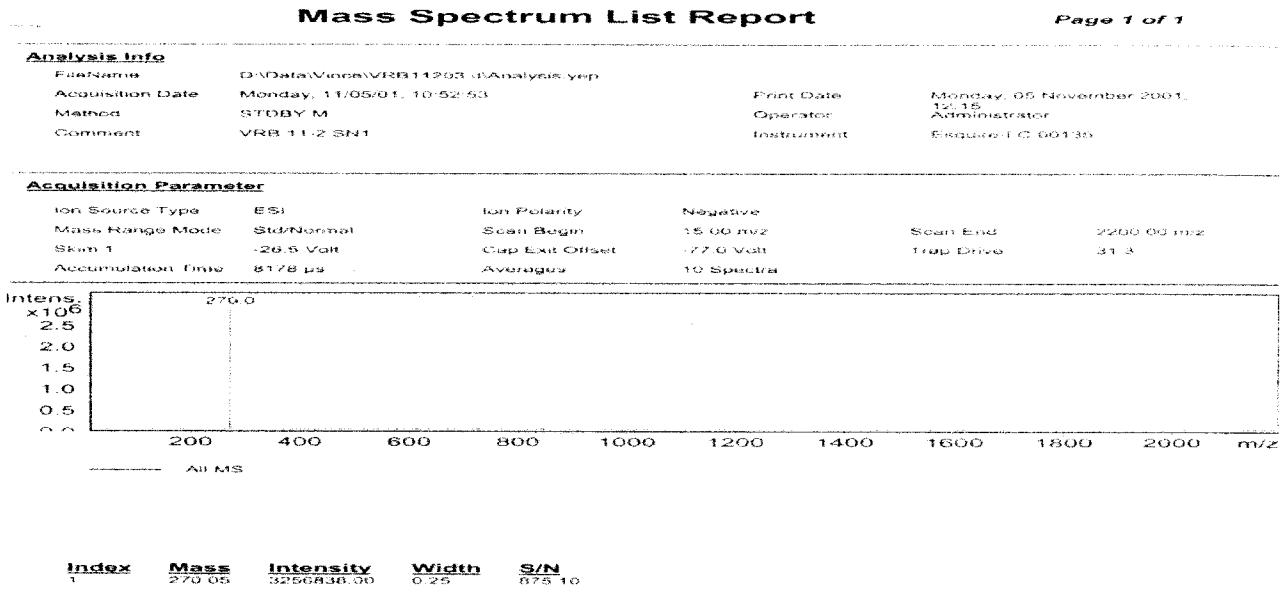



**Figure 3-3.** The mass spectral data above is the picture depicting the mass of the first portion of the synthesis.

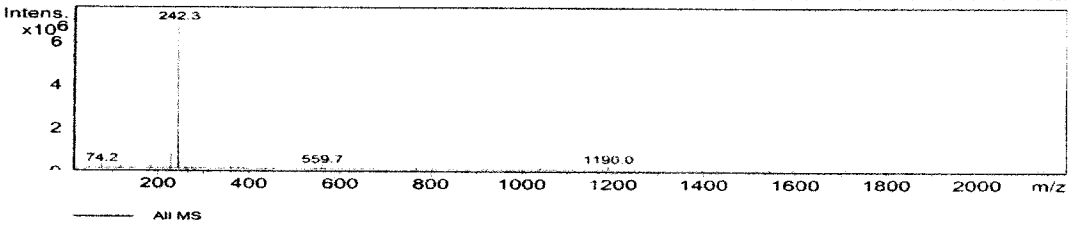
This was the key step in that this product will be used in the further synthetic steps of the experiment. It is from this product that the other analogs will be derived from. The ester created at C4 of the alkyl ligand makes for an ideal site for stereo specific chemistry. With this functional group in place several reactions to lengthen the linker arm can be run simultaneously to ensure a ligand of the appropriate length is used in column construction and to test binding affinity to the enzyme.

**Analysis Info**

| | | | |
|---|---|---|---|
| FileName | D:\Data\Vince\VRB11205.d\Analysis.yep | | |
| Acquisition Date | Monday, 11/05/01, 11:20:44 | Print Date | Monday, 05 November 2001, 12:18 |
| Method | STDBY.M | Operator | Administrator |
| Comment | VRB 11-2 SN1 | Instrument | Esquire-LC 00135 |

**Acquisition Parameter**

| | | | | | |
|---|---|---|---|---|---|
| Ion Source Type | ESI | Ion Polarity | Positive | | |
| Mass Range Mode | Std/Normal | Scan Begin | 15.00 m/z | Scan End | 2200.00 m/z |
| Skim 1 | 15.0 Volt | Cap Exit Offset | 77.0 Volt | Trap Drive | 37.0 |
| Accumulation Time | 1489 µs | Averages | 10 Spectra | | |




| Index | Mass | Intensity | Width | S/N |
|---|---|---|---|---|
| 1 | 46.32 | 181573.00 | 0.02 | 171.17 |
| 2 | 74.16 | 220884.00 | 0.27 | 208.23 |
| 3 | 182.14 | 207167.00 | 0.37 | 195.30 |
| 4 | 226.11 | 983772.00 | 0.31 | 927.41 |
| 5 | 242.34 | 7567962.00 | 0.32 | 7134.39 |
| 6 | 243.20 | 1468684.00 | 0.34 | 1384.54 |
| 7 | 244.03 | 310778.00 | 0.38 | 292.97 |
| 8 | 272.06 | 2661998.00 | 0.30 | 2509.49 |
| 9 | 273.04 | 374344.00 | 0.31 | 352.90 |
| 10 | 289.09 | 1537379.00 | 0.30 | 1449.30 |
| 11 | 290.07 | 211346.00 | 0.31 | 199.24 |
| 12 | 294.01 | 200126.00 | 0.27 | 188.66 |
| 13 | 358.15 | 229385.00 | 0.35 | 216.24 |
| 14 | 559.66 | 183974.00 | 0.15 | 173.43 |
| 15 | 559.98 | 177558.00 | 0.39 | 167.39 |
| 16 | 1189.99 | 218466.00 | 0.14 | 205.95 |

Bruker Daltonics DataAnalysis 2.0

**Figure 3-4** This is the mass spectral data of the product dubbed product 1b. It is the hydrolyzed form of the product 1 in figure 3-3. As can be seen the change in mass represents the loss of an ethanol group, replaced by the addition of a hydroxyl group to give the final compound the carboxylic acid portion.

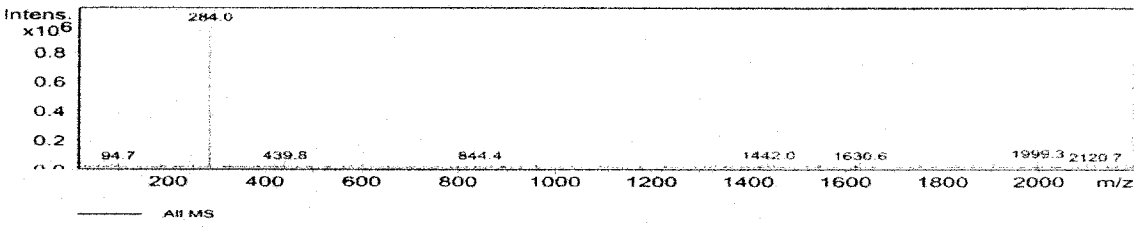
**Mass Spectrum List Report** *Page 1 of 1*

**Analysis Info**

| | | | |
|---|---|---|---|
| FileName | D:\Data\Barnes\EDAFC293.d\Analysis.yep | | |
| Acquisition Date | Friday, 11/30/01, 11:23:15 | Print Date | Friday, 30.November 2001, 15.23 |
| Method | STDBY.M | Operator | Administrator |
| Comment | N3 EDAFC 29 | Instrument | Esquire-LC 00135 |

**Acquisition Parameter**

| | | | | | |
|---|---|---|---|---|---|
| Ion Source Type | ESI | Ion Polarity | Negative | | |
| Mass Range Mode | Std/Normal | Scan Begin | 15.00 m/z | Scan End | 2200.00 m/z |
| Skim 1 | -24.0 Volt | Cap Exit Offset | -77.0 Volt | Trap Drive | 35.0 |
| Accumulation Time | 4396 µs | Averages | 10 Spectra | | |




| Index | Mass | Intensity | Width | S/N |
|---|---|---|---|---|
| 1 | 94.65 | 37191.00 | 0.20 | 206.99 |
| 2 | 210.52 | 35376.00 | 0.02 | 196.89 |
| 3 | 284.02 | 1097211.00 | 0.29 | 6106.61 |
| 4 | 284.68 | 135635.00 | 0.35 | 754.89 |
| 5 | 439.82 | 42590.00 | 0.03 | 237.04 |
| 6 | 844.42 | 42173.00 | 0.03 | 234.72 |
| 7 | 1442.05 | 46842.00 | 0.19 | 260.70 |
| 8 | 1550.32 | 41585.00 | 0.02 | 231.44 |
| 9 | 1630.65 | 41936.00 | 0.20 | 233.40 |
| 10 | 1906.23 | 50118.00 | 0.13 | 278.94 |
| 11 | 1919.92 | 33113.00 | 0.03 | 184.29 |
| 12 | 1999.32 | 59122.00 | 0.04 | 329.05 |
| 13 | 2120.72 | 32134.00 | 0.04 | 178.84 |
| 14 | 2187.95 | 45138.00 | 0.19 | 251.22 |

**Figure 3-5.** The mass spectral data of product 2. Although the synthesis was a success the testing carried out to check the affinity of the protein for this derivative showed it was not as efficient as product 1b. For that reason it was not used in the attempts made towards the purification of IDCase.

## Cell Growth Procedures

For our research, IDCase was obtained from *Rhodotorula glutinis* yeast cells. *R. glutinis* has no telemorphic stage, has been studied little in the past, and many questions are still unanswered concerning the yeast. It is known, however, that it is a non-fermenting yeast, which is red-pink in color when grown at between 25-30°C. It has been previously isolated in a variety of conditions and areas worldwide.[10-12] Because

thymine-7-hydroxylase has previously been isolated from *R. glutinis*, it was believed that it could also be used to isolate the IDCase enzyme.[13]

Two types of media were utilized in the growth of *R. glutinis*; YM, a rich medium, and thymine, a restrictive medium. YM medium allows for the best growth of the *R. glutinis* cells. In the restrictive media, thymine is the only source of nitrogen, therefore presumably IDCase must be present for the cells to grow. The rich media was used to obtain viable cells, which were inoculated into the restrictive media, and the protein isolated from these cells.

**Materials and Methods**

10 mL of autoclaved YM media (Table 3-3) was inoculated with yeast freezer stock, and the inoculated media was shaken for 24 hours in a 28°C incubator. A fresh, 200 mL flask of autoclaved YM media was inoculated with the entire 10 mL culture and incubated, with shaking, for 24-36 hours at 28°C. The yeast cells were removed from the YM media by centrifugation, and the resultant pellet was used to inoculate 1 L thymine media (Table 3-2) and was shaken in a 28°C incubator for 18-24 hours.

Table 3-1. YM Media.

| Components | Amount |
|---|---|
| Yeast Extract (Amresco) | 3.0 g |
| Malt Extract (BD) | 3.0 g |
| Peptone (Amresco) | 5.0 g |
| Glucose (Amresco) | 10 g |
| Agar, Bacteriological (if necessary) (Amresco) | 20 g |
| Dissolve in 950 mL $dH_2O$, adjust to pH 6.5, and dilute to 1 L | 1.0 L |

Table 3-2. Thymine Media.

| Components | Amount |
|---|---|
| Yeast Carbon Base (Difco) | 11.7 g |
| Thymine (Sigma) | 1.0 g |
| Dissolve in 950 mL $dH_2O$, adjust to pH 6.5, and dilute to 1 L | 1.0 L |

**Isolation**

Yeast cells were separated from media by centrifugation for 15 minutes at 10,000 rpm. The cell pellet was resuspended in a small volume (about 2.0 mL per 1.0 g wet cell mass) of lysis buffer (10% v/v glycerol, 50 μM β-mercaptoethanol, 20 mM Tris pH 7.4 buffer). Also added to the lysis buffer were protease inhibitors: phenylmethyl-sulfonylfluoride (PMSF, 0.4 mM), leupeptin (1.2 μM), and pepstatin-A (4 μM). This solution was transferred to a screwcapped container containing 1.0 mm glass beads (Biospec), which was then blended using a blender (Biospec Bead Beater) three times for one-minute intervals, with five minutes in-between blendings to keep the cell lysate cool. The liquid was then removed from the glass beads, and centrifuged at 10,000 rpm to separate the cell material from the soluble proteins.

**$AmSO_4$ Fractionation**

50-70% $AmSO_4$, at 5% increments, was added to total soluble protein over a 10 minute interval. (Table 3-3) Protein/$AmSO_4$ solution was then mixed by magnetic stirring at 4°C for 30 minutes. The solution was then transferred to a 30 mL glass centrifuge tube (Corex) and centrifuged at 12,000 rpm for 20 minutes. The supernatant was decanted and saved for later quantification by $^{14}C$ assay.

Table 3-3. $AmSO_4$ fractionation (grams per 100 mL)[14]

| | 50 | 55 | 60 | 65 | 70 |
|---|---|---|---|---|---|
| 0 | 29.1 | 32.6 | 36.1 | 39.8 | 43.6 |

To the total soluble protein taken from broken cells, 50% $AmSO_4$, was added over a 10 minute interval, while mixing by magnetic stirring at 4°C. The resultant mixture was stirred for 30 minutes, then centrifuged at 12,000 rpm for 20 minutes. The supernatant was collected, and $AmSO_4$ was added over 10 minutes, to bring the final $AmSO_4$ concentration to 70%. Again, this was mixed for 30 minutes, then centrifuged at 12000 rpm for 20 minutes. The supernatant was decanted, and the pellet kept.

The pellet was dissolved in a minimal (about 1.0 mL/g) volume of buffer, and pipetted into a selective membrane dialysis bag, and placed into about 1 L buffer, slowly stirred at 4°C, and dialyzed for about 8-12 hours. After the dialysis the protein is subjected to $^{14}C$ and Bradford assays

**Custom Affinity Column Chromatography**

One possible way to purify IDCase is the use of custom affinity chromatography. As stated previously, Brody and Westheimer noted a 3200 fold purification of ODCase after one pass over their column of substrate-binding inhibitor.[7]

For an affinity column to work, an active-site binding inhibitor is needed. This inhibitor must have a linker arm attached, one which will not interfere with binding to the protein. The linker arm must have an end which can selectively bind to the matrix. The matrix must be inert as not to interfere with the protein, nor interact with the column buffer. The matrix must also be porous, as to allow free flow of buffer and unwanted proteins.

Custom affinity chromatography is a selective technique to purify IDCase. Many factors can affect its effectiveness however. While it is possible to determine how many molecules of inhibitor are attached to the column, it is impossible to know how many molecules are attached to an agarose bead, or the position of the inhibitor in relation to another molecule of inhibitor or another agarose bead. If the linker arm is not long enough, the protein will not be able to bind. If the linker arms are bound too closely together, there will not be enough room for the protein to bind.

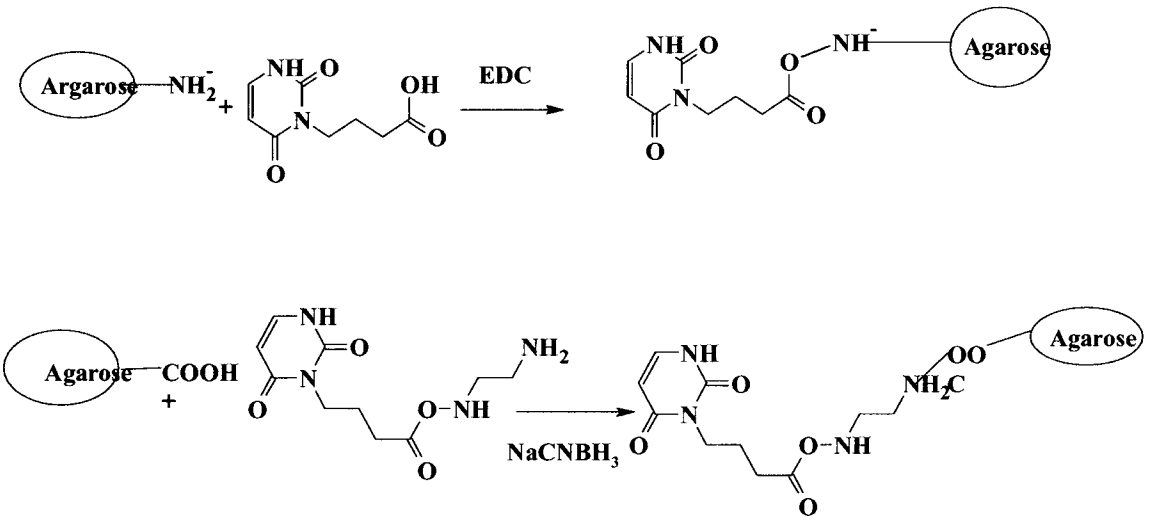
**Materials/Methods**

Product 1b and Product 2 were attached to a AminoLink activated agarose resin, using the protocol described by the manufacturer (Pierce, Figures 3-6 and 7).[16] The gel slurry is first poured into a 15 ml syringe and the packing solution drained. It is then washed with 5 x 10 mL portions of water followed by 5 x 10 mL washings with the conjugation buffer (0.1M MES, 0.9% NaCl, pH 4.7). 20 mg Product 1b was dissolved in 2 mL conjugation buffer and OD at 282nm was determined. Buffer plus Product 1b mixture was added to gel and shaken. 60 mg EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) was dissolved in 0.5 mL conjugation buffer, then added to the column gel. The mixture was gently shaken at room temperature for three hours. The solution was drained to gel bed top and the volume recorded. The slurry is then transferred to a new tube and 10.0 mL wash buffer (1.0 M NaCl) was added and allowed to drain until the liquid descends to the top of the gel bed. A polyethylene disk, supplied by Pierce, was inserted 10.0 mm above bed, and solution drained. The column material was transferred

to fresh tube and washed 12 times with 10 mL of the conjugation buffer. These washing were collected and the OD of fractions was determined by UV spectroscopy.

The preparation for the product 2 column begins with the same method of washing as does product 1b. After washing, the gel is placed in a clean, dry screw-cap tube, the gel was mixed with an equivalent volume of inhibitor. In a hood, 0.5 mL of 1M $NaCNBH_3$ per mL of slurry was mixed in. The solution was capped and mixed for about 2 hours by gentle inversion. After the mixing process the solution was allowed to sit for four hours, then drained by vacuum filtration and washed twice with 1M Tris·HCl (pH 7.4). The resultant cake was returned to the screw-cap tube and washed twice with an equivalent volume of 1M Tris·HCl. 0.05 mL of 1M $NaCNBH_3$ per mL of slurry was added and mixed for 30 minutes at room temperature. The gel was drained by vacuum filtration and washed with 10 volumes of 1M NaCl, then 10 volumes of 0.05% $NaN_3$. The fractions collected from the final washings were subjected to UV analysis to determine the amount of inhibitor attached to the column.

The column was washed with buffer (10%glycerol, 50mM pH 7.4 Tris, 50 μM β–ME) to ensure any salt, protein, or unattached inhibitor from previous use was removed. Crude protein, having been subjected to $AmSO_4$ precipitated and dialyzed, was placed on top of the column bed. Column buffer was run over the column at 4.0 mL/min. Fractions were tested by 900 μL Bradford reagent and 100 μL fractionate. Once no color change was noted by ocular detection, 50 mL of 0.2M NaCl in buffer was run over the




Figures 3-6 AND 3-7. Depiction of the difference in the attachment point of the resin, this would entail which process of attachment to use. Product 1b would be the top figure, and product 2 would be the bottom figure.

**Results/Discussion**

A modest increase in specific activity was seen after using the custom affinity column. Use of UV spectroscopy determined that 440 mM of the inhibitor Product 1b remained on the column. The analysis of the column prepared with product 2 showed little retention of the inhibitor to the column and was not used in isolation attempts. About 10 g of *R. glutinis* cells were obtained from 1 L of thymine media, and total protein was isolated as previously described. Table 3-4 shows the total and specific activities of crude (total) protein isolated from the *R. glutinis* cells, protein after $AmSO_4$ precipitation and dialysis, an early column fraction, and fraction after 0.8M NaCl was applied to column. A 5-fold increase in specific activity of the IDCase protein, with a 7.3% recovery of protein was determined after the one pass over the column.

| Purification Step | Volume (mL) | Total Activity (nmol/min) | Total Protein (mg) | Specific Activity (nmol $min^{-1}$ $mg^{-1}$) | Percent Recovery % |
|---|---|---|---|---|---|
| 1. Crude Protein | 32.5 | 8391.6 | 171 | 49.1 | 100 |
| 2. 50-70% $AmSO_4$ | 7.5 | 4062.0 | 70.1 | 58.0 | 48.4 |
| 3. Fraction 8 | 7.5 | 2429.9 | 30.0 | 81.1 | 28.9 |
| 4. Fraction 20 | 7.5 | 613.38 | 2.53 | 242 | 7.31 |

Table 3-4. Stepwise table of purification of IDCase. 1) unaltered protein, 2) protein after precipitation and dialysis, 3) protein not binding to column, 4) protein after high salt elution.

As the purity of the protein remained unknown the actual concentration of IDCase was unknown. The change in activity versus the protein concentration reveals that the protein is present but again in an unknown quantity. In an attempt to improve the quality of the purification process another form of chromatography was used.

## Chapter IV: IDCase Activity in Rhodotorula glutinis and Conventional Chromatography

### Introduction

#### $^{14}C$ Assay Cell Growth and Harvesting

As the protein of interest is a decarboxylating protein, assaying for the conversion of IOA to uracil can be easily achieved through the use of the isotope labeled substrates. By labeling the leaving group, in this case $^{14}C$ labeled $CO_2$ and counting the radioactivity released we can calculate the activity of the protein. These assays will be applied to the IDCase produced from cultures of *R. glutinis*. This sensitive, specific assay can provide data for the purification of native or recombinant IDCase.

$^{14}C$ assays were performed by adding [carboxy-$^{14}C$] IOA to the protein solution. If present, the IDCase removes the carboxyl group from the IOA, leaving $^{14}CO_2$ and uracil. In an assay vial, 25 µL Tris buffer pH 7.4, 50 µL [carboxy-$^{14}C$] IOA (5000 cpm/assay), and 375 µL $dH_2O$ were combined. Wicks were made of filter paper (Whatman) were moistened with 2M KOH to collect the $CO_2$ driven off by the reaction. To the assay vial, 50 µL of protein solution was added, then the reaction capped with a rubber septum and basket containing the KOH dampened wick. The reaction was run for 5 minutes in a 30°C shaking water bath, then quenched with 0.1 mL 2M HCl. The quenched reaction was allowed to sit for 1 hour, allowing time for the wick to absorb any $^{14}CO_2$ released. The wicks were then removed and placed in a 70°C oven for about twenty minutes, or until dry but not to the point of discoloration. Once dry, each wick was individually inserted into a pre-counted vial containing scintillation fluid (ScintiSafe

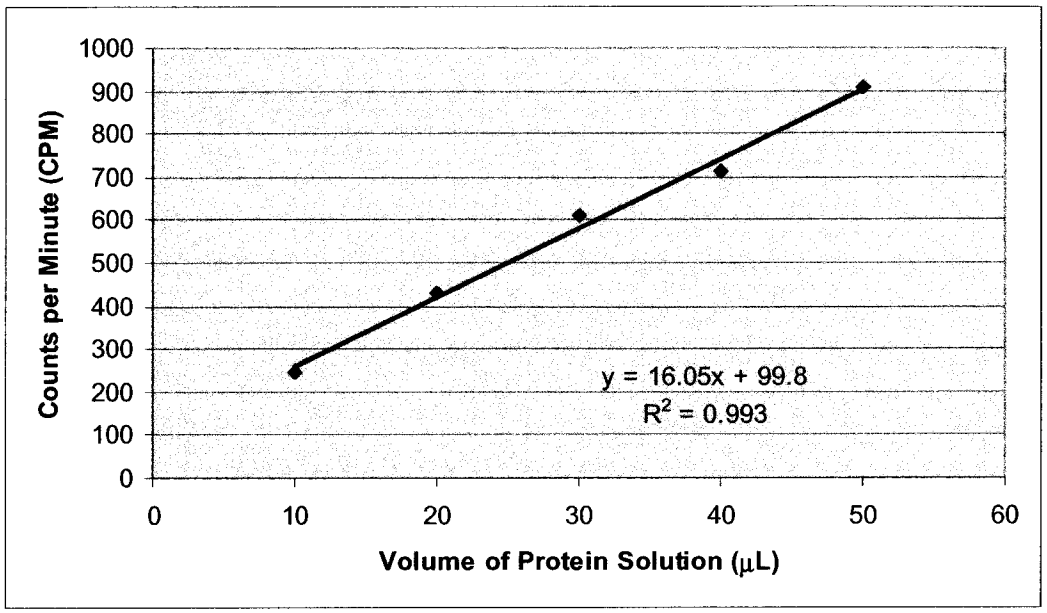
Econo 1) and the amount of $^{14}C$ present was quantitated with a scintillation counter (Packard 1900CA Liquid Scintillation Analyzer).

**Bradford Assay**

Following the standard procedure for a Bradford assay and using a 80:20 dilution of the colouring solution the assays are carried out in duplicate. Five different concentrations of BSA are added to 0.9 mL of Bradford Assay Reagent (Pierce), and diluted to 1 mL with 0.15 M NaCl. Amounts of 0, 5, 10, 15, and 20 µL of 0.5 mg/mL BSA were added.[16] An amount of sample protein was added to 0.9 mL Bradford Assay reagent, and diluted to 1 mL with 0.15 M NaCl. The intent is to use an appropriate amount of protein so that the absorbance is between the values from the standard. A graph of the µg protein vs. absorbance was plotted, and from this graph, µg sample protein can be determined. Specific activity of the protein can be calculated once µg protein and the cpm's of the proteins conversion in the $^{14}C$ assay have been determined.

**Assay *R. glutinis* procedures**

The protein solution isolated from broken cells of the *R. glutinis* culture was assayed by $^{14}C$ assay (Graph 4-2). A linear relationship between volume of protein solution and cpm measured can be seen, and all volumes were below 50% of total $^{14}C$ counts. Therefore, a volume of up to 50 µL protein solution could be used without exceeding the range measurable by the previous assay technique.




Graph 4-2 . Comparison of Volume of protein added vs. CPM.

In attempts to purify the protein, the slurry from the harvesting procedure was divided equally into 6 aliquots, containing 50, 55, 60, 65, and 70 percent $AmSO_4$ respectively. No $AmSO_4$ was added to the $6^{th}$ aliquot. It was determined that IDCase from *R. glutinis* precipitates out of solution between 50 and 70 % $AmSO_4$ (Table 4-1). Also, it was determined that only a minimal amount of IDCase protein was lost when a 50-70% ramp of $AmSO_4$ was utilized (Table 4-2). Therefore, 50% $AmSO_4$ was added to total protein, with the supernatant kept, and the $AmSO_4$ concentration increased to 70%. The pellet could then be kept and dialyzed to remove the $AmSO_4$.

| Sample | CPM | % conversion |
|---|---|---|
| Direct Spot | 4042 | |
| Total Protein – No $AmSO_4$ | 2328 | 57.6 |
| 50% supernatant | 1704 | 42.1 |
| 55% supernatant | 282 | 7.0 |
| 60% supernatant | 192 | 4.8 |
| 65% supernatant | 76 | 1.9 |
| 70% supernatant | 195 | 4.8 |
| No Protein | 70 | 1.7 |

Table 4-1. $^{14}C$ counts of various percentages of $AmSO_4$ added to protein slurry containing IDCase

| | cpm | % conversion |
|---|---|---|
| Direct Spot | 1901 | - |
| Total Protein – No $AmSO_4$ | 1936 | 100 |
| 50-70% supernatant | 112 | 5.9 |
| No Protein | 52 | - |

Table 4-2. $^{14}C$ counts of total protein and 50-70% $AmSO_4$, indicating that 5.9% of IDCase is lost in $AmSO_4$ fractionation.

The assay on the protein from this step showed a modest increase in activity after dialysis, but due either to growth techniques or time between precipitation and assays the concentration of the protein are not sufficient for continuing on with this method. Presumably, enough protein could be obtained by cultivating very large cultures (20 L or more), but gene cloning and over production of recombinant IDCase should provide more convenient conditions.

**Ion Exchange Chromatography: Protein concentration and IDCase activity.**

Proteins have a wide variety of distinguishing characteristics, including ionic strength. This characteristic allows their purification by ion exchange chromatography. In ion exchange chromatography, molecules are separated based on their charge. In ion exchange, the stationary phase is a bead or resin with a fixed charge. If that charge is negative, then the process is called cation-exchange chromatography, and if the charge is positive, then it is anion-exchange chromatography. Two common resins are carboxymethyl (CM) cellulose (cation exchange) and diethylamino-ethyl (DEAE) cellulose (anion exchange).

These fixed charges on the beads will interact with molecules of opposite charge in the buffering solution (mobile phase). For example, the fixed negative charge of a cation exchange resin will always be associated with a positively charged species in the buffer solution. If a protein of positive charge is passed through the column, it may bind to the matrix, and it can subsequently be eluted by increasing the ion concentration of the mobile phase. (The elution can be looked on as a competition for binding sites on the matrix. As the ionic strength of the buffer increases, it becomes more likely that a salt ion from the buffer will take the site on the resin and the protein will be eluted from the column.)

There are 2 common ways that ion exchange columns are eluted. In a gradient elution, the salt concentration increases constantly and gradually during the elution process, for example, gradually moving from no NaCl to 1 M NaCl over the course of the elution. The second method is a step-wise elution, where the change in salt concentration

is abrupt, for example, switching from the no-salt loading buffer to the 1 M elution buffer with no intermediate salt concentrations.

The pH of the mobile phase solution is critical in ion exchange chromatography. The pH must be such that the stationary phase resin has the correct charge. For example, at very low pH, the carboxymethyl species would be protonated and, hence, would not have the required negative charge to attract positively charged molecules in the system. Additionally, the protein that one wishes to bind the resin must be at the pH that will give it the appropriate charge, i.e., below the pI of the protein in order to have a net positive charge.

**Materials and Methods**

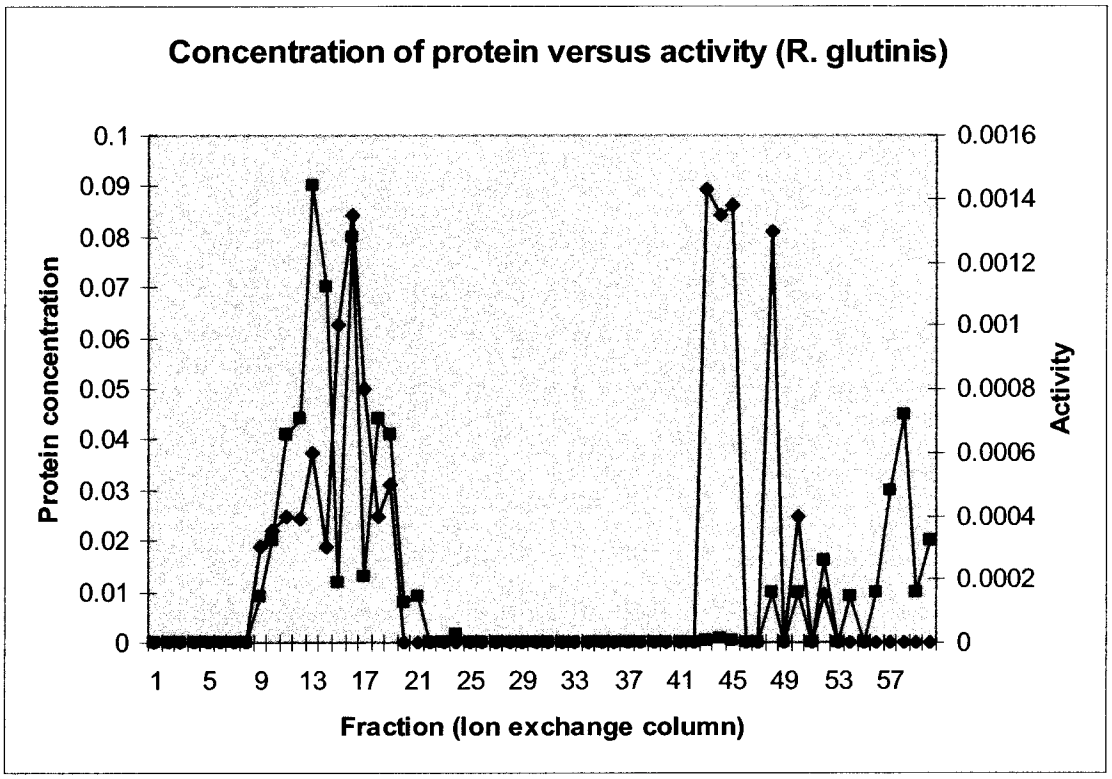
CM Sepharose gel was purchased from Sigma. A volume 75 ml of the gel is transferred to an Econo column and washed 3 times with 100ml of dialysis buffer. (Same buffer used for cell dialysis.) After washing the column protein is applied and allowed to flow over the column at a rate of 1 ml per min to ensure time for reaction and to coat the resin evenly. As the protein is loaded on the column, samples collected by the fraction collector are tested using the Bradford Assay Method. The fractions are collected and checked for visual color change and then on the spectrophotometer to calculate absorbance and concentration. As the color change lessens the pH of the buffering solution is changed. Using a MOPS buffer at a pH of 8.5 set in a gradient the protein eventually changes enough physiologically that it is released from the stationary phase and collected.

With the protein totally removed from the column the fractions are now ready for the various assays used to determine the purity, concentration and activity of the protein. For concentration of protein in each of the samples UV absorbance was used. These samples are the same used in the previous step to determine the changes in the buffering system. After the concentration is determined the samples are subjected to a $^{14}C$ assay to measure the amount of IDCase activity. This data is then applied to a double Y-axis graph using the Bradford data to determine protein concentration and the $^{14}C$ assay data to measure IDCase activity nmol $min^{-1}$ $ug^{-1}$. It is from this data that the purity and efficiency of the enzyme can be determined, as well as insight into the effectiveness of the procedure being used.

**Results and Discussion**

The protein used for the ion exchange column was prepared using the same methods as were employed for the Affinity Chromatographic procedure. The only change was that of the buffering system used as the mobile phase of the column. For the ion exchange portion of the experiments a 0.5 M MOPS buffer was used at a pH of 8.5. The flow rate was adjusted due to the change in the size of the column. The protein once loaded onto the column was eluted using the MOPS buffer and an increasing gradient of NaCl. The first and unwanted protein to come off the column showed some activity when assayed, but the activity was low in relation to this concentration. The later proteins, as was predicted contained the most activity. (Graph 4-1) This coincides with the predicted results as well as the intended functionality of the ion exchange column. The drawback to

this attempt at the purification lies in the fact that there were very low concentrations of the protein acquired by this procedure as a whole and even though there existed a modest increase in the activity of the enzyme, this preparation method and the procedures followed simply did not yield enough protein to work with (Graph 4-2). From our best results, the cell prep only yielded five fractions of activity after passage over the column. With concentrations this low it would not be efficient to pass the semi-purified protein over the affinity column for further purification. However, this data should be very useful for future purification efforts with the recombinant IDCase, which should be present in much higher concentrations.




Graph 4-2. Data gathered from the CM cellulose ion exchange column. Series 1 is indicative of the activity present in the $^{14}C$ assay, while the series 2 points are the resulting concentrations as given from the analysis of the Bradford Assay method.

It was evident from the results of the affinity column and the ion exchange column that a more efficient method of cultivating the protein was needed. For this opportunity several techniques were attempted to gauge the most effective procedure in attaining the protein that would allow for testing and measurement of the catalytic efficiency possessed by this enzyme.

## Chapter V: Conclusion

The quest to isolate, purify and characterize the enzyme IDCase has been an in-depth learning experience and it has linked the use of old and new techniques employable in the search or new answers. During the course of the experiment many techniques where used, all aimed at a specific portion of the total process. There are several instances were a portion of the research had to be based on the acceptance of the work of others, and some where new and improved methods had to be devised. Overall the experimentation has proved successful and will probably lead to more in-depth and interesting studies.

The studies have shown that under the conditions used for isolating the enzyme from natural sources are not the most efficient. Although activity showed an increase the quantity of protein gathered from these sources was not enough to continue forward with further studies. This shows that the isolation techniques work, but may need refinement in order to use the growth, harvesting and isolation from natural sources.

The synthesis of the inhibitors was a success and can be used to test and compare similarities and differences not only in this pathway but many others where natural products look like analogs of synthesized products. This also allows for the speculation of the mechanistic reaction behind the enzyme's actions. The knowledge of the inhibitor synthesis and the types of reaction may lead to the development of new assays that allow for the ease of protein isolation.

The studies on the recombinant protein gave great insight into some of the kinetic properties of the enzyme. The next step in the long list of things to accomplish would be

the testing of the enzymes specific activity, which of course requires the use of purified protein. Studies into the structure of the enzyme may also be accomplished on pure protein with the use of NMR and crystallography. For an enzyme first studied in the 1970's and allowed to lay dormant for over thirty years the new data and classification may prove to be more useful and efficient than the researchers who originally discovered it would have believed possible.

# References


1. Jones, M.E. *Annu. Rev. Biochem.* **49,** 253-279.

2. Voet, D., and Voet, J.G. *Biochemistry: 2nd Ed.* (1995), 804.

3. Palmatier, R. D., McCroskey, R. P., and Abbott, M. T. *J. Biol. Chem.* **245,** 6706-6710.

4. Smiley, J.A., Angelot, J.M., Cannon, R.C., Marshall, E.M., and Asch, D.K. *Analytical Biochemistry,* **266**, 85-92.

5. Landfried, D.A., Barnes, V.R., and Smiley, J.A. Poster presentation.

6. O'Leary, M.H. *The Enzymes, vol. XX.* (1992), 235-269.

7. Brody, R. S., and Westheimer, F. H. *J. Biol. Chem.* **254,** 4238-4244.

8. Yun, Danny. Master's Thesis. Youngstown State University, 1999.

9. Theisen, M., Kelln, R. A., and Neuhard, J. *Eur. J. Biochem.*, **164**, 613-619.

10. Barnett, J.A., Payne, R.W. and Yarrow, D. (2000) *Yeasts: Characteristics and Identifcation*. 3rd edn. Cambridge University Press, Cambridge.

11. Fell, J.W. and Statzell-Tallman, A. (1998) Rhodotorula F.C. Harrison. In: *The Yeasts, a Taxonomic Study, 4th edn.*, pp. 800-827. Elsevier Science B.V., Amsterdam. B.V., Amsterdam.

12. Gadanho, M., J.P. Sampaio. *FEMS Yeast Research* **2**. 47-58.

13. Wondrack, L.M., Hsu, C-A., Abbott, M.T. *J. Biol. Chem.* **253,** 6511-6515.

14. Segel, p 400. – footnote.

15. Bradford, M.M. *Anal. Biochem.* **72**, 248-254.

16. Pierce – *EDC/Diaminodipropylamine Immobilization Kit* 44899. 2000.

17. Clontech - *λTriplEx & λTriplEx2 Libraries User Manual.* PT3003-1 (PR09529), p. 43, 2000.

18. Wood, R. I., Gitschier, J., Lasky, L. A. and Lawn, R. M. *Proc. Natl Acad. Sci.,* **82**, 1585-1588.

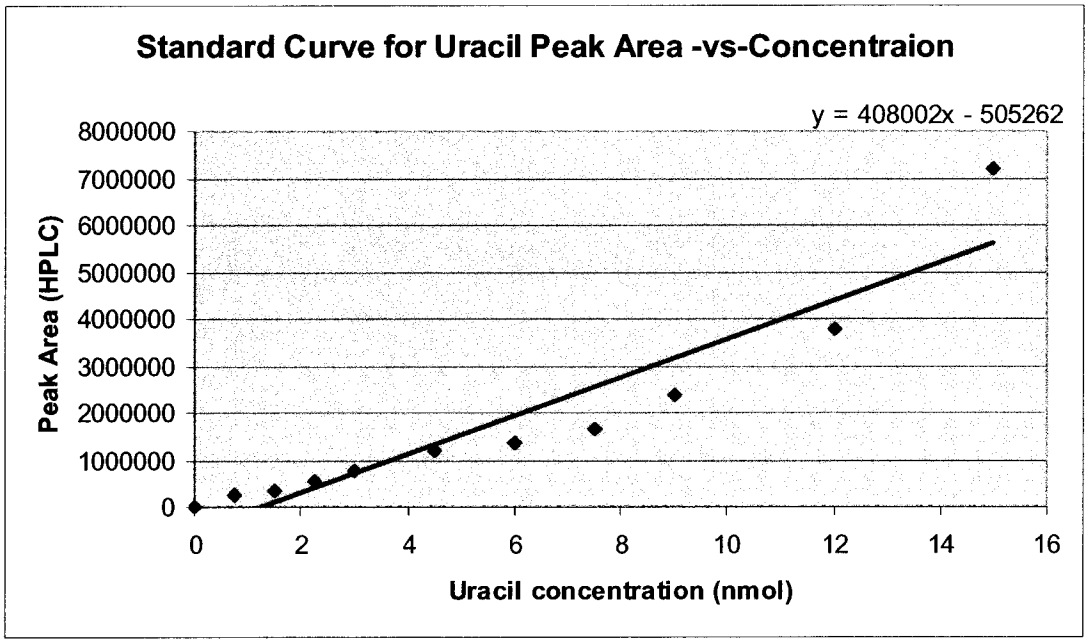
19. Palazzolo, M. J., Hamilton, B. A., Ding D. L., Martin, C. H., Mead, D. A., Mierendorf, R. C., Raghavan, K. V., Meyerowitz, E. M., and Lipshitz, H. D. *Gene*, **1**, 25-36.

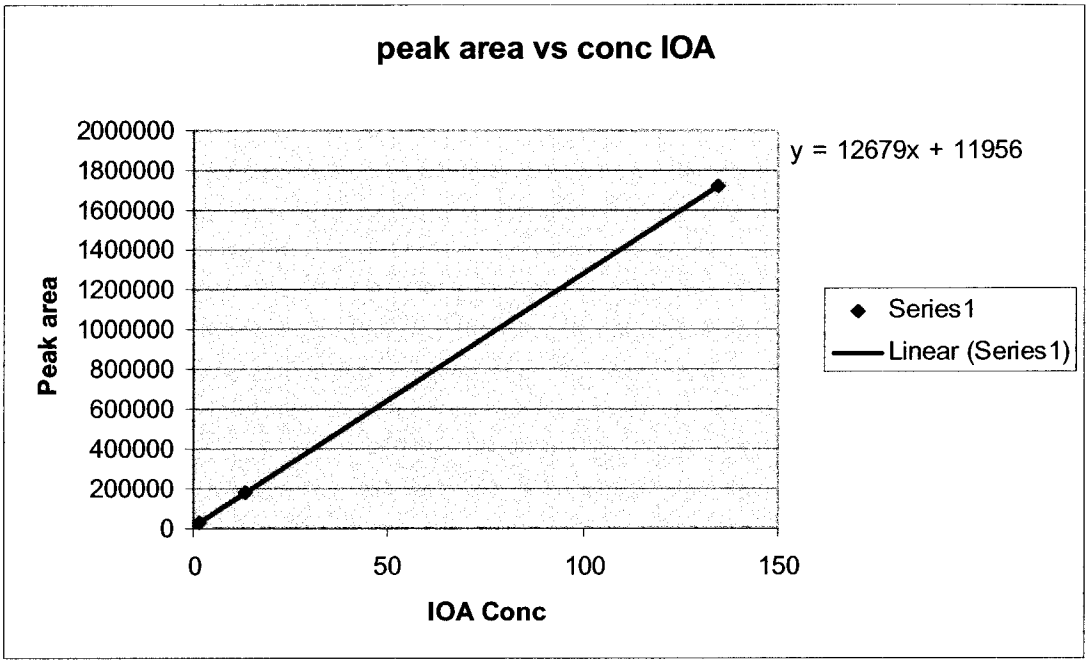
## Appendix A: Non Enzymatic data

Table of the molar concentration injected to establish the standard curve for uracil.

| Uracil Std molar injection | peak area |
|---|---|
| 0 | 0 |
| 0.75 | 247800 |
| 1.5 | 358565 |
| 2.25 | 557986 |
| 3 | 751147 |
| 4.5 | 1189257 |
| 6 | 1377731 |
| 7.5 | 1677149 |

| IOA Std Molar injection nmol | PA |
|---|---|
| 0 | 0 |
| 0.75 | 247800 |
| 1.5 | 358565 |
| 2.25 | 557986 |
| 3 | 751147 |
| 4.5 | 1189257 |
| 6 | 1377731 |
| 7.5 | 1677149 |
| 9 | 2401502 |
| 12 | 3775608 |
| 15 | 7197497 |





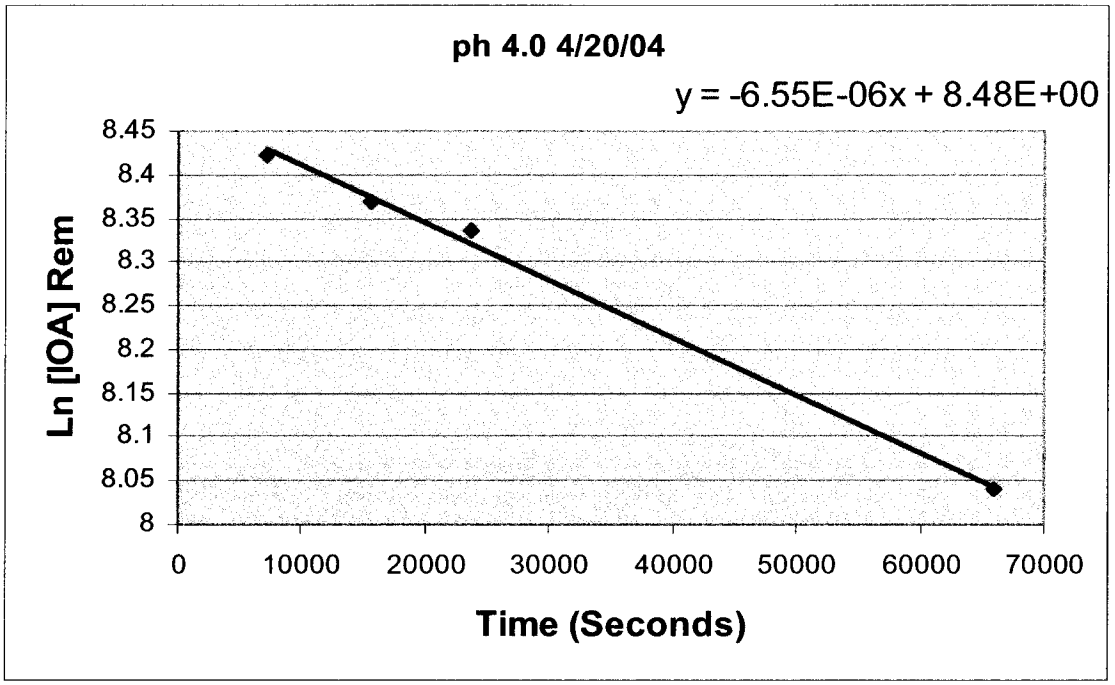

Graph of the injection showing direct proportionality of the concentration injected and the area of the HLPC peak.

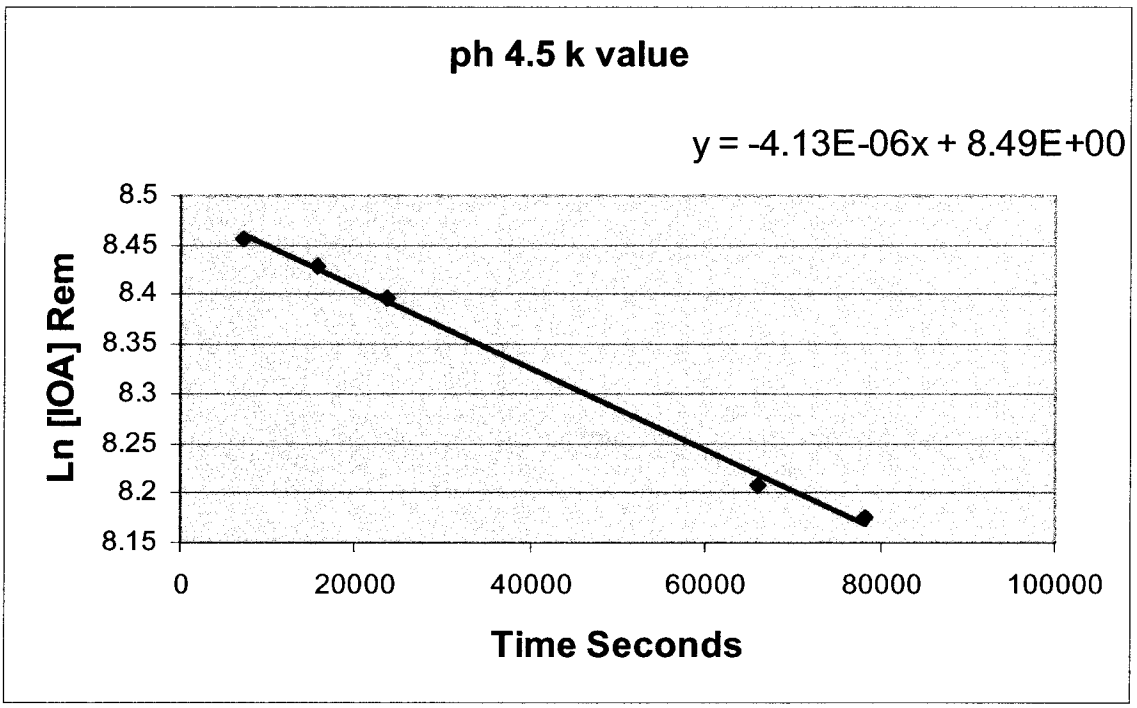
Data gathered from experimental reaction and HPLC.

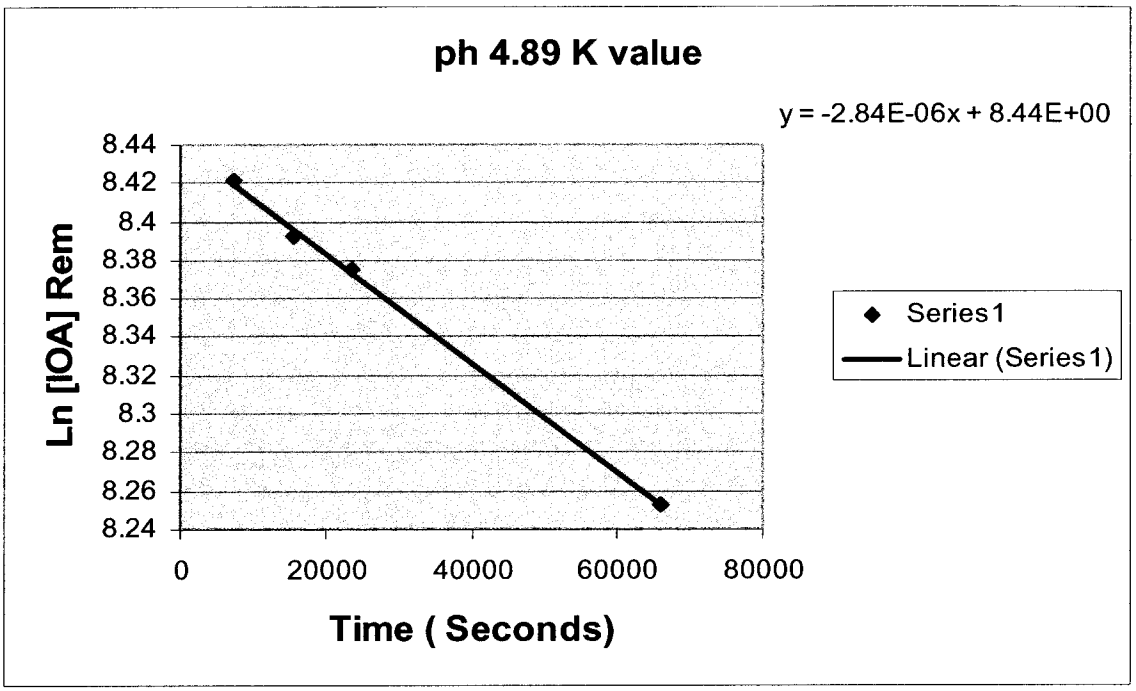
| pH | Peak Area | Std Curve | nmoles uracil | Uracil tube(uM) | IOA REM uM | Ln IOA rem | Min | SEC. | Ln ioa rem |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| 4 | 1175984 | 7.675941 | 2302.782293 | 767.5940976 | 4232.4059 | 8.3505259 | 120 | 7200 | 8.3505259 |
| 4 | 2067246 | 11.74511 | 3523.533064 | 1174.511021 | 3825.489 | 8.2494416 | 135 | 15300 | 8.2494416 |
| 4 | 2570511 | 14.04283 | 4212.849042 | 1404.283014 | 3595.717 | 8.1874987 | 135 | 23400 | 8.1874987 |
| 4 | 2667615 | 14.486171 | 4345.851215 | 1448.617072 | 3551.3829 | 8.1750924 | 280 | 40200 | 8.1750924 |
| 4 | 3256064 | 17.172809 | 5151.842687 | 1717.280896 | 3282.7191 | 8.0964274 | 660 | 79800 | 8.0964274 |
| 4 | 4235828 | 21.646045 | 6493.813576 | 2164.604525 | 2835.3955 | 7.9499367 | 145 | 88500 | 7.9499367 |
| | | | | | | | | | |
| 5.4 | 1978676 | 11.340733 | 3402.219808 | 1134.073269 | 3865.9267 | 8.2599567 | 230 | | 8.2599567 |
| 5.4 | 560598 | 4.8663185 | 1459.895538 | 486.6318462 | 4513.3682 | 8.414799 | 380 | 22800 | 8.414799 |
| 5.4 | 995174 | 6.8504301 | 2055.129025 | 685.0430082 | 4314.957 | 8.3698426 | 640 | 61200 | 8.3698426 |
| 5.4 | 1610540 | 9.6599613 | 2897.988385 | 965.9961283 | 4034.0039 | 8.3025147 | 1189 | 132540 | 8.3025147 |
| 5.4 | 1964084 | 11.274111 | 3382.233322 | 1127.411107 | 3872.5889 | 8.2616785 | 1324 | 211980 | 8.2616785 |

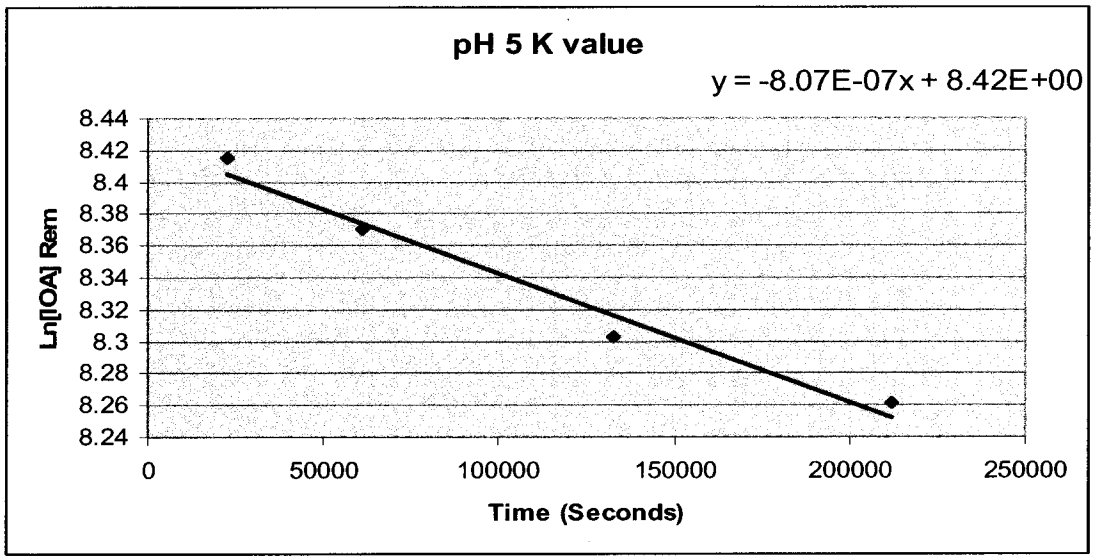
| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| 6.34 | 457861 | 4.3972597 | 1319.177913 | 439.7259711 | 4560.274 | 8.425138 | 230 | 13800 | 8.425138 |
| 6.34 | 1056590 | 7.1308326 | 2139.249776 | 713.0832588 | 4286.9167 | 8.363323 | 380 | 36600 | 8.363323 |
| 6.34 | 1606253 | 9.6403884 | 2892.116533 | 964.0388443 | 4035.9612 | 8.3029998 | 640 | 75000 | 8.3029998 |
| 6.34 | 2311747 | 12.86141 | 3858.423124 | 1286.141041 | 3713.859 | 8.2198268 | 1189 | 146340 | 8.2198268 |
| 6.34 | 895926 | 6.3973008 | 1919.190241 | 639.7300802 | 4360.2699 | 8.3802892 | 1324 | | 8.3802892 |
| | | | | | | | | | |
| 7 | 691536 | 0.2449133 | 73.47398862 | 24.49132954 | 6402.526 | 8.7644479 | 66 | 3960 | 8.7644479 |
| 7 | 11880332 | 2.6700923 | 801.0276799 | 267.0092266 | 5674.9723 | 8.643821 | 455 | 31260 | 8.643821 |
| 7 | 12777579 | 2.8645712 | 859.371345 | 286.457115 | 5616.6287 | 8.6334869 | 210 | 43860 | 8.6334869 |
| 7 | 18171600 | 4.0337288 | 1210.118636 | 403.3728788 | 5265.8814 | 8.5690038 | 250 | 58860 | 8.5690038 |
| 7 | 21448098 | 4.7439119 | 1423.173572 | 474.3911907 | 5052.8264 | 8.5277031 | 350 | 79860 | 8.5277031 |
| | | | | | | | | | |
| 8 | 1206376 | 0.3565052 | 106.9515623 | 35.65052077 | 6369.0484 | 8.7592054 | 66 | | 8.7592054 |
| 8 | 666856 | 0.2395639 | 71.8691667 | 23.9563889 | 6404.1308 | 8.7646985 | 455 | 27300 | 8.7646985 |
| 8 | 1579922 | 0.4374716 | 131.2414654 | 43.74715515 | 6344.7585 | 8.7553843 | 210 | 39900 | 8.7553843 |
| 8 | 2743301 | 0.6896347 | 206.8904169 | 68.96347231 | 6269.1096 | 8.7433896 | 250 | 54900 | 8.7433896 |
| 8 | 3407956 | 0.8336991 | 250.1097409 | 83.36991362 | 6225.8903 | 8.7364717 | 350 | 75900 | 8.7364717 |
| | | | | | | | | | |
| 9 | 155229 | 3.0155551 | 904.6665267 | 301.5555089 | 5571.3335 | 8.6253897 | 66 | 3960 | 8.6253897 |
| 9 | 1102000 | 7.3381577 | 2201.447304 | 733.815768 | 4274.5527 | 8.3604347 | 455 | 31260 | 8.3604347 |
| 9 | 1298048 | 2.2332396 | 2469.9718 | 823.3239333 | 4006.0282 | 8.2955556 | 210 | 43860 | 8.2955556 |
| 9 | 1530843 | 9.2609456 | 2788.828 | 929.6093333 | 3687.172 | 8.212615 | 250 | 58860 | 8.212615 |
| 9 | 2295307 | 12.786352 | 3835.905455 | 1278.635152 | 2640.0945 | 7.87857 | 350 | 79860 | 7.87857 |
| | | | | | | | | | |
| 10 | 1379215 | 5.9223159 | 1776.69476 | 592.2315868 | 4407.7684 | 8.3911238 | 90 | 5400 | 8.3911238 |
| 10 | 1697396 | 7.3750114 | 2212.503424 | 737.5011414 | 4262.4989 | 8.3576109 | 180 | 10800 | 8.3576109 |
| 10 | 2411299 | 10.634426 | 3190.327721 | 1063.442574 | 3936.5574 | 8.2780619 | 270 | 16200 | 8.2780619 |
| 10 | 2872991 | 12.742339 | 3822.701664 | 1274.233888 | 3725.7661 | 8.2230278 | 360 | 21600 | 8.2230278 |
| | | | | | | | | | |
| pH | Peak Area | Std Curve | nmoles Uracil | Uracil in tube | IOA Rem | Ln IOA Rem | Time Min | Time Sec | Ln IOA Rem |
| 11 | 440381 | 1.6359507 | 490.7851964 | 163.5950655 | 4836.4049 | 8.4839269 | 90 | 5400 | 8.4839269 |
| 11 | 740803 | 3.0075652 | 902.2695728 | 300.7565243 | 4699.2435 | 8.4551568 | 180 | 10800 | 8.4551568 |
| 11 | 2237900 | 9.8427507 | 2952.82521 | 984.2750699 | 4015.7249 | 8.2979732 | 270 | 16200 | 8.2979732 |
| 11 | 4397040 | 9.8427507 | 5910.173129 | 1970.05771 | 3029.9423 | 8.0162989 | 360 | 21600 | 8.0162989 |
| | | | | | | | | | |
| 12 | 440381 | 4.3174526 | 1295.235769 | 431.7452563 | 4568.2547 | 8.4268865 | 115 | 6900 | 8.4268865 |
| 12 | 740803 | 5.6890672 | 1706.720145 | 568.9067151 | 4431.0933 | 8.3964016 | 115 | 13800 | 8.3964016 |
| 12 | 2237900 | 12.524253 | 3757.275782 | 1252.425261 | 3747.5747 | 8.2288642 | 142 | 22320 | 8.2288642 |
| 12 | 4397040 | 22.382079 | 6714.623701 | 2238.2079 | 2761.7921 | 7.9236351 | 149 | 31260 | 7.9236351 |
| 12 | 5295800 | 26.485479 | 7945.643708 | 2648.547903 | 2351.4521 | 7.7627883 | 138 | 39540 | 7.7627883 |
| | | | | | | | | | |
| 13 | 910395 | 6.4633608 | 1939.008255 | 646.3360849 | 4353.6639 | 8.378773 | 60 | 3600 | 8.378773 |
| 13 | 2223158 | 12.456946 | 3737.083843 | 1245.694614 | 3754.3054 | 8.2306586 | 120 | 10800 | 8.2306586 |
| 13 | 2874999 | 15.433009 | 4629.90257 | 1543.300857 | 3456.6991 | 8.1480694 | 115 | 17700 | 8.1480694 |
| 13 | 7516582 | 36.624742 | 10987.42261 | 3662.474204 | 1337.5258 | 7.1985768 | 353 | 38880 | 7.1985768 |
| 13 | 7504849 | 36.571174 | 10971.35206 | 3657.117355 | 1342.8826 | 7.2025738 | 540 | 71280 | 7.2025738 |

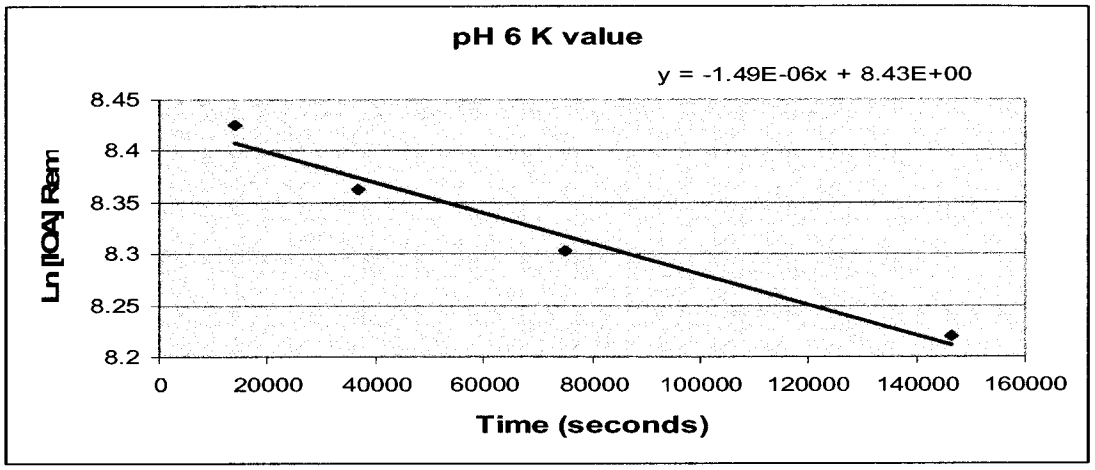
The graphs and the correspond equation of the line printed on the graph represent the K value for that pH trial.

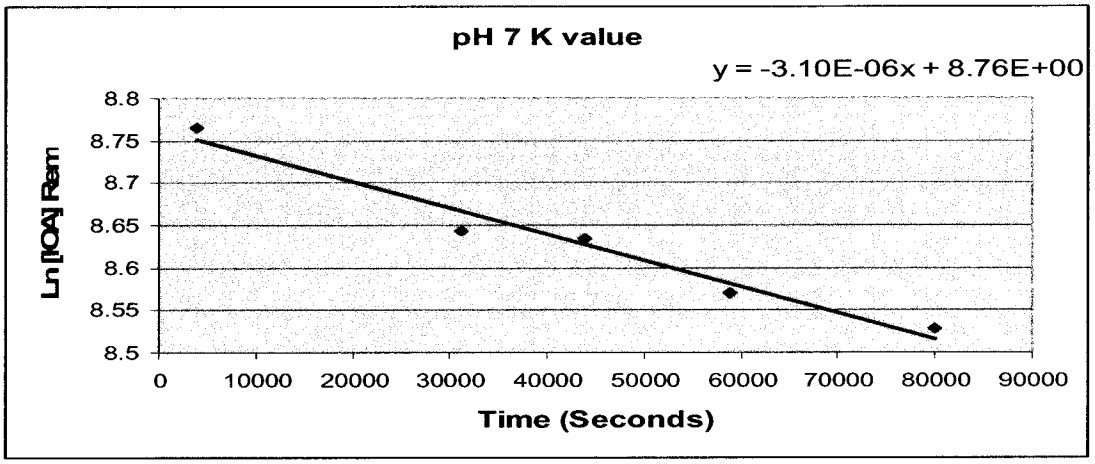


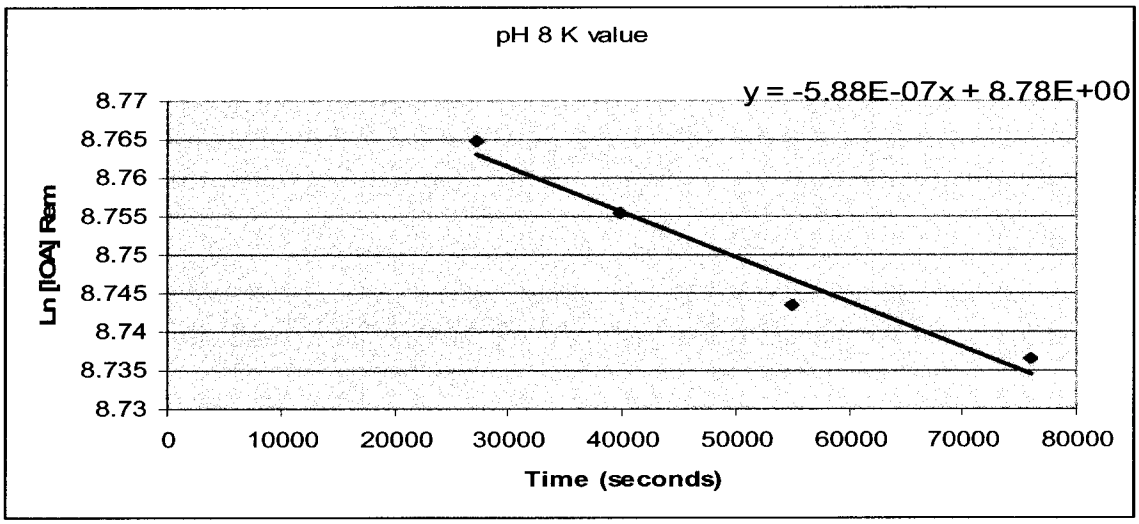



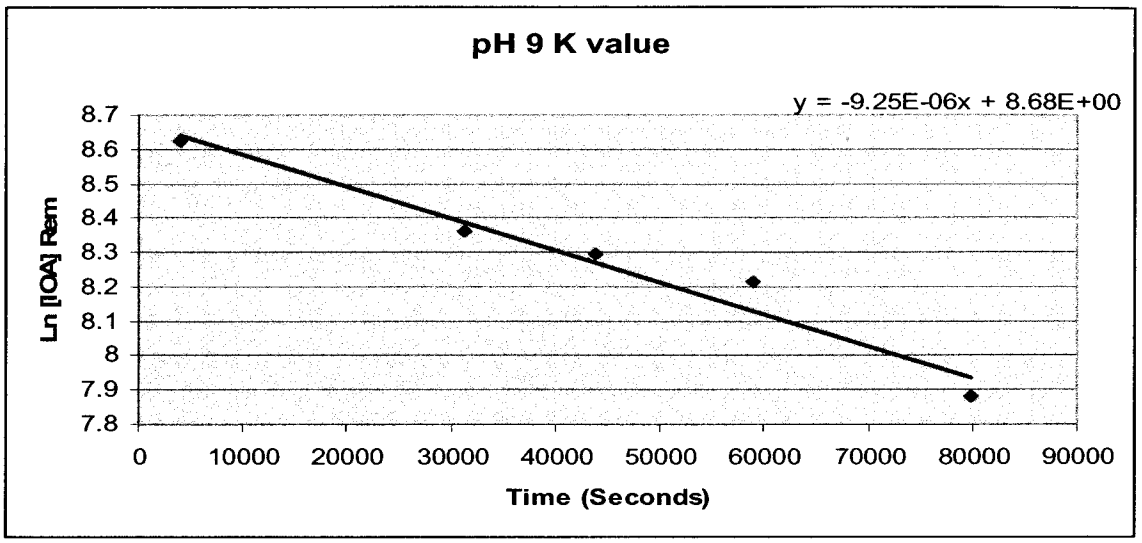

ph 4.89 K value
y = -2.84E-06x + 8.44E+00
Ln [IOA] Rem
8.44
8.42
8.4
8.38
8.36
8.34
8.32
8.3
8.28
8.26
8.24
0
20000
40000
60000
80000
Time ( Seconds)
Series1
Linear (Series1)

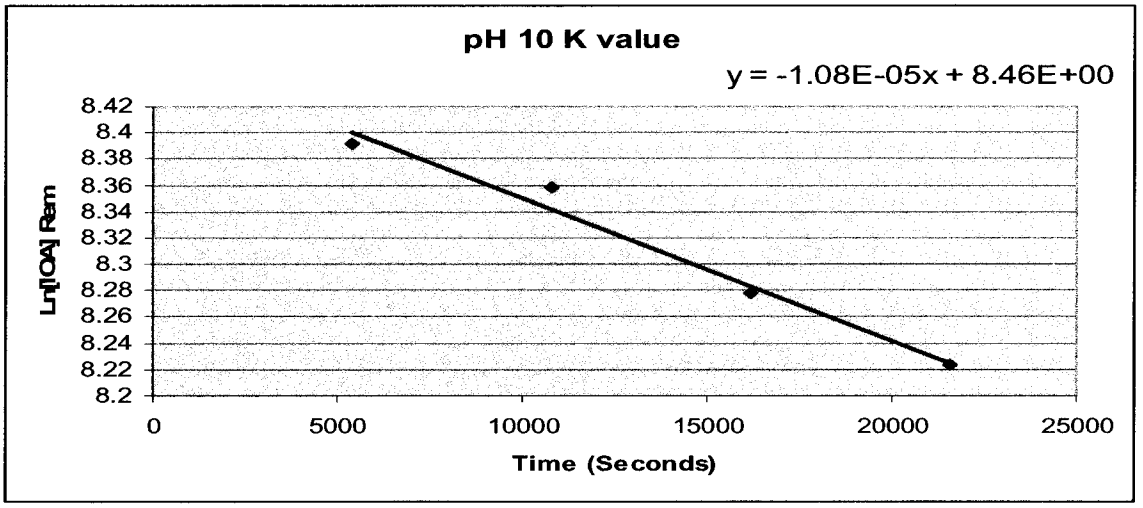

pH 5 K value
y = -8.07E-07x + 8.42E+00
Ln[IOA] Rem
8.44
8.42
8.4
8.38
8.36
8.34
8.32
8.3
8.28
8.26
8.24
0
50000
100000
150000
200000
250000
Time (Seconds)

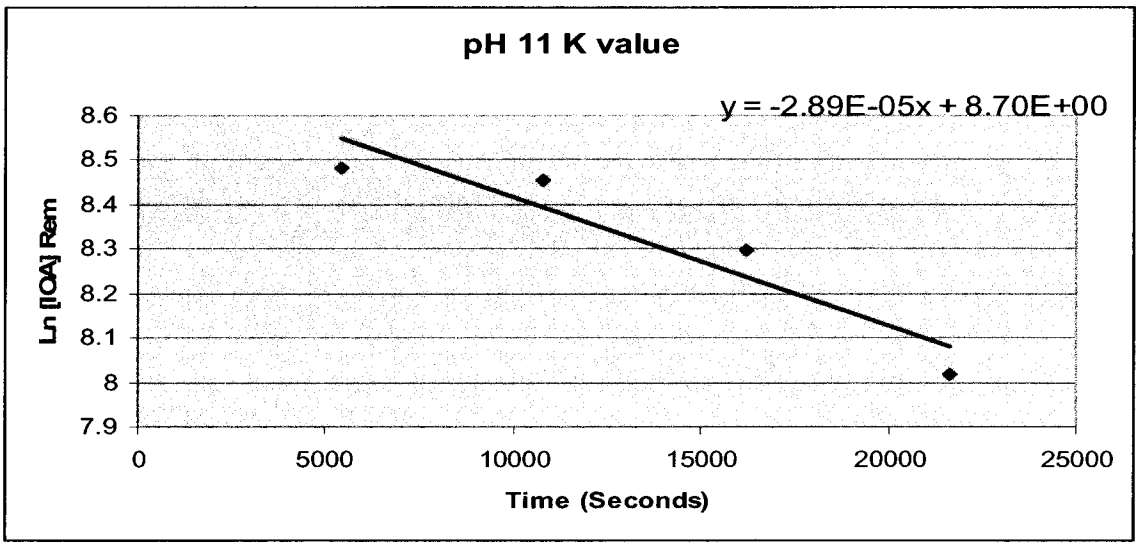

pH 6 K value
y = -1.49E-06x + 8.43E+00
Ln [IOA] Rem
8.45
8.4
8.35
8.3
8.25
8.2
0
20000
40000
60000
80000
100000
120000
140000
160000
Time (seconds)

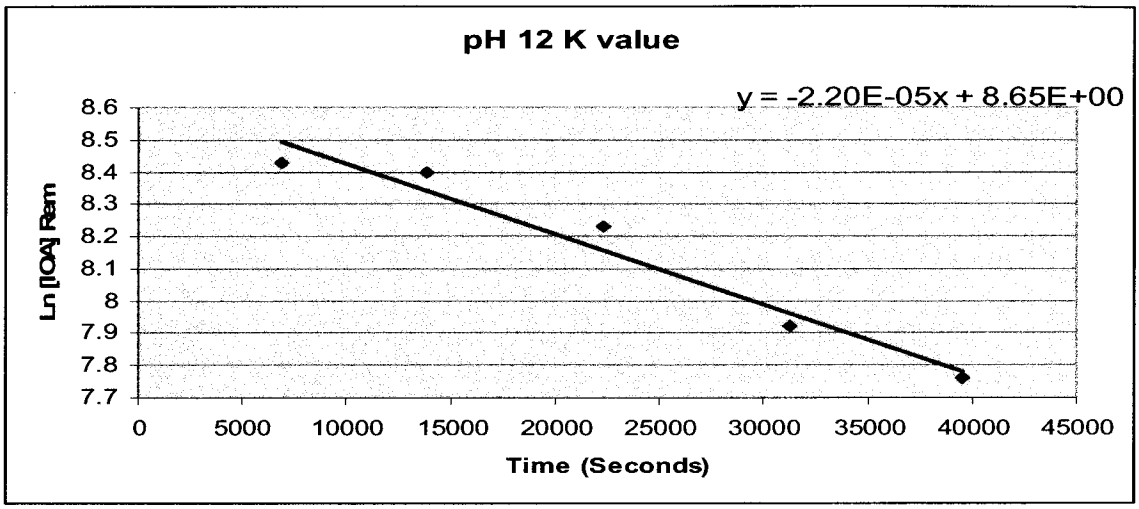

pH 7 K value
y = -3.10E-06x + 8.76E+00
Ln [IOA] Rem
8.8
8.75
8.7
8.65
8.6
8.55
8.5
0
10000
20000
30000
40000
50000
60000
70000
80000
90000
Time (Seconds)

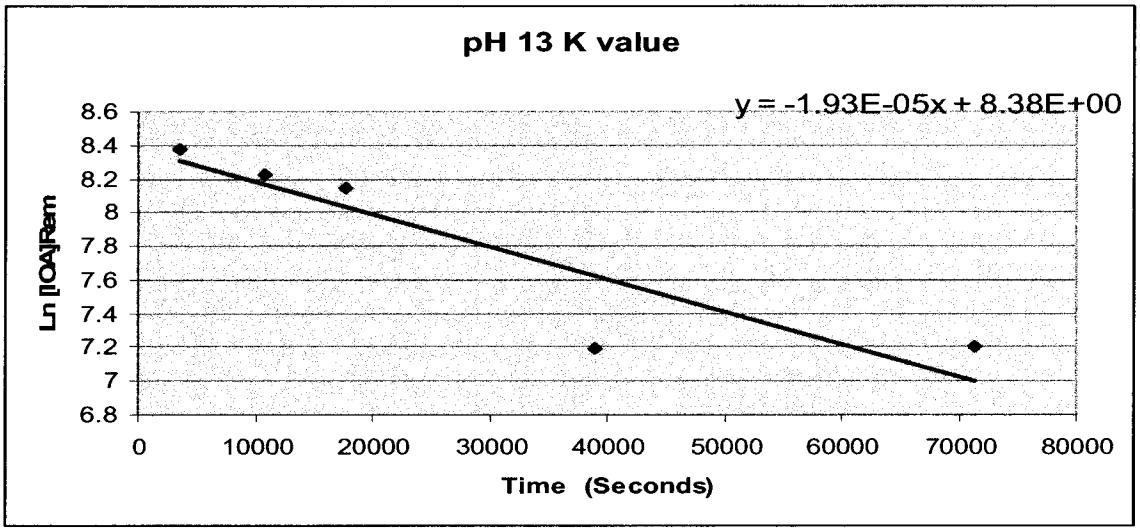

pH 8 K value
y = -5.88E-07x + 8.78E+00
Ln [IOA] Rem
8.77
8.765
8.76
8.755
8.75
8.745
8.74
8.735
8.73
0
10000
20000
30000
40000
50000
60000
70000
80000
Time (seconds)


pH 9 K value
y = -9.25E-06x + 8.68E+00
Ln [IOA] Rem
8.7
8.6
8.5
8.4
8.3
8.2
8.1
8
7.9
7.8
0
10000
20000
30000
40000
50000
60000
70000
80000
90000
Time (Seconds)


pH 10 K value
y = -1.08E-05x + 8.46E+00
Ln[IOA] Rem
8.42
8.4
8.38
8.36
8.34
8.32
8.3
8.28
8.26
8.24
8.22
8.2
0
5000
10000
15000
20000
25000
Time (Seconds)

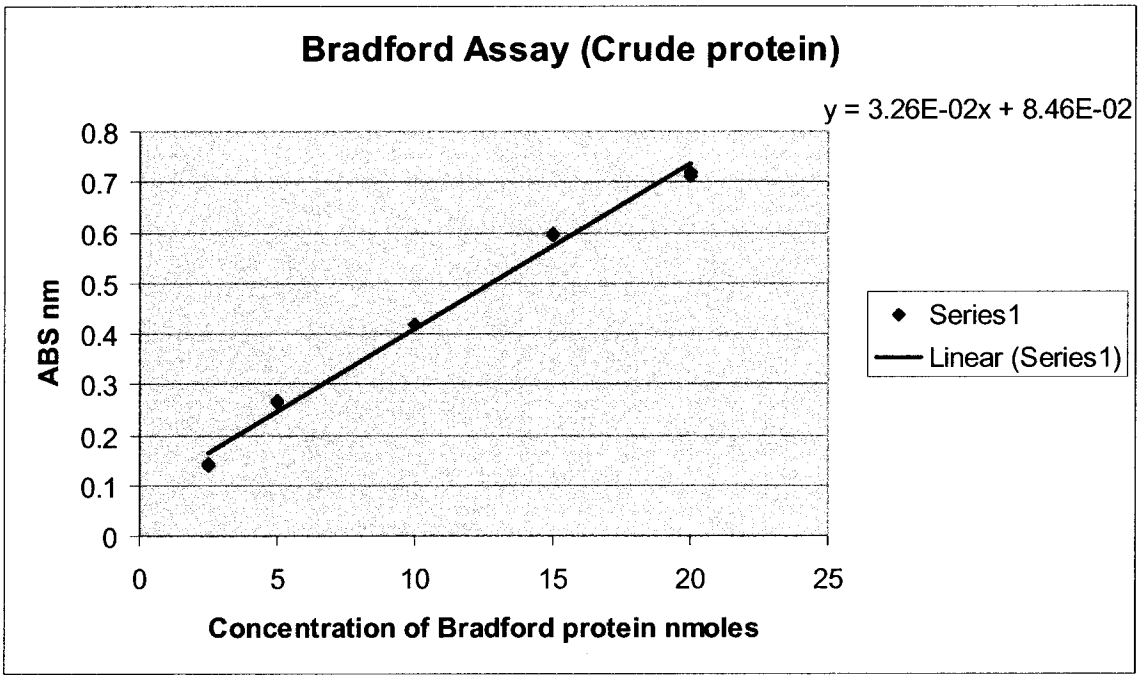

pH 11 K value
y = -2.89E-05x + 8.70E+00
Ln [IOA] Rem
8.6
8.5
8.4
8.3
8.2
8.1
8
7.9
0
5000
10000
15000
20000
25000
Time (Seconds)


pH 12 K value
y = -2.20E-05x + 8.65E+00
Ln [IOA] Ram
8.6
8.5
8.4
8.3
8.2
8.1
8
7.9
7.8
7.7
0
5000
10000
15000
20000
25000
30000
35000
40000
45000
Time (Seconds)


pH 13 K value
y = -1.93E-05x + 8.38E+00
Ln [IOA]Ram
8.6
8.4
8.2
8
7.8
7.6
7.4
7.2
7
6.8
0
10000
20000
30000
40000
50000
60000
70000
80000
Time (Seconds)

Appendix B: The Enzymatic data

| | |
|---|---|
| 2.5 | 0.14424 |
| 2.5 | 0.13822 |
| 5 | 0.26394 |
| 5 | 0.26609 |
| 10 | 0.41792 |
| 10 | 0.41843 |
| 15 | 0.59301 |
| 15 | 0.59642 |
| 20 | 0.71338 |
| 20 | 0.72017 |




**Error! Not a valid link.**

| Sample | [Ioa] | cpm/ul | total | cpm Counted | (-) background | nmol | nmol/min | ul in assay | 1/s |
|---|---|---|---|---|---|---|---|---|---|
| ph 5.5 | 71 | 140 | 9940 | 633 | 583 | 5.83 | 1.166 | 5 | 0.01 |
| 2 | 71 | 140 | 9940 | 506 | 456 | 4.56 | 0.912 | 5 | 0.01 |
| 3 | 36 | 140 | 5040 | 333 | 283 | 2.83 | 0.566 | 5 | 0.02 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 4 | 36 | 140 | 5040 | 365 | 315 | 3.15 | 0.63 | 5 | 0.02 |
| 5 | 18 | 466 | 8388 | 737 | 687 | 2.064 | 0.412861 | 5 | 0.04 |
| 6 | 18 | 466 | 8388 | 583 | 533 | 1.602 | 0.320313 | 5 | 0.04 |
| 7 | 12 | 466 | 5592 | 518 | 468 | 1.406 | 0.28125 | 5 | 0.05988 |
| 8 | 12 | 466 | 5592 | 468 | 418 | 1.256 | 0.251202 | 5 | 0.05988 |
| 9 | 9 | 466 | 4194 | 519 | 469 | 1.409 | 0.281851 | 5 | 0.08 |
| | | | | | | | | | |
| 10 | 9 | 466 | 4194 | 476 | 426 | 1.28 | 0.25601 | 5 | 0.08 |
| 11 | 7.1 | 466 | 3308.6 | 382 | 332 | 0.998 | 0.199519 | 5 | 0.1 |
| 12 | 7.1 | 466 | 3308.6 | 406 | 356 | 1.07 | 0.213942 | 5 | 0.1 |
| | | | | | | | | | |
| ph 6.0 | 71 | 140 | 9940 | 170 | 120 | 1.2 | 0.24 | 5 | 0.01 |
| 14 | 71 | 140 | 9940 | 160 | 110 | 1.1 | 0.22 | 5 | 0.01 |
| 15 | 36 | 140 | 5040 | 167 | 117 | 1.17 | 0.234 | 5 | 0.02 |
| 16 | 36 | 140 | 5040 | 149 | 99 | 0.99 | 0.198 | 5 | 0.02 |
| 17 | 18 | 466 | 8388 | 657 | 607 | 1.824 | 0.364784 | 5 | 0.04 |
| 18 | 18 | 466 | 8388 | 282 | 232 | 0.697 | 0.139423 | 5 | 0.04 |
| 19 | 12 | 466 | 5592 | 205 | 155 | 0.466 | 0.093149 | 5 | 0.05988 |
| 20 | 12 | 466 | 5592 | 304 | 254 | 0.763 | 0.152644 | 5 | 0.05988 |
| 21 | 9 | 466 | 4194 | 211 | 161 | 0.484 | 0.096755 | 5 | 0.08 |
| 22 | 9 | 466 | 4194 | 324 | 274 | 0.823 | 0.164663 | 5 | 0.08 |
| 23 | 7.1 | 466 | 3308.6 | 276 | 226 | 0.679 | 0.135817 | 5 | 0.1 |
| 24 | 7.1 | 466 | 3308.6 | 279 | 229 | 0.688 | 0.13762 | 5 | 0.1 |
| | | | | | | | | | |
| ph 6.5 | 71 | 140 | 9940 | 535 | 485 | 4.85 | 0.97 | 5 | 0.01 |
| 26 | 71 | 140 | 9940 | 306 | 256 | 2.56 | 0.512 | 5 | 0.01 |
| 27 | 36 | 140 | 5040 | 399 | 349 | 3.49 | 0.698 | 5 | 0.02 |
| 28 | 36 | 140 | 5040 | 382 | 332 | 3.32 | 0.664 | 5 | 0.02 |
| 29 | 18 | 466 | 8388 | 1083 | 1033 | 3.104 | 0.620793 | 5 | 0.04 |
| 30 | 18 | 466 | 8388 | 555 | 505 | 1.517 | 0.303486 | 5 | 0.04 |
| 31 | 12 | 466 | 5592 | 830 | 780 | 2.344 | 0.46875 | 5 | 0.05988 |
| 32 | 12 | 466 | 5592 | 907 | 857 | 2.575 | 0.515024 | 5 | 0.05988 |
| 33 | 9 | 466 | 4194 | 583 | 533 | 1.602 | 0.320313 | 5 | 0.08 |
| 34 | 9 | 466 | 4194 | 617 | 567 | 1.704 | 0.340745 | 5 | 0.08 |
| 35 | 7.1 | 466 | 3308.6 | 594 | 544 | 1.635 | 0.326923 | 5 | 0.1 |
| 36 | 7.1 | 466 | 3308.6 | 707 | 657 | 1.974 | 0.394832 | 5 | 0.1 |
| | | | | 0 | -50 | -0.15 | | | |
| ph 10.0 | 71 | 140 | 9940 | 146 | 96 | 0.96 | 0.192 | 5 | 0.01 |
| 38 | 71 | 140 | 9940 | 120 | 70 | 0.7 | 0.14 | 5 | 0.01 |
| 39 | 36 | 140 | 5040 | 110 | 60 | 0.6 | 0.12 | 5 | 0.02 |
| 40 | 36 | 140 | 5040 | 122 | 72 | 0.72 | 0.144 | 5 | 0.02 |
| 41 | 18 | 466 | 8388 | 184 | 134 | 0.403 | 0.080529 | 5 | 0.04 |
| 42 | 18 | 466 | 8388 | 272 | 222 | 0.667 | 0.133413 | 5 | 0.04 |
| 43 | 12 | 466 | 5592 | 183 | 133 | 0.4 | 0.079928 | 5 | 0.05988 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 44 | 12 | 466 | 5592 | 128 | 78 | 0.234 | 0.046875 | 5 | 0.05988 |
| 45 | 9 | 466 | 4194 | 169 | 119 | 0.358 | 0.071514 | 5 | 0.08 |
| 46 | 9 | 466 | 4194 | 183 | 133 | 0.4 | 0.079928 | 5 | 0.08 |
| 47 | 7.1 | 466 | 3308.6 | 170 | 120 | 0.361 | 0.072115 | 5 | 0.1 |
| 48 | 7.1 | 466 | 3308.6 | 155 | 105 | 0.316 | 0.063101 | 5 | 0.1 |

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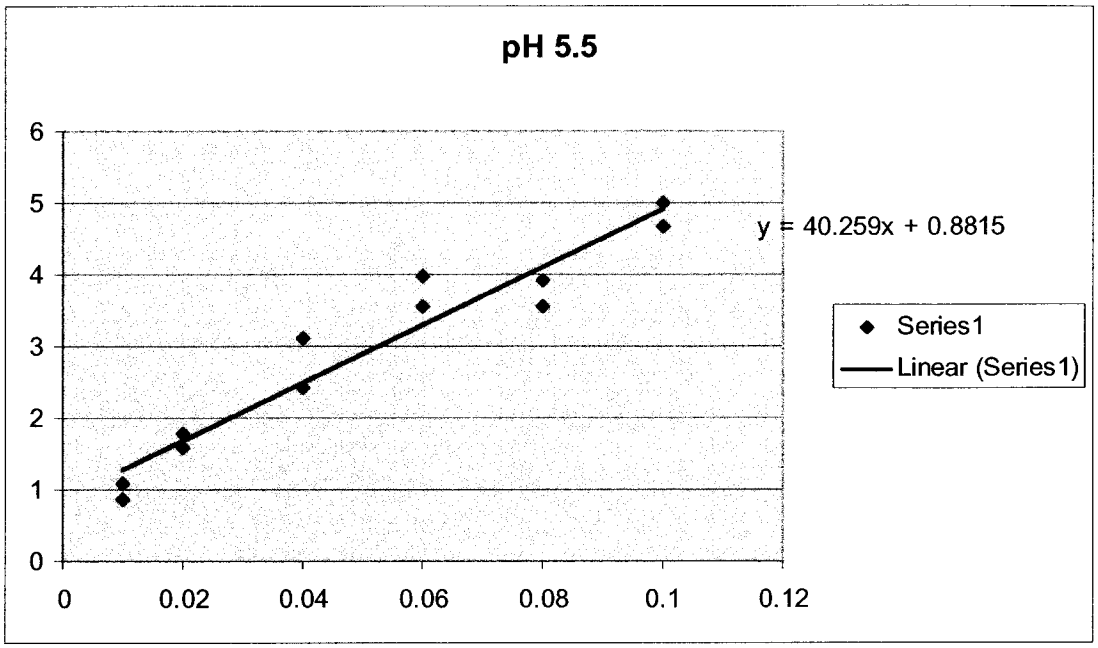
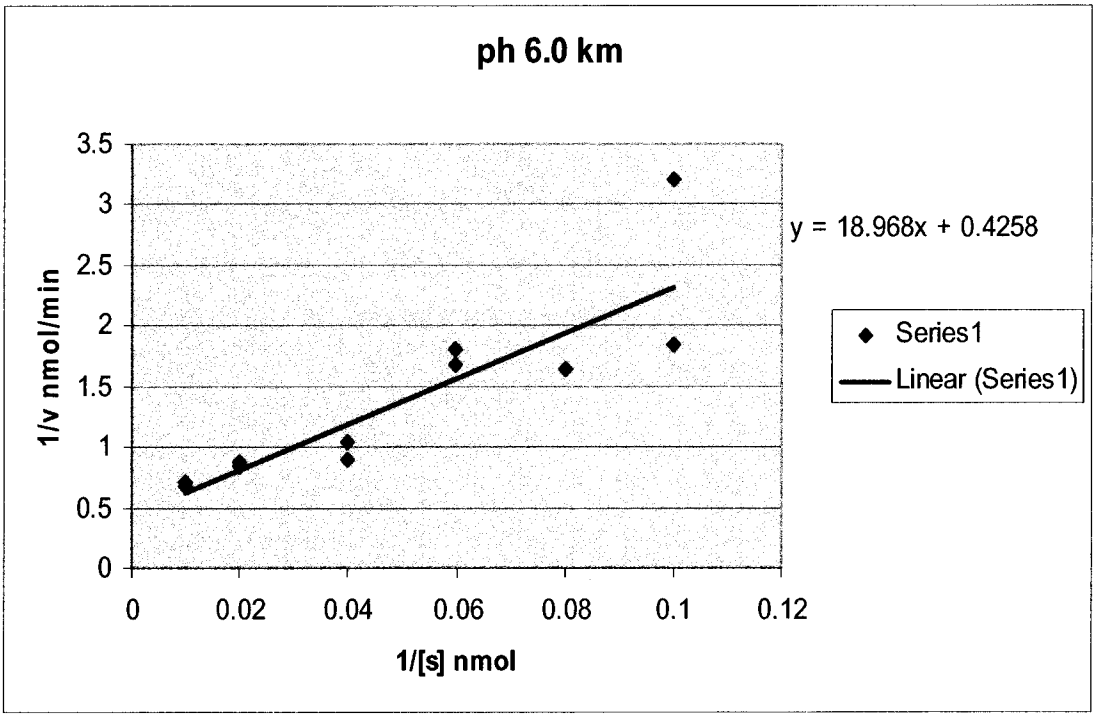
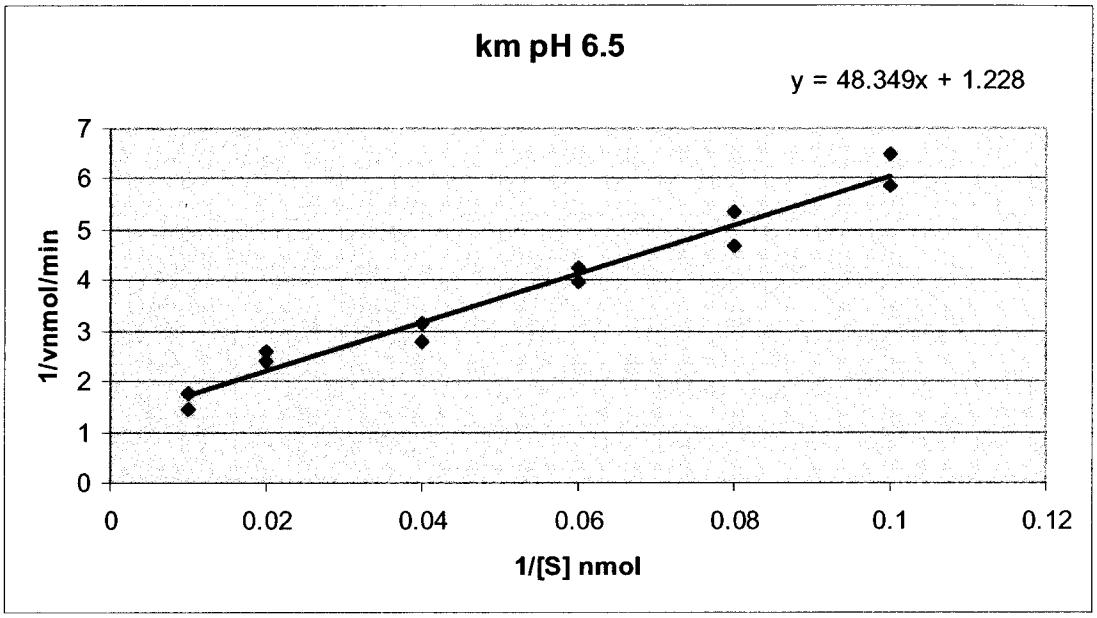
| pH | Km | 1/Vmax | Vmax | log (v/k) | log Vmax |
|---|---|---|---|---|---|
| 5.5 | 45.671 | 1.763 | 0.567215 | -1.90589 | -0.24625 |
| 6 | 44.548 | 0.8516 | 1.17426 | -1.57906 | 0.069764 |
| 6.5 | 15.279 | 2.3423 | 0.426931 | -1.55374 | -0.36964 |
| 7 | 50.923 | 1.334 | 0.749625 | -1.83207 | -0.12516 |
| 8 | 35.611 | 1.5249 | 0.655781 | -1.73483 | -0.18324 |
| 9 | 121.176 | 1.0634 | 0.94038 | -2.11011 | -0.0267 |
| 10 | 16.562 | 11.47 | 0.087184 | -2.27868 | -1.05956 |

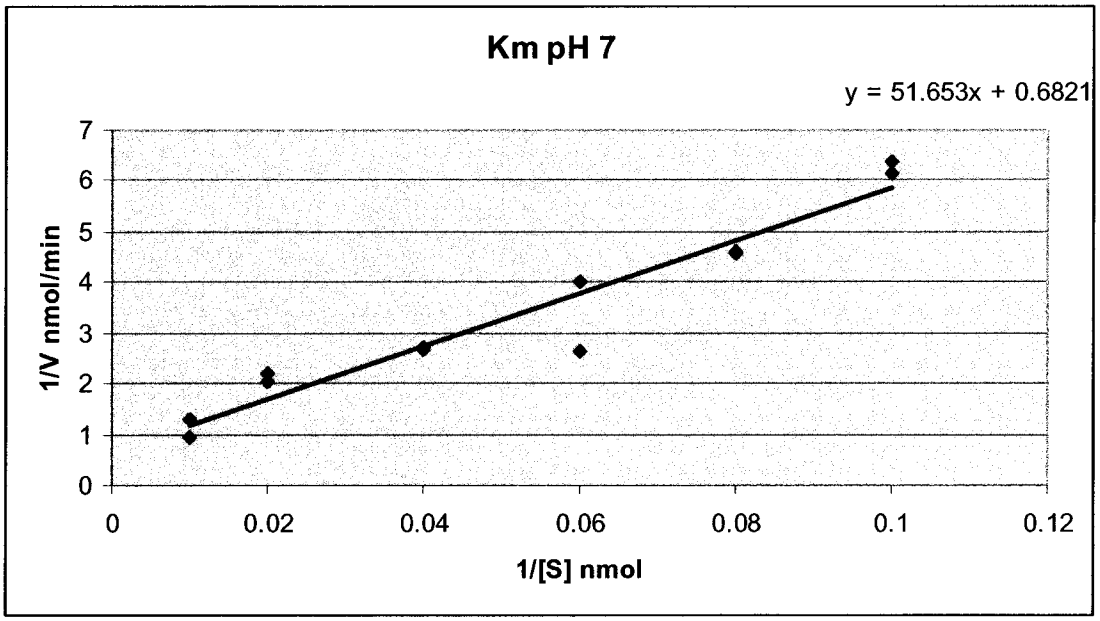
Table of the data, original and converted, for use in plotting the kinetic properties of the enzymatic reactions.
The following graphs represent the data from the assaying of the crude IDCase protein

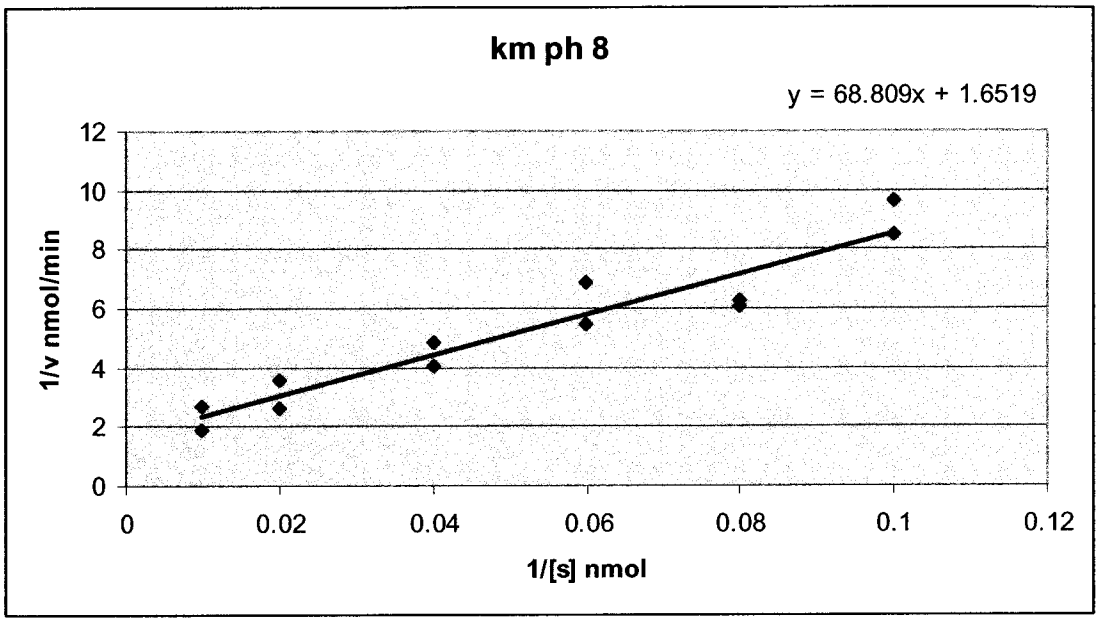
and the equations in the right hand corner are the equations used to interpret the results.

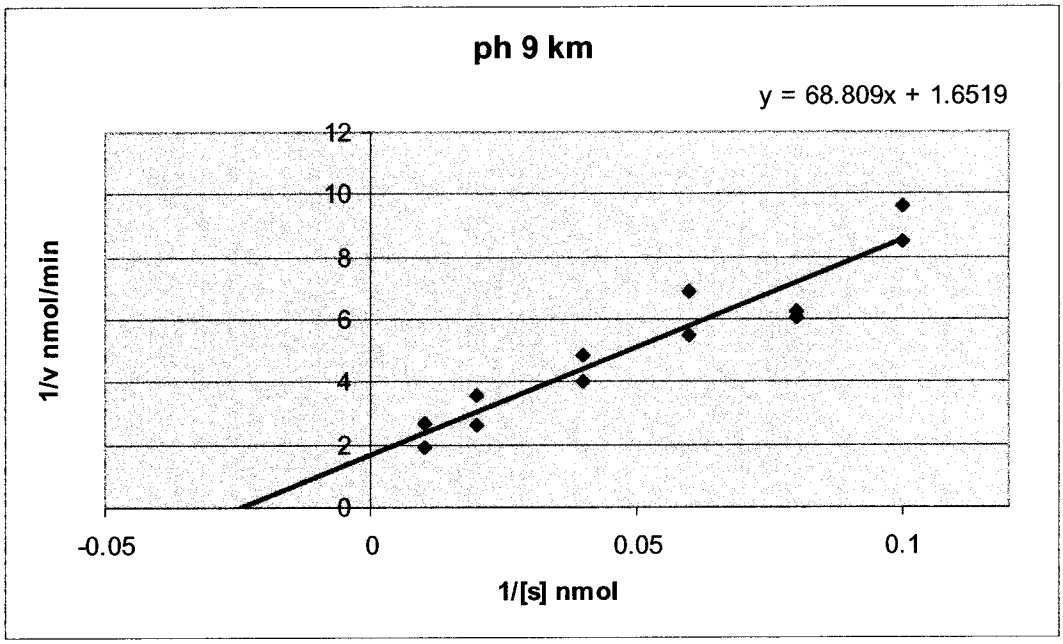

pH 5.5
y = 40.259x + 0.8815
Series1
Linear (Series1)
0
1
2
3
4
5
6
0
0.02
0.04
0.06
0.08
0.1
0.12

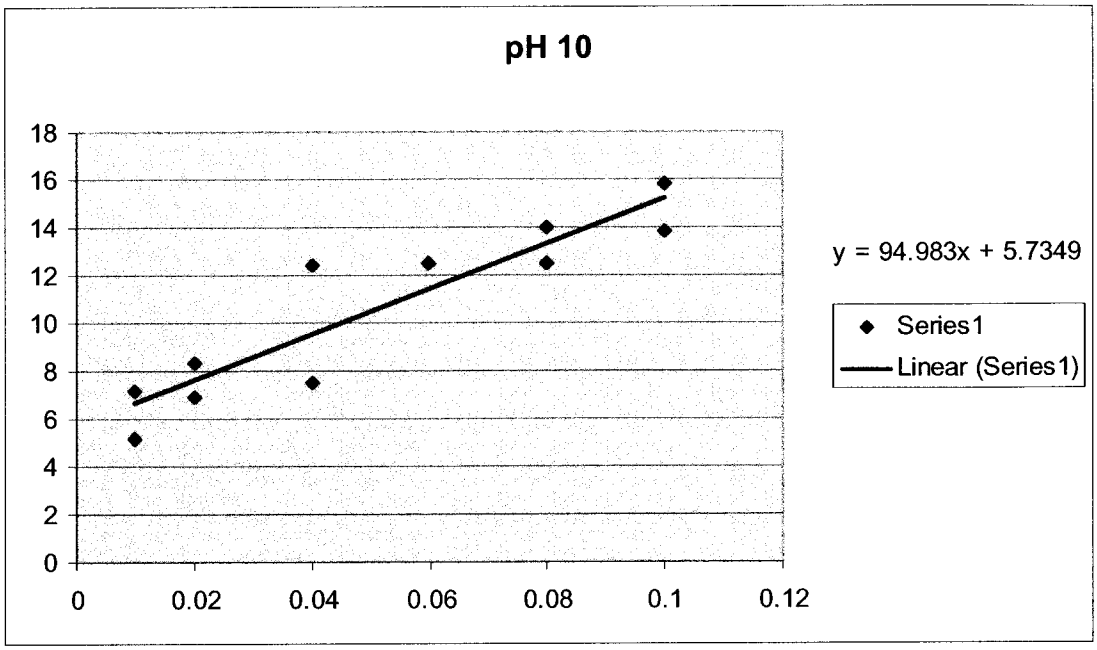

ph 6.0 km
y = 18.968x + 0.4258
Series1
Linear (Series1)
1/v nmol/min
1/[s] nmol
0
0.5
1
1.5
2
2.5
3
3.5
0
0.02
0.04
0.06
0.08
0.1
0.12


km pH 6.5
y = 48.349x + 1.228
1/vnmol/min
1/[S] nmol
0
1
2
3
4
5
6
7
0
0.02
0.04
0.06
0.08
0.1
0.12


Km pH 7
y = 51.653x + 0.6821
1/V nmol/min
1/[S] nmol
0
1
2
3
4
5
6
7
0
0.02
0.04
0.06
0.08
0.1
0.12


km ph 8
y = 68.809x + 1.6519
12
10
8
6
4
2
0
1/v nmol/min
0
0.02
0.04
0.06
0.08
0.1
0.12
1/[s] nmol


ph 9 km
y = 68.809x + 1.6519
12
10
8
6
4
2
0
1/v nmol/min
-0.05
0
0.05
0.1
1/[s] nmol


pH 10
18
16
14
12
10
8
6
4
2
0
0
0.02
0.04
0.06
0.08
0.1
0.12
y = 94.983x + 5.7349
Series1
Linear (Series1)

Appendix C: $^{14}C$ assay preparations

## IDCase Assay Mixes

Low Specific Activity (for high conc. assays):

300 µL 50 mM iso-orotate
13.6 µL [$^{14}C$] iso-orotate
$dH_2O$ to ~14 mL
Add 100 µL 2 M HCl; check acidity with pH paper; let sit in hood for 1 hour
Add 100 µL 2 M NaOH; check neutral pH; adjust to neutral pH as needed
Bring to final volume of 15 mL

Expected IOA conc.: 1 mM
Check with UV spec: dilute 50 µL into 950 µL buffer or water
Expected $A_{270} = 0.5$
Calculate exact conc. based on $A_{270}$

Expected cpm: 6000 cpm per 50 µL

Expected cpm in high conc. assays:

| Final conc.: | 100 µM | 75 µM | 50 µM |
|---|---|---|---|
| Volume in 500 µL: | 50 µL | 37.5 µL | 25 µL |
| cpm in assay | 6000 | 4500 | 3000 |

High Specific Activity (for low conc. assays):

300 µL 50 mM iso-orotate
68 µL [$^{14}$C] iso-orotate
$dH_2O$ to ~14 mL
Add 100 µL 2 M HCl; check acidity with pH paper; let sit in hood for 1 hour
Add 100 µL 2 M NaOH; check neutral pH; adjust to neutral pH as needed
Bring to final volume of 15 mL

Expected IOA conc.: 1 mM
Check with UV spec: dilute 50 µL into 950 µL buffer or water
Expected $A_{270} = 0.5$
Calculate exact conc. based on $A_{270}$

Expected cpm: 3000 cpm per 5 µL

Expected cpm in low conc. assays:

| Final conc.: | 10 µM | 12.5 µM | 16.7 µM | 25 µM |
|---|---|---|---|---|
| Volume in 500 µL: | 5 µL | 6.25 µL | 8.33 µL | 12.5 µL |
| cpm in assay | 3000 | 3750 | 5000 | 7500 |

IDCase Assays : each assay is run in duplicate =, with the 20 µL buffer being the desired pH.

| | |
|---|---|
| 100 µM Assay: **#1 #2**<br>879 µL dH2O<br>20 µL PO4 buffer<br>71 µL low s.a. assay mix<br>30 µL enzyme<br><br>50 µM Assay: **#3 #4**<br>914 µL dH2O<br>20 µL PO4 buffer<br>36 µL low s.a. assay mix<br>30 µL enzyme<br><br>25 µM Assay: **#5 #6**<br>932 µL dH2O<br>20 µL PO4 buffer<br>18 µL high s.a. assay mix<br>30 µL enzyme | 16.7 µM Assay: **#7 #8**<br>938 µL dH2O<br>20 µL PO4 buffer<br>12 µL high s.a. assay mix<br>30 µL enzyme<br><br>12.5 µM Assay: **#9 #10**<br>941 µL dH2O<br>20 µL PO4 buffer<br>9 µL high s.a. assay mix<br>30 µL enzyme<br><br>10 µM Assay: **#11 #12**<br>943 µL dH2O<br>20 µL PO4 buffer<br>7.1 µL high s.a. assay mix<br>30 µL enzyme |

Direct spots: 10 µL each of high and low s.a. assay mixes run each assay in duplicate

Blanks: each assay mix, 970 µL dH2O, 20 µl buffer (desired pH), 10 µL assay mix, no enzyme

Each assay, 5 minutes
Pre-warm assay mixes to 30°C briefly before adding enzyme