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Studies of Alkaline Phosphatase Inhibition by Metal Chelators using Capillary Electrophoresis

Courtney A. Neel
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Courtney A. Neel entitled "Studies of Alkaline Phosphatase Inhibition by Metal Chelators using Capillary Electrophoresis." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemistry.

S. Douglass Gilman, Major Professor

We have read this thesis and recommend its acceptance:

Kelsey D. Cook, Bin Zhao

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Studies of Alkaline Phosphatase Inhibition by Metal Chelators using Capillary Electrophoresis

A Thesis presented for the Master of Science Degree

The University of Tennessee, Knoxville

Courtney A. Neel

May 2005

Acknowledgements

I would like to thank everyone who has helped me get to this point. First and foremost, I thank my mom and dad, who taught me that I could be whatever I chose. Without them, none of this would have been possible. I also want to thank my Aunt Jackie and Kevin. I am extremely blessed to have such a wonderful family. I thank my friends for all of the love and support I have received over the years. I also thank Dr. Gilman for all of his encouragement. I would like to thank the rest of the Gilman group, especially Yohannes Rezenom and Amber Wellman, without whom I would have been lost.

Abstract

Alkaline phosphatase inhibition by metal chelators has been studied using capillary electrophoresis. The enzyme-inhibition assays were performed by electrophoretically mixing enzyme and inhibitor zones in a substrate filled capillary. Enzyme inhibition could be seen as a decrease in product formation as detected using laser-induced fluorescence. The enzyme-inhibition assays were adapted so they could be performed using a commercial CE system. Use of a commercial system is desirable for these assays due to ease of use and system features such as autosamplers and capillary and sample cooling. This technique could prove useful for pharmaceutical industries as a screening tool for new drug therapies.

Six metal chelators were studied as enzyme inhibitors using CE enzyme-inhibition assays. EDTA inhibition assays performed previously on a laboratory constructed CE system were compared to those using a commercial system. The assays performed on the commercial system showed irreversible inhibition at concentrations of 1.0 mM or less and activation at 2.0 mM. The opposite trend was seen with the laboratory constructed system. One hypothesis to explain this discrepancy is that two different water sources contained different concentrations of metal contaminants. The two water sources were used due to equipment failure making one source unavailable. The common metal chelators EGTA, NTA, and 1,10-phenanthroline all exhibited reversible inhibition of alkaline phosphatase at concentrations of 0.08 to 2.0 mM. Crown ethers, which are not commonly used as enzyme inhibitors, were also studied. 12-crown-4 and 18-crown-6 both reversibly inhibited alkaline phosphatase at concentrations of 0.08 to 0.3 mM. The reversible inhibition exhibited by the various chelators examined (excluding EDTA) could be due to a time dependent mechanism in which Zn^{2+}, which is required by ALP, is slowly removed, rendering the enzyme inactive.

Alkaline phosphatase inhibition was also studied using on-column reagent addition. The enzyme was added on-column, through a gap reactor and mixed

with the fluorescent enzyme substrate, Attophos, and zones of inhibitor. Theophylline, a reversible noncompetitive inhibitor, and sodium vanadate, a reversible competitive inhibitor were used. Enzyme inhibition could be easily seen as a decrease in product formation detected by laser-induced fluorescence.

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Nomenclature

Å	angstroms
cm	centimeter
°	degrees
°C	degrees Celsius
kV	kilovolt
μm	micrometer
μM	micromolar
mM	millimolar
mW	milliwatt
min	minute
M	molar
nL	nanoliter
nm	nanometer
nM	nanomolar
psi	pounds per square inch
s	second
V	volts

Abbreviations

ADA	adenosine deaminase
ADP	adenine diphosphate

ALP	alkaline phosphatase
AMP	adenine monophosphate
ATP	adenine triphosphate
AttoPhos	[2,2'-bibenzothiazol]-6-hydroxy-benzathiazole phosphate
CE	capillary electrophoresis
CE-LIF	capillary electrophoresis with laser-induced fluorescence detection
DEA	diethanolamine
E	enzyme
[E]	concentration of enzyme
EC	electrochemical detection
EDTA	ethylenediaminetetraacetic acid
EGTA	[ethylenebis(oxyethylenenitrilo)]tetraacetic acid
EI	enzyme-inhibitor complex
[EI]	concentration of enzyme-inhibitor complex
EMMA	electrophoretically mediated microanalysis
ES	enzyme-substrate complex
[ES]	concentration of enzyme-substrate complex
ESI	enzyme-substrate-inhibitor complex
[ESI]	concentration of enzyme-substrate-inhibitor complex
FTIR	Fourier transform infrared spectroscopy
HK	hexokinase
H_2O_2	hydrogen peroxide
I	inhibitor

[I]	concentration of inhibitor
i.d.	inner diameter
K_i	dissociation constant of the enzyme-inhibitor complex
K_m	Michaelis-Menten constant
LDH	lactate dehydrogenase
LIF	laser-induced fluorescence
MS	mass spectrometry
N_2	nitrogen gas
NAD^+	nicotinamide adenine dinucleotide
NTA	nitrilotriacetic acid
o.d.	outer diameter
P	product
[P]	concentration of product
PMT	photomultiplier tube
RSD	relative standard deviation
S	substrate
[S]	concentration of substrate
TPEN	N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine
UV	ultraviolet
V_0	initial reaction rate
V_{max}	maximum reaction rate of an enzyme reacting with substrate
Zn^{2+}	zinc with 2+ charge

Chapter 1

Introduction

1.1 Enzyme Assays

Enzymes are biological catalysts that are essential in the biochemical reactions that sustain life in virtually all known species.[1, 2] Accordingly, enzyme assays are one of the most common procedures in biochemistry, molecular biology, and diagnosis of disease.[1] Enzymes in biological fluids are not usually measured by mass since they are found in low concentrations and samples often contain large amounts of other proteins. Therefore, enzymes are frequently identified and measured by their catalytic activity.[1, 3, 4] In an enzyme assay, the rate at which an enzyme catalyzes the conversion of a substrate into product provides information about the enzyme activity and the reaction kinetics. An enzyme-catalyzed reaction occurs within a pocket on the enzyme known as the active site. A substrate will bind to the active site and be acted upon by the enzyme. The surface of the active site is lined with amino acid residues whose functional groups bind the substrate and catalyze product formation.[1] There are many factors that may affect the reaction rate such as the need for cofactors, temperature, buffers, pH, and enzyme stability.[1, 2]

Enzymatic catalysis can be described as the reaction of an enzyme (E) and a substrate (S) to form an intermediate enzyme-substrate complex (ES), which then breaks down to product (P) and free enzyme.[1, 2, 5-7] At any time in an enzyme catalyzed reaction, the enzyme must exist in one of two forms, as free enzyme (E) or the ES complex. At low substrate concentrations most of the enzyme is found in the free form. The reaction rate would be proportional to [S] because the equilibrium is pushed toward ES formation as [S] increases.[1] At high substrate concentrations it is assumed that all of the enzyme present is in the ES intermediate form. The enzyme is saturated with substrate so that the free enzyme concentration is negligible compared to the substrate concentration.[1, 5, 6] Further increases in [S] have no effect on the reaction rate. It is at this point that the

reaction has achieved a steady state in which [ES] remains constant over time. It is here that the initial rate (V_0) of the catalyzed reaction reaches a maximum (V_{max}).[1]

Enzyme assays are performed by monitoring product formation or substrate depletion.[2] The reaction mixture is created by adding to the reaction vessel all the required components; substrate, enzyme, buffer, and cofactors or coenzymes. The reaction is initiated by adding either enzyme or substrate to the mixture. There are two methods for following the reaction after initiation. Continuous measurements may be made or a sampling scheme can be created. The continuous method follows the reaction for a set period of time with continuous measurements being made to determine the rate. With the sampling method, samples are removed from the reaction mixture at set time intervals and separate measurements are taken from each aliquot. The progress of the reaction can be monitored using several different methods, but photometric and radiometric assays are the most common.[2]

Photometric assays are based on changes in the optical properties of the system that arise from the chemical transformations that occur during the conversion of substrate to product.[2] Absorbance detection has been frequently used to monitor assays and requires only a simple spectrophotometer. Fluorescence can also be used to detect product formation. However, components may need to be labeled with fluorophores since few substances are naturally fluorescent. Most photometric assays are performed using the continuous method.[2]

Radiometric enzyme assays are based on the conversion of radio-labelled substrates to radio-labelled products.[2] These assays are performed using the sampling method. Samples are removed at time intervals and the radioactivity of the product is measured. For these assays to be successful a suitably labeled substrate of known specific activity is needed.[2]

The kinetics of enzymatic reactions under these conditions can be described mathematically using the Michaelis-Menten equation[1, 2, 5, 6]:

$$V_0 = (V_{max} [S]) / (K_m + [S]) \qquad (1a)$$

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} E + P$$

Figure 1.1. General enzyme assay reaction scheme

where K_m is the Michaelis-Menten constant. The basic hypothesis behind this treatment is that the rate-limiting step in the enzymatic reaction is the breakdown of ES complex to product and free enzyme.[1] It is assumed that no reverse reaction between E + P occurs due to a negligible concentration of P initially.[1] Therefore, the reaction rate V_0 is defined by the following rate limiting step:

$$V_0 = k_2 [ES] \qquad (1b)$$

The Michaelis-Menten constant is equal to

$$K_m = (k_{-1} + k_2) / k_1 \qquad (1c)$$

where the k values are rate constants for each step in the reaction scheme shown in Figure 1.1. K_m is equivalent to the substrate concentration at which V_0 is one-half $V_{max.}$ K_m is not an actual dissociation constant of the enzyme substrate complex; however, it reflects the stability of the complex and has practical value.[1] The value of K_m can be determined experimentally by plotting V_0 versus [S] which results in a hyperbolic curve that is known as a Michaelis-Menten plot as shown in Figure 1.2.[1] It is more common to use the Lineweaver-Burk plot to determine K_m, which can be obtained by taking the reciprocal of both sides of the Michaelis-Menten equation[1, 2, 5, 6]:

$$1 / V_0 = 1 / V_{max} + (K_m / V_{max}) / [S] \qquad (1d)$$

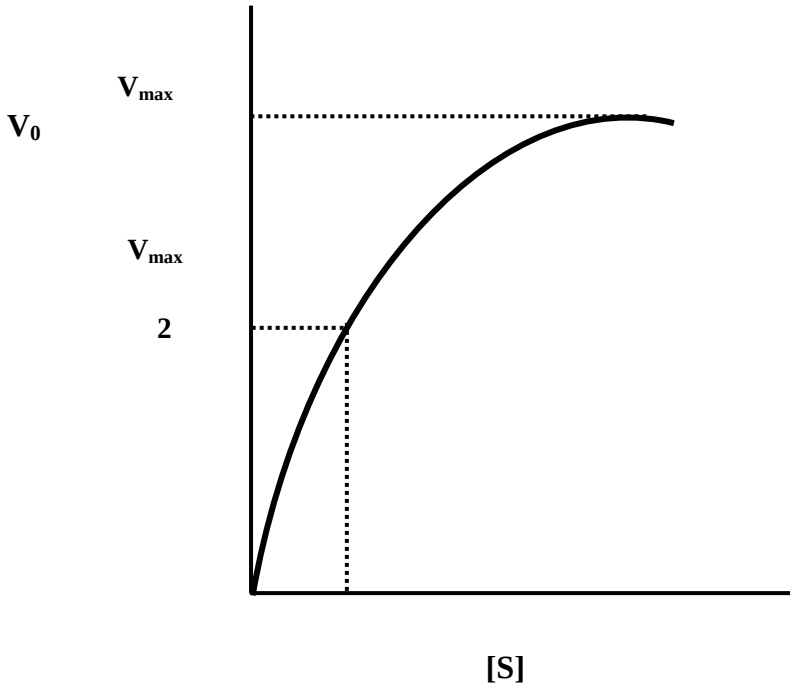


Figure 1.2. General Michaelis-Menten plot

In a Lineweaver-Burk plot, $1/V_0$ is plotted versus 1/[S] at varying substrate concentrations. This yields a linear relationship from which K_m and V_{max} may be determined directly where $-1/K_m$
is the abscissa intercept, $1/V_{max}$ is the ordinate intercept, and K_m/V_{max} is the slope of the line (Figure 1.3).[1, 2, 5, 6]

1.2 Electrophoretically Mediated Microanalysis (EMMA)

Due to the biological importance of enzymes, assay methods should be specific, sensitive, rapid, simple, quantitative and unaffected by side reactions or complicated biological matrices.[8] Enzyme assays, in general, have three major required steps; mixing of reagents to initiate the reaction, progress of the reaction that may include a time period of incubation to allow product to accumulate, and detection of the reaction product.[7, 8]

Capillary electrophoresis (CE) is a powerful analytical separation technique whose small dimensions are well suited for the steps required to perform enzyme assays.[8] CE has several advantages over other enzyme assay formats. CE provides fast analysis times and requires only
small amounts of material.[8-10] CE also has the ability to efficiently separate and quantify substrates and products that may be similar in structure.[8, 10] Several sensitive detection methods may be used in conjunction with CE such as UV-Vis spectrophotometry, laser-induced fluorescence detection (LIF), electrochemical detection (EC), and mass spectrometry.[8, 10]

CE enzyme assays may be classified in three different categories. In pre-column assays, the enzymatic reaction occurs before separation of substrate and product by CE. This type of assay requires larger sample volumes and relevant kinetic information may be missed since the reaction begins in the sample container rather than on-column. In on-column assays, the enzymatic reaction occurs in the capillary, followed by CE separation. In post-column assays, the enzymatic reaction occurs post CE separation.[6, 8] In the post-column approach, the CE instrument may need to be modified in order to incorporate the sampling step and additional sample handling is needed. On-column, or on-line, enzyme assays are particularly useful since they tend to circumvent some of the problems

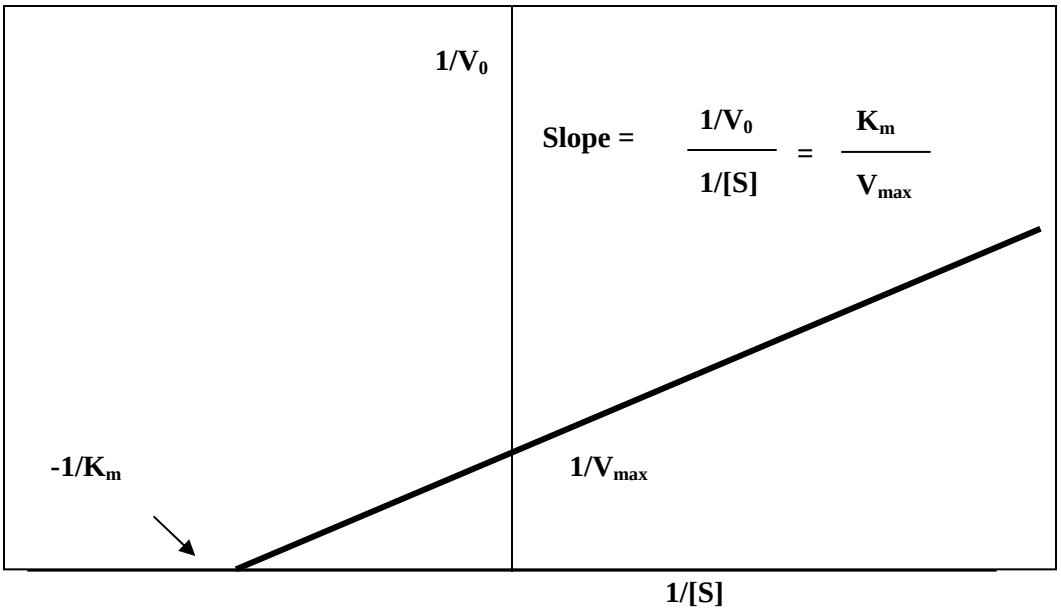


Figure 1.3. General Lineweaver-Burk plot

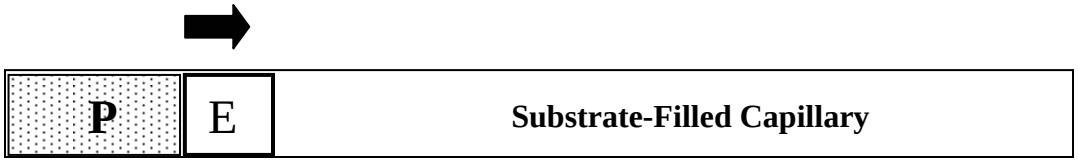
associated with traditional enzyme assays. All processes needed for the reaction occur inside the capillary, therefore the volume of the assay is in the nanoliter range, mixing is achieved rapidly, and band broadening is minimized.[7-9]

Originally, CE was used only as a separation technique in enzyme assays.[9, 11] In these assays, the enzyme reaction occurred outside the capillary in a separate reaction vessel much like a traditional assay. After an incubation period, the mixture was injected into the capillary and the species were separated and detected.[9, 11] CE enzyme assays have many advantages over traditional assays. Depletion of substrate and formation of product can be monitored simultaneously. Only a small volume of sample is required, and the analysis is fast. There is also no need for radiolabeled components. High sensitivity and resolution can be achieved with quantitation possible.[11] These assays do have several disadvantages as well. The sample is still mixed outside of the capillary as with traditional assays. There is a lag time between the start of the reaction and the start of the analysis. For some kinetic studies, the reaction must be stopped during this period by adding a reagent or changing solution conditions such as cooling the mixture.[11] Even though CE only requires nL volumes for analysis, a larger amount of starting material is still needed to carry out the reaction. Adsorption of biological components to the capillary walls is also a problem leading to an inaccurate analysis.

Bao and Regnier[7] described the first on-line CE enzyme assays in 1992 using the enzyme glucose-6-phosphate dehydrogenase. The authors later termed the method electrophoretically mediated microanalysis or EMMA. EMMA utilizes the differences in electrophoretic mobilities of enzyme, substrate, and product. There are various modes of EMMA based on zone engagement, sample introduction, etc.; however, there are two basic formats that exist for mixing of reaction components, continuous engagement and transient engagement.[6, 8, 9, 11-13] The basic schemes for both are shown in Figure 1.4.

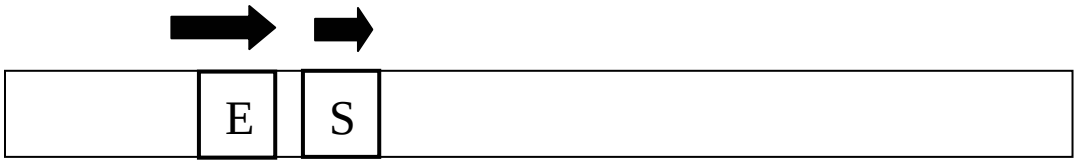
In continuous engagement EMMA, the capillary is filled with running buffer, which typically contains an enzyme substrate at a saturating concentration. A plug of enzyme is introduced into the capillary by an electrokinetic or pressure injection. Upon application of a potential, the enzyme continuously mixes with substrate as it moves through the capillary, and product formation occurs until the enzyme exits the capillary.

Continuous-engagement EMMA

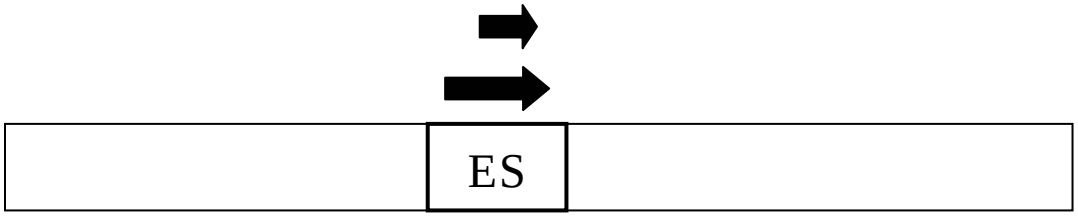


A zone of enzyme is injected onto a substrate-filled capillary. Product forms continuously as the enzyme migrates through the capillary to the detector.

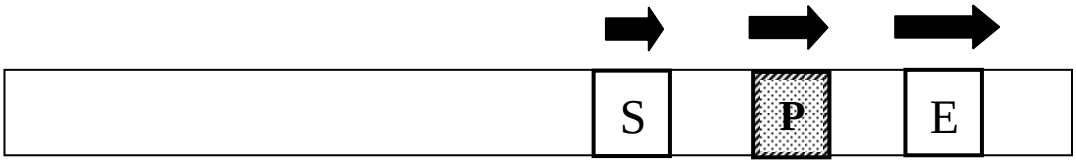
Transient-engagement EMMA



Enzyme and substrate are injected onto the capillary as separate zones.



Enzyme and substrate electrophoretically mix and form product.



The zones are electrophoretically separated and detected.

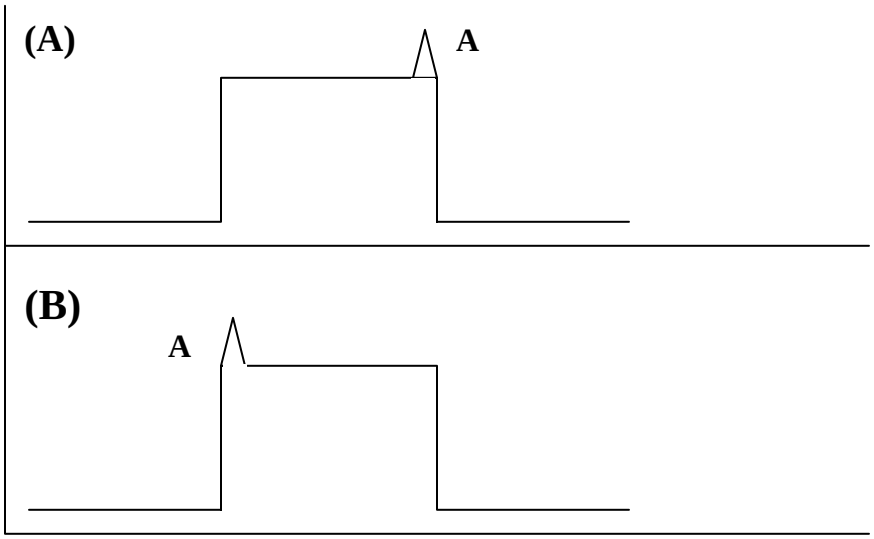
Figure 1.4. Schemes for the two types of EMMA

The product accumulates and appears as a flat plateau at the detector.[7, 9] When the electrophoretic mobility of the ES complex is greater than that of the product, the first product detected will be that which was formed as the enzyme migrates past the detector (Figure 1.5). The product at point A is due to the enzyme that was introduced at the inlet. This point has a peak shape on top of the plateau as a result of product that was formed after the enzyme was injected but before the potential was reapplied. If the electrophoretic mobility of the product is greater than that of the ES complex the electropherogram will be reversed (Figure 1.5).[7, 9]

In transient engagement EMMA, or the plug-plug format, small plugs of enzyme and substrate are injected separately into a capillary filled with buffer. When a potential is applied the zones are electrophoretically mixed due to a difference in mobility. The reagent with the slowest electrophoretic mobility is injected first. When the faster moving reagent is injected, it will overtake the slower zone, and the reaction occurs. Product will be formed and separated from the reagents and later will be detected downstream. The electropherogram will appear as a conventional CE separation experiment, and there will be no plateau as seen in the continuous engagement format (Figure 1.5).[8, 9]

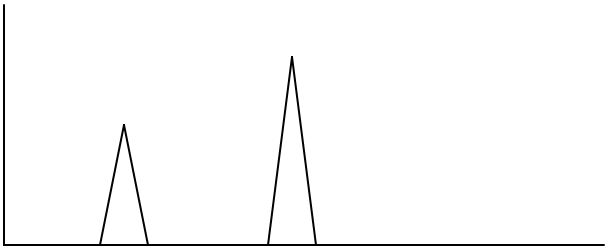
Various enzymes have been studied using EMMA. The enzyme lactate dehydrogenase (LDH) has been studied using various substrates with both UV and EC detection.[8, 14] Regeher and Regnier[15] studied the enzymatic formation of H_2O_2 by glucose oxidase and galactose oxidase. Catalase was studied by detecting the consumption of H_2O_2. All three assays utilized the chemiluminescent reaction of H_2O_2 and luminol for detection. Jin et al.[16] also studied glucose oxidase using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). Conversion of adenosine diphosphate to adenosine triphosphate by creatine kinase was studied using UV detection. Gomez et al.[8] used EMMA with UV detection to study dual enzyme systems utilizing hexokinase (HK) and apyrase in the conversion of adenine triphosphate (ATP) to adenine diphosphate (ADP) and adenosine monophosphate (AMP) in the presence of glucose. Xu et al.[6] used EMMA to perform a Michaelis-Menten analysis of ALP by CE-UV. EMMA has also been used for a range of enzyme kinetics studies,

Continuous-engagement EMMA



Theoretical electropherograms for continuous-engagement EMMA. (A) The electrophoretic mobility of the ES complex is greater than that of the product. (B) The electrophoretic mobility of the product is greater than that of the ES complex.

Transient-engagement EMMA



Theoretical electropherogram for transient-engagement EMMA.

Figure 1.5. Model electropherograms for the two types of EMMA

particularly for determining K_m values and the extent of inhibition by various compounds (K_i) values. Several reviews have been written discussing the broad spectrum of enzymes that have been assayed using EMMA.[8, 9, 11]

Both types of EMMA may be run in zero potential mode where no electrophoretic separation occurs. The enzyme and substrate will stay mixed in one zone and product will accumulate. Upon application of the potential, the product will be swept to the detector. When the continuous engagement format is run in zero potential mode, the product plateau will have an extra peak corresponding to the product accumulation. Zero potential mode is more difficult in the transient engagement format because the potential must be stopped at exactly the moment that the two zones merge. This mode of EMMA is very useful when using dilute enzyme solutions as it enhances the sensitivity of the assay.[9, 11] Saevels et al.[5] used zero potential EMMA to determine the inhibition constant for *erythro*-9-(2-hydroxy-3-nonyl)adenine, which is a competitive inhibitor of adenosine deaminase (ADA). The Yeung group separated and detected isoenzymes of LDH from single human erythrocytes using CE-LIF to monitor the product from the reaction between lactate and NAD^+.[17] Craig et al.[18] were able to use the technique combined with CE-LIF to measure the reaction rate of a single alkaline phosphatase (ALP) molecule using AttoPhos, a fluorogenic substrate.

1.3 Enzyme Inhibition

Enzyme inhibitors are molecules or ions that either slow down or stop enzyme catalysis by preventing the formation of the ES complex or preventing it's breakdown to E + P (Figure 1.6).[1, 14] Enzyme inhibitors are important compounds that serve as control mechanisms in biological systems.[14] Therefore, most clinical drug therapy is based on the inhibition of specific enzymes. Analysis of enzyme reactions has been fundamental to the modern design of many pharmaceuticals. Enzyme inhibitors have served as drugs to treat cancer, hypertension, and arthritis among other conditions.[12] Enzyme inhibition studies can provide valuable insight into the mechanisms of enzyme activity and have defined some important metabolic pathways.[1, 8]

Competitive Inhibition

$$E + S \leftrightarrow ES \leftrightarrow E + P$$
$$+$$
$$I$$
$$\Updownarrow K_i$$
$$EI$$

Noncompetitive Inhibition

$$E + S \leftrightarrow ES \leftrightarrow E + P$$
$$+ \qquad +$$
$$I \qquad I$$
$$\Updownarrow K_i \qquad \Updownarrow K_i$$
$$EI + S \leftrightarrow ESI$$

Uncompetitive Inhibition

$$E + S \leftrightarrow ES \leftrightarrow E + P$$
$$+$$
$$I$$
$$\Updownarrow K_i$$
$$ESI$$

Figure 1.6. Three types of reversible inhibition

There are two main classes of enzyme inhibitors, reversible and irreversible. The distinguishing characteristic for all reversible inhibitors is that when the inhibitor concentration drops, enzyme activity is resumed. These inhibitors usually bind to the enzyme by non-covalent bonds, and the inhibitor maintains a reversible equilibrium with the enzyme.[1, 14] Reversible inhibitors can be classified as competitive, noncompetitive, or uncompetitive.[1, 5, 14] A competitive inhibitor often resembles the size and shape of the substrate and competes with that substrate for the enzyme's binding sites or active sites.[1, 5, 14] The inhibitor occupies the active site, forming an EI complex and thus preventing substrate from binding to the enzyme.[1] Competitive inhibition can be described using a form of the Michaelis-Menten equation[1, 3-5, 14, 19]

$$V_0 = (V_{max} [S]) / ([S] + K_m (1 + [I] / K_i)) \qquad (1e)$$

where

$$K_i = [E] [I] / [EI] \qquad (1f)$$

K_i represents the inhibition constant for the dissociation of the EI complex. Since the inhibition is competitive, increasing substrate concentration can reverse the effect of the inhibitor. When [S] exceeds [I], binding of the substrate to the enzyme active site is far more probable than inhibitor binding. The enzyme will then exhibit a typical V_{max}. The apparent K_m, or the [S] at which V_0 is equivalent to one half V_{max}, will increase in the presence of inhibitor.[1, 3-5, 14, 19]

Noncompetitive inhibitors can bind to both the enzyme itself and the ES complex. Inhibition is caused by binding of the inhibitor to a site on the enzyme other than the active site.[1, 5, 14] Substrate binding to the enzyme is unaltered, but an ESI complex is created that cannot form product. Noncompetitive inhibition can also be characterized using a form of the Michaelis-Menten equation[1, 3-5, 14, 19]:

$$V_0 = V_{max} [S] / K_m ((1 + [I]/K_i) + [S] (1 + [I]/K_i)) \qquad (1g)$$

Since substrate binding to the enzyme is unaltered for this inhibition type, increasing [S] concentration will not influence the effect of the inhibitor. Therefore, the K_m value will not be altered.[1] ESI complexes are formed, but they are unable to progress to product because the effect of a noncompetitive inhibitor is to reduce the concentration of ES complex that can create product. This will cause a decrease in V_{max}.[1]

Uncompetitive inhibitors bind only to the ES complex itself.[1, 14] In the presence of an uncompetitive inhibitor the Michaelis-Menten equation becomes[1, 3, 4, 14, 19]:

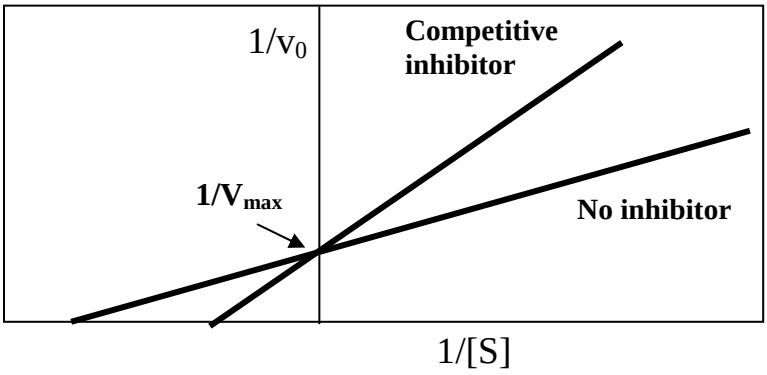
$$V_0 = V_{max} [S] / K_m + [S] (1 + [I]/K_i) \qquad (1h)$$

Since the inhibitor binds only ES, V_{max} is decreased and will not be reversed by the addition of more substrate. K_m is also decreased. In practice, uncompetitive inhibition only occurs with enzymes having two or more substrates.[1] Lineweaver-Burk plots of all three types of inhibition can be seen in Figure 1.7.

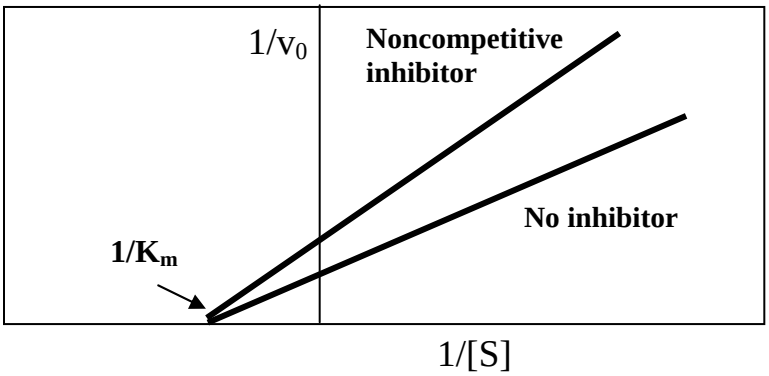
Irreversible inhibitors do not allow enzyme activity to resume even after the concentration of inhibitor is decreased. They are characterized by covalent bond formation with the enzyme active site and time-dependent inactivation.[4] Irreversible inhibitors are further classified as either active site-directed, also known as affinity labels, or mechanism based, which are also known as k_{cat} inhibitors or suicide inhibitors.[3, 4, 20-24]

Active site-directed inhibitors have been used extensively to identify functional groups contained in the enzyme active site.[22] These inhibitors are structural analogs of the normal enzyme substrate that possess a chemically reactive group. [20-22, 24] The similarity of the inhibitor to the substrate allows it to bind to the active site of the enzyme. The functional group can then cause modification of the active site, rendering the enzyme inactive. Affinity labeling contains two distinct steps, selective binding to the active site and covalent bond formation.[24] Inhibitors bind to an enzyme before formation of the covalent enzyme-inhibitor complex which is represented by the following:[3]

Competitive Inhibition



Noncompetitive Inhibition



Uncompetitive Inhibition

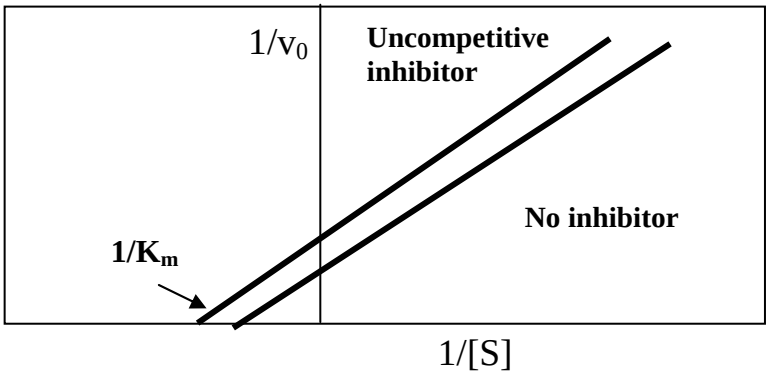


Figure 1.7. Lineweaver-Burk plots for reversible inhibition

$$E + I \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} EI \xrightarrow{k_2} E\text{-}I \qquad (1i)$$

It is assumed that the initial binding of the inhibitor to the enzyme is not interrupted by the formation of the covalent bond because this bond formation is relatively slow.[3, 4, 24] Using this assumption, a kinetic treatment was determined by Kitz and Wilson in 1962.[3] The rate of formation of the inhibited enzyme is given by:

$$dx/dt = k_2 e / (1 + K_i/i) \qquad (1j)$$

where x is the concentration of the E-I complex and K_i is the inhibitor dissociation constant previously mentioned in equation 1f. Integration of the equation gives:

$$k't = \ln e - \ln (e-x) \qquad (1k)$$

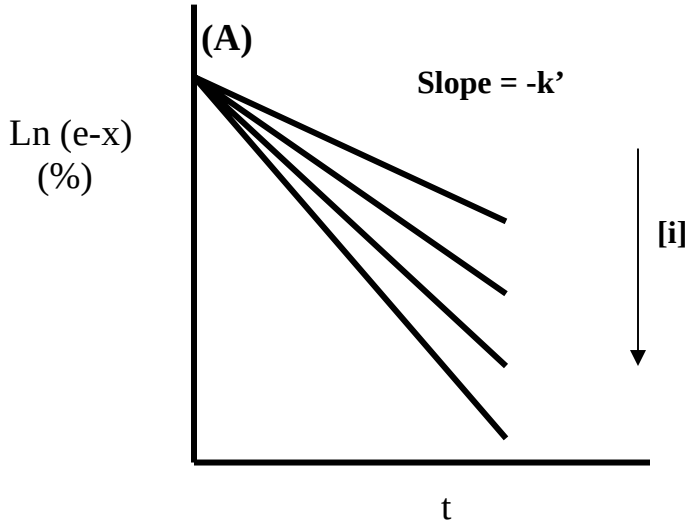
where

$$k' = k_{+2} / (1 + K_i/i) \qquad (1l)$$

As already mentioned, this inhibition is time-dependent with a first order rate constant. Graphing ln (e-x) versus time (t) will give a hyperbolic dependence on the concentration of inhibitor.[3, 21, 24] A plot of the reciprocal form of equation 1l[3, 25]:

$$1 / k' = (((K_i / k_2) (1/i)) + 1/ k_2) \qquad (1m)$$

will give values for both K_i and k_2 as shown in Figure 1.8. One other important characteristic of these inhibitors is protection against inactivation will occur in the presence of substrate.[3, 4, 22-25] Since the first step in this type of inhibition is formation of a bond to the active site, the presence of substrate would slow down this process due to the displacement of inhibitor by substrate[3, 24, 25]. Substrate protection can be described by the following:



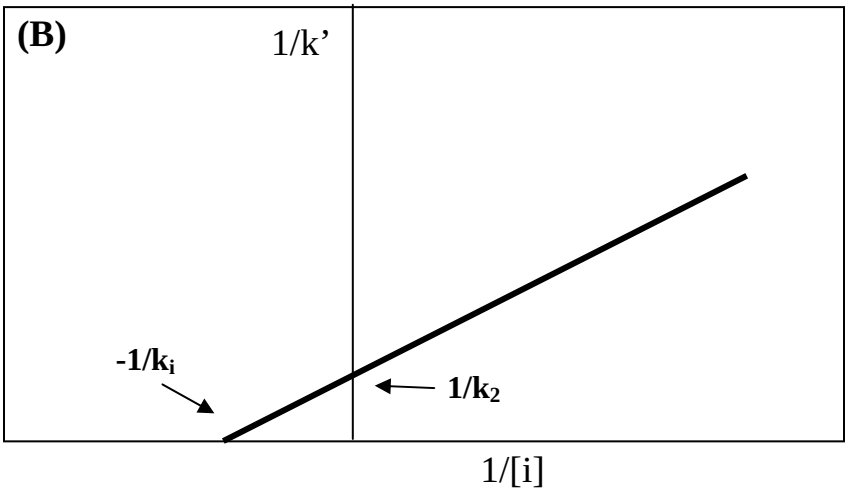


Figure 1.8. Kinetics of irreversible inhibitors. (A) Semi-logarithmic plot of reaction rate at various inhibitor concentrations. (B) Plot of the reciprocal of the first-order rate constant.

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} EI \xrightarrow{k_{+2}} E\text{-}I \qquad (1n)$$

$$+ \; S$$

$$ES \quad (k_{+3},\ k_{-3})$$

and the value of k' will change.

$$k' = k_2 / (1 + K_i/i)(1 + s/K_s) \qquad (1o)$$

$$K_s = k_{-3} / k_{+3} \qquad (1p)$$

A plot of the reciprocal of equation 1o (1/k' vs. 1/i) will give values of the rate constants K_s, K_i, and K_{+2}.

As previously mentioned, active site-directed inhibitors have been extremely valuable in identifying the functional groups contained at enzyme active sites and providing mechanistic details about the catalytic process.[22, 24] More importantly, these inhibitors can be used for drug design once a target enzyme has been selected. Since the inhibition is irreversible, the drugs are less susceptible to displacement by normal metabolites found in the body and can be very effective treatments.[4] However, it is rare to find one of these inhibitors that is selective enough to be used in multi-component systems. The active functional groups are present on the molecule before reaching the target enzyme, and the possibility that they may react nonspecifically is great.[3, 20-22, 24]

Mechanism based inhibitors utilize an enzyme's normal reaction mechanism to inactivate the enzyme.[1] These inhibitors are relatively inert molecules that resemble the natural enzyme substrate and therefore will react with the enzyme's active site. Once the molecule binds in the active site, a functional group contained within the active site will modify the inhibitor. It is converted into a compound which reacts with the enzyme leading to inactivation.[1, 4, 20-24, 26] The initial encounter between the enzyme and inhibitor is indistinguishable from that of enzyme and substrate. By reacting with the "faux" substrate, the enzyme brings about its own inactivation and consequently, these inhibitors are known as suicide inhibitors.[23] The success of mechanism based inhibitors is

dependent on three basic factors.[20, 24, 26] First, the molecule must be relatively inert to maintain specificity for the target enzyme. Second, the molecule must resemble the natural substrate of the enzyme. The fewer chemical modifications the inhibitor must undergo, the more effective an inhibitor it will be. This is key for the specificity of the inhibitor for the enzyme. If an inhibitor is structurally simple, many enzymes will use it as a substrate and it is less likely to be specific. Lastly, the inhibitor must be converted into a chemically reactive substance. The reactive molecule must be formed within bonding distance to an active site functional group capable of reacting with it. The dissociation rate of the enzyme inhibitor complex must also be relatively slow. Rapid dissociation would preclude a reaction with an active site functional group.

The scheme for mechanism-based inhibition is as follows[4]:

$$E + I \leftrightarrow \underset{k_{-1}}{\overset{k_1}{EI}} \rightarrow \overset{k_2}{EI^*} \rightarrow \overset{k_3}{E - I} \qquad (1q)$$

where the enzyme inhibitor complex (EI) becomes an activated species (EI*) that produces the inhibited species. Steady-state kinetics cannot distinguish between active site-directed and mechanism based inhibition, so the mathematical treatment is the same for both with the constants k' and K_i slightly more complex for mechanism based inhibition[3, 4] with

$$k' = ((k_2\, k_3)\,(k_2 + k_3))/\,(1 + K_i/i\,) \qquad (1r)$$

where

$$K_i = ((k_{-1}\, k_3) + (k_2\, k_3))\,/\,((k_1)\,(k_2 + k_3)) \qquad (1s)$$

A plot of the reciprocal of equation 1s will give the values of the rate constants K_i, k', and k_2 as stated previously for active site-directed inhibitors (Figure 1.8). Protection against inactivation in the presence of substrate is also a characteristic of these inhibitors.[3, 4] The kinetic treatment is the same as that for active site-directed inhibitors.

Mechanism based inhibitors are particularly useful for in-vivo applications since they are chemically inert until they reach the active site of the enzyme for which they are

specific.[22-24, 26] This minimizes the possibility of nonspecific reactions in the body, which is an important consideration in drug design.

1.4 Enzyme Inhibition Assays

Capillary electrophoresis has been used to evaluate the effects of inhibitors on enzyme activity. Saevels et al.[5] used transient engagement EMMA to study the deamination of adenosine to inosine by adenosine deaminase (ADA). In these experiments, the buffer contained a competitive inhibitor of ADA, erythro-9-(2-hydroxy-3-nonyl)adenine. Enzyme and substrate were injected as separate plugs in the inhibitor filled capillary. When the two plugs were fully mixed, the potential was turned off for 5 min to allow product buildup. Product was detected by CE-UV. Lineweaver-Burk plots were used to determine K_i.

Whisnant et al.[12] developed a method for studying inhibition of ALP using CE-LIF. The capillary was filled with running buffer containing the fluorogenic substrate AttoPhos. Theophylline, a competitive inhibitor of ALP, and enzyme were injected as separate zones into the column. This experimental design is considered a combination of transient and continuous-engagement EMMA. The zones migrated through the capillary at constant potential and product was formed continuously. When the inhibitor and enzyme zones electrophoretically mixed, product formation decreased. The inhibition could be seen on an electropherogram as a negative peak on the product plateau. The K_i value was calculated using the Michaelis-Menten treatment. In a related series of studies, using the same variation of EMMA, the group studied reversible and irreversible inhibition and activation of ALP.[27] Reversible inhibitors included theophylline, sodium vanadate, and sodium arsenate. EDTA was found to be an irreversible inhibitor at concentrations of 1 mM and higher and an activator at concentrations from 20 to 400 μM. The K_i values for the reversible inhibitors were determined by Michaelis-Menten treatment and were within the ranges reported in the literature. Using this technique, reversible and irreversible inhibition and activation can all be easily distinguished upon visual inspection of the electropherograms.

Glatz et al.[14] used a combination of EMMA and a partial filling technique to study the inhibition of rhodanese by 2-oxoglutarate. Rhodanese catalyzes the conversion of thiosulfate into thiocyanate using cyanide as a co-substrate. In this method, part of the capillary is filled with the appropriate buffer for the enzymatic reaction and the rest is filled with the optimal background electrolyte for the separation of the substrate and product. The inhibition constants were determined for 2-oxoglutarate with respect to both thiosulfate and cyanide. The type of inhibition was also determined. 2-oxoglutarate was found to be a competitive inhibitor with respect to cyanide and an uncompetitive inhibitor with respect to thiosulfate.

EMMA has also been used in conjunction with microchips to study enzyme inhibition. The inhibition of β-galactosidase by phenylethyl-β-D-thiogalactoside was studied using continuous-engagement EMMA on a microchip with LIF detection.[28] Substrate, buffer, enzyme, and inhibitor were all held in separate reservoirs on the microchip and mixed electrophoretically. Substrate and buffer were first mixed in a channel, and enzyme and inhibitor were mixed with the resultant running buffer solution. The K_i of phenylethyl-β-D-thiogalactoside was determined. The inhibition of protein kinase A by H-89, a competitive inhibitor, was studied using a microchip.[29] The inhibitor was first diluted on the chip and mixed with enzyme and substrate. Plugs of this sample mixture were injected into a separate channel for separation and detection. Acetylcholinesterase inhibition has also been studied using this method.[30] Enzyme was continuously pumped through the chip and substrate was mixed in with this stream. Inhibitor zones were injected before mixing with substrate. The non-fluorescent product was derivatized on-column after the reaction for LIF detection. Inhibition could be distinguished by a decrease in fluorescence and a K_i value was determined for the competitive inhibitor, tarcine.

1.5 Alkaline Phosphatase

Alkaline phosphatase (ALP) is an enzyme found in a wide majority of living organisms and is prevalent in tissues that have an absorptive or transport function such as intestine, kidney, placenta, and liver.[31] ALP nonspecifically hydrolyzes phosphate esters to produce an alcohol

and an inorganic phosphate at alkaline pH.[18, 32-35] ALP is classified as a zinc metalloprotein because zinc is required for activity of the enzyme.[12, 18, 31, 33-35] It is an enzyme that contains 4 mol of zinc ions per mol of ALP. Though the exact structure of ALP is still under investigation, it is generally accepted that it is homodimeric in nature with each active site containing a phosphorylatable serine and three metal binding sites, two for zinc and one for magnesium.[33]

While the exact role of metals in enzymes is not fully understood, in general, divalent metal ions tend to increase the structural stability of the active conformation of an enzyme or take part in the catalytic process itself.[33] The structure of ALP varies between species and other divalent metals have been found in addition to zinc and magnesium. *Micrococcus sodonensis* ALP has been found to require calcium to be active.[35] Another form found in yeast requires iron while *B. subtilis* ALP seems to need cobalt.[35] The focus of the remainder of this discussion will be on mammalian ALP, which requires zinc and magnesium only.

A general mechanism of the dephosphorylation of substrates by alkaline phosphatase has been proposed.[18, 31, 34, 35]

$$\mathrm{EH + R\text{-}OP \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} EH'R\text{-}OP \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} EH^{*'}R\text{-}OP \xrightarrow{k_3} EP + ROH} \qquad \text{(1t)}$$

slow

$$EP \xrightarrow{k_4} EH + P$$

EH is free enzyme, R-OP is the substrate, EH'R-OP is the enzyme substrate complex, EH*'R-OP is the activated enzyme substrate complex, EP is the phosphorylated enzyme, ROH is the alcohol and P is the inorganic phosphate.[18] In the first step, free enzyme binds to a phosphomonoester substrate to form the enzyme substrate complex.[18, 34, 35] The second step is the catalytic reaction, where a nucleophilic attack on the phosphate group of the substrate causes formation of a phosphoryl enzyme intermediate.[18, 34] Hydrolysis transforms the covalent intermediate into a noncovalent enzyme phosphate complex that decomposes into the reaction product and free enzyme.[31, 34]

The ALP active site is open to the surface of the enzyme and is found in a pocket formed from the ends of a number of helices and sheets.[33] Magnesium does not seem to participate in the catalysis reaction itself but instead stabilizes the structure of the

enzyme. A serine residue found in the active site is phosphorylated in the reaction.[31, 33, 35] One of the zinc ions interacts with the serine in order to stabilize the residue for nucleophilic attack on the phosphate portion of the substrate. The second zinc ion binds to the substrate, neutralizing the negatively charged phosphate and exposing the phosphorus atom to nucleophilic attack. This model of the mechanism at the active site matches well with what is known about other hydrolytic enzymes.[31, 33, 35]

While this mechanism is widely accepted, it is not known whether or not both subunits of the dimer function identically. There has been evidence to support theories of both negative cooperativity and independence.[31, 35] Negative cooperativity is the case in which only one dimer actively functions.[31, 35] It has been suggested that once a molecule of substrate binds to one active site, the other active site becomes incapable of functioning due to conformational changes in the subunit.[31] There is also a body of evidence supporting the independence of the subunits.[35] Molecular hybrids were created consisting of a wild-type subunit and an inactive mutant subunit. The observed activity of the hybrid indicated that the subunits acted independently based on the idea that the activity of the hybrid should be the mean of the activities of the two subunits. If the situation were based on negative cooperativity , the activity would be considerably less.[35] More studies are needed before a consensus can be reached on this issue.

1.6 Enzyme Inhibition by Metal Chelators

Metal ions, particularly transition metals, have empty orbitals and are capable of accepting electron pairs.[36] Therefore, metal ions are Lewis acids and accept electron pairs from electron-donating ligands that are Lewis bases. A ligand that attaches to metals through only one atom is called a monodentate. Most transition metals can bind to six ligand atoms. A ligand that attaches to a metal ion through more than one atom to form a five or six membered ring is called multidentate or chelating agent.[36, 37] The chelate effect is the ability of multidentate ligands to form more stable metal complexes than those formed by monodentates.[37] This effect can be understood by looking at the thermodynamics of the reaction based on the equation for standard free energy change[36, 37]:

$$\Delta G = \Delta H - T\Delta S \qquad (1u)$$

Two processes that drive a chemical reaction are decreasing enthalpy, which is a negative ΔH value (release of heat), and increasing entropy, which is a positive ΔS value (more disorder in a system). A reaction is thermodynamically favorable if $\Delta G < 0$. More order is created when metals complex with monodentates than multidentates. The enthalpy change for both is approximately the same. The change in entropy then becomes the most important factor in the chelate effect.[37]

Ethylenediaminetetraacetic acid, or EDTA as it is commonly known, is one of the most widely used chelators.[36-38] EDTA has six potential binding sites for metal ions, four carboxyl groups and two amino groups.[38] It is a hexadentate ligand. EDTA combines with metal ions in a 1:1 ratio regardless of the charge on the cation.[37, 38] In general the reaction of EDTA with a metal ion can be described as[37, 38]:

$$M^{n+} + Y^{4-} \leftrightarrow MY^{(n-4)+} \qquad (1v)$$

EDTA forms very stable complexes with metals due to the complexing sites within the molecule that give rise to a cagelike structure, which surrounds the cation and prevents side reactions.[38] Due to this structural stability, EDTA is used widely as a preservative in foods and biological samples. It is often added to mayonnaise, salad dressings, and oils to prevent oxidation of fats. It is also used to protect stored blood from oxidizing and is an important reagent for blood banks, hospitals, and crime labs.[38]

Treatment of enzymes with chelators removes metal atoms essential for enzyme activity and thus leads to the formation of a metal-free inactive apoenzyme.[33, 35] EDTA is frequently used with ALP to create the apoenzyme for kinetic studies. The effect of chelators on enzymes is dependent on several factors including concentrations of chelator and enzyme, time interval of treatment, pH, and enzyme source.[35] Enzymes from different organisms respond differently to EDTA treatment.

It is generally accepted that EDTA reacts with an enzyme by removing Zn^{2+}, but the exact mechanism is not fully known. Zhang et al. used green crab ALP from the species *Scylla serrata* to study the reaction kinetics of inactivation by EDTA.[39, 40] A reaction scheme was determined which includes conformational changes of the enzyme and a loss of the zinc ions contained in the active site. The first step is a fast and reversible formation of a complex between ALP, more specifically Zn^{2+} contained in ALP, and EDTA. In the second step, the complex irreversibly changes from a strained, active state to a conformationally stable inactive state. This is the rate-limiting step and represents the inactivation reaction rate K_i. In the final step, Zn^{2+} is removed from the active site of the enzyme and a free Zn^{2+}- EDTA complex is formed. This study correlates with another study done by Bortolato et al. which used Fourier transform infrared spectroscopy (FTIR) to probe the structure of the apoenzyme of bovine intestinal mucosa ALP.[33] The findings suggest that the zinc ions in ALP are also necessary for stabilization of the secondary and tertiary structures of the enzyme. The removal of the metal ions induced conformation changes that could correspond to disappearance of α-helices and formation of an unordered structure. While these are two of the most recent studies of EDTA induced inactivation, there are still some concerns with adopting this as a general mechanism. Most important is the fact that these studies were performed on only two species of organisms. Many more studies are needed including a wide range of organisms before a consensus on the general mechanism of EDTA inactivation can be reached.

Chapter 2

Analysis of Alkaline Phosphatase Inhibition by Metal Chelators Using Capillary Electrophoresis

2.1 Introduction

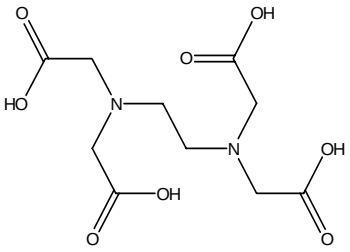
Enzyme inhibition by metal chelators was studied using an automated capillary electrophoresis system and alkaline phosphatase as the model enzyme. As was previously discussed, metal chelators are thought to bind to a metalloenzyme's active site and remove metals essential for enzyme activity, thus inhibiting the enzyme.[35, 39, 40] Therefore, metal chelators could serve as useful clinical compounds for drug therapy based on the inhibition of certain enzymes.

The exact mechanism of enzyme inhibition by metal chelators has been widely debated.[35, 39-44] EDTA is one of the most commonly used chelators and has been extensively studied[36-38]; however, in order to expand the knowledge base for these types of assays, other classes of metal chelators need to be studied. EDTA inhibition had previously been studied in our laboratory using a laboratory constructed CE-LIF system and this work provided a starting point for this series of experiments.[27] It was found that irreversible inhibition of alkaline phosphatase occurred at concentrations of 1.0 mM EDTA or higher. Activation of alkaline phosphatase was observed at concentrations of 20 to 400 μM EDTA. Few studies in the literature give a clear and consistent picture of the mechanism of EDTA inhibition. This is due in part to the use of various forms of enzyme, purity and preparation methods.[35] In order to obtain information about the overall inhibition mechanism a series of inhibitors should be used with the same form and purity of enzyme. Chelators with structures different from EDTA can help to determine if the inhibition is specific to that particular structure or is due to a more generic chelator form. Chelators with varying affinities for zinc ions should also be included to develop an understanding of how their ability to complex affects the mechanism. One would

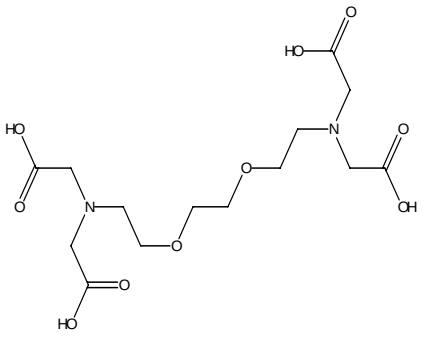
assume that a chelator with a higher affinity would be a more potent inhibitor. A description of the inhibition trends based on structure and affinity for zinc should give a more concrete representation of the mechanism of enzyme inhibition by metal chelators. The studies presented here include a range of metal chelators. Structures of the inhibitors used in this study can be seen in Figure 2.1.

To expand the utility of these enzyme inhibition studies, we have also demonstrated the use of an automated CE system in conjunction with these assays. Conventional enzyme assays can be performed in a microtitre plate quickly and in parallel. However, they are limited by the amount of material required for an assay. The most effective way to reduce the amount of material needed for an assay is to reduce the concentration and allow longer incubation periods for product buildup or reduce the total volume of the assay. CE is a technique well suited to performing enzyme assays because it only requires nL size samples.[8-10] CE enzyme-inhibition assays also provide more information about an assay compared to traditional methods. CE allows the progress of the entire assay to be monitored. The activity of the enzyme can be measured before, during and after interaction with the inhibitor. With traditional assays, only the activity of the enzyme during interaction with the inhibitor is observed. The type of inhibition (reversible or irreversible) or activation can be readily distinguished by visual inspection of electropherograms. Other kinetic parameters that can be obtained from an electropherogram include velocities of ES complex, product and inhibitor, the time at which the enzyme and inhibitor interact, and the location in the capillary where the interaction occurs.

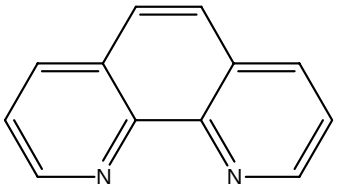
CE enzyme assays have proved to be very advantageous in reducing the amount of material needed to the nL scale; however, laboratory constructed CE instruments still have several disadvantages. Optical components may be difficult to align initially and generally need to be realigned on a day-to-day basis. Experiments must be performed in a darkened area to prevent ambient light from reaching the detector. Software design and coupling to the system may also prove complicated. Assays performed this way may also be time consuming since each solution, (enzyme, inhibitor, etc.) is manually injected and



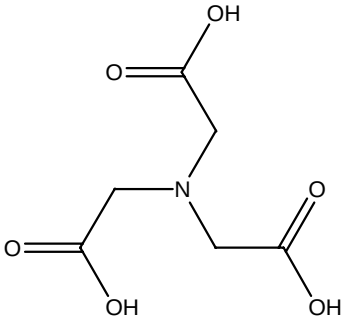
EDTA



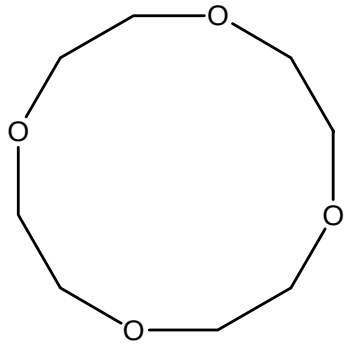
[ethylenebis(oxy-ethylenenitrilo)]tetraacetic acid



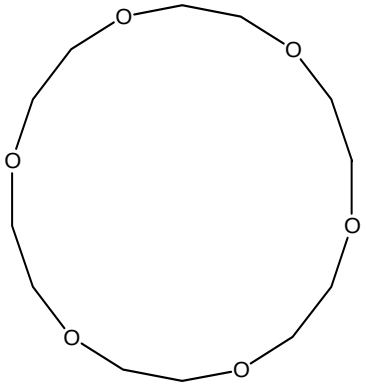
1,10-phenanthroline



nitrilotriacetic acid



12-crown-4



18-crown-6

2.1 Metal chelator structures

each run must be started by a technician. This laboratory constructed system would not be practical on an industrial scale where hundreds to thousands of assays must be performed on a short time scale.

Several commercial CE systems are available. These feature easy to use software systems with data analysis packages, capillary and sample cooling features and autosamplers with high sample capacity.[45-47] Most of these instruments come with a variety of detectors that may be interchanged and can also be coupled to other systems such as MS. Automated capillary arrays, which can analyze hundreds of samples simultaneously, are also commercially available. These automated systems could be valuable to the pharmaceutical and biotechnology industries by permitting large scale screening of compounds for inhibitory effects in order to create new drug therapies.[46]

2.2 Experimental

2.2.1 Reagents

AttoPhos ([2,2'-bibenzothiazol]-6-hydroxy-benzathiazole phosphate) was purchased from Promega (Madison, WI). Calf intestinal alkaline phosphatase (EC 3.1.3.1) was supplied by ICN Biomedicals (Aurora, OH). DEA (diethanolamine), Theophylline (99%), EGTA ([ethylenebis(oxyethylenenitrilo)]tetraacetic acid), 1,10-phenanthroline (99%), NTA (nitrilotriacetic acid) (99%), 12-Crown-4, and 18-Crown-6 were obtained from Acros (Pittsburgh, PA). Disodium EDTA was purchased from Fisher Scientific (Norcross, GA). All solutions were prepared in distilled, deionized water bought from VWR (West Chester, PA).

2.2.2 CE-LIF Instrumentation and Experimental Conditions

A Beckman Coulter P/ACE™ MDQ Capillary Electrophoresis System (Fullerton, CA) was used in this study. The instrument was equipped with a LIF detector module to allow for CE-LIF detection. The 457.9 nm line of a Melles Griot 43 Series argon ion laser was used for excitation. A 475 nm long pass filter from Edmund Optics (Barrington, NJ) and a 560 ± 10 nm bandpass filter from Andover (Salem, NH) were

used as emission filters. The power of the laser beam coupled to the instrument was approximately 3 mW. Fused silica capillaries with a 50 μm i.d. and 220 μm o.d. were used. The capillary total length was 60 cm and the length to the detection window, from which the polyimide coating was removed using a low flame, was 50 cm. Both the sample chamber and capillary cartridge temperatures were held constant at 25 °C. 32 Karat™ Software version 5.0 from Beckman Coulter (Fullerton, CA) was used for data acquisition and analysis.

The running buffer consisted of 50 mM DEA at pH 9.5 and 0.10 mM AttoPhos, a fluorogenic alkaline phosphatase substrate. The enzyme solution contained 0.18 nM alkaline phosphatase and 50 mM DEA buffer at pH 9.5. Running buffer and enzyme solution were prepared fresh daily. The inhibitor solutions contained 50 mM DEA buffer, 0.10 mM AttoPhos and inhibitor at selected concentrations. All injections were performed by pressure at 0.5 psi. The applied electric field for all separations was 310 V/cm. A 50 mM DEA rinse buffer was used to wash the electrode and capillary in order to prevent cross contamination of enzyme and inhibitor solutions. This was done by a 1.0 s injection of rinse buffer at 0.1 psi in order to immerse the electrode and capillary in the solution without injecting a large amount of material.

2.3 Results and Discussion

2.3.1 On-Column Assay of Alkaline Phosphatase

Alkaline phosphatase is a metalloenzyme that is found in most living organisms.[31] Therefore, ALP is a relatively cheap, widely available enzyme to use for kinetic studies. It has been used in conjunction with the EMMA technique to study the catalyzed reaction between ALP and several substrates, including p-aminophenylphosphate and p-nitrophenylphosphate.[39, 40, 43, 44] Here, ALP was used to study the catalyzed reaction that occurs with the substrate AttoPhos. This assay serves as a control for comparison of enzyme-inhibition assays using metal chelators.

These assays were first performed on a laboratory constructed CE system.[27] Briefly, the 457.9 nm line of an argon ion laser was focused onto the capillary using a fused-silica plano convex lens. Fluorescence was collected at 90° to the laser by a 20 X

microscope objective and was filtered by both a 560 ± 10 nm bandpass filter and 1 mm diameter aperature. Fluorescence was detected using a PMT. The laser power at the capillary was 35 mW. The capillary was thermostatted at 40° C by enclosing it from injection end to detection window in Teflon tubing and flowing heated N_2 gas through the tubing. The thermostatting prevented fluctuations in the reaction rate due to temperature changes down the length of the capillary. The temperature of N_2 was previously optimized for maximum enzyme activity. These assays were performed by injecting a 3 s zone of 0.18 nM ALP at 17.8 kV into a capillary containing 0.10 mM AttoPhos, a fluorogenic substrate. Then a constant potential of 17.8 kV (310 V/cm) was applied. Product formation was continuous as the ALP-AttoPhos complex migrated through the buffer filled capillary and was detected using LIF. The fluorescence signal is indicative of the reaction rate of the system. The results of an ALP enzyme assay (commercial system) are shown in Figure 2.2. The fluorescent product accumulates and appears as a flat plateau. The small peak at point A on the plateau results from product formation that occurs after the enzyme has been injected, but before the separation potential is applied. It is a zero field incubation phenomenon.[7] This artifact is observed at the end of the product plateau, which is the opposite of where it might be expected. Since the mobility of the ALP-AttoPhos complex is greater than that of the fluorescent product, the ES complex will migrate to the detector faster. The first product detected is product that is produced at the end of the experiment as the ES complex migrates past the detector. The product produced when the enzyme is first injected into the capillary, or the peak, will be detected last. Therefore, all the electropherograms presented will be reversed in time.[12] Relative standard deviation (RSD) values were used as a measure of reproducibility of enzyme activity and thus, system reliability. RSD values for the ALP assays on the laboratory constructed system were 2% or less.

These assays were then performed on a commercial automated CE system. There are a few differences between the laboratory constructed and commercial systems. The commercial system was equipped with both electrokinetic and pressure injection capability. It also was equipped with capillary and sample chamber thermostatting. The

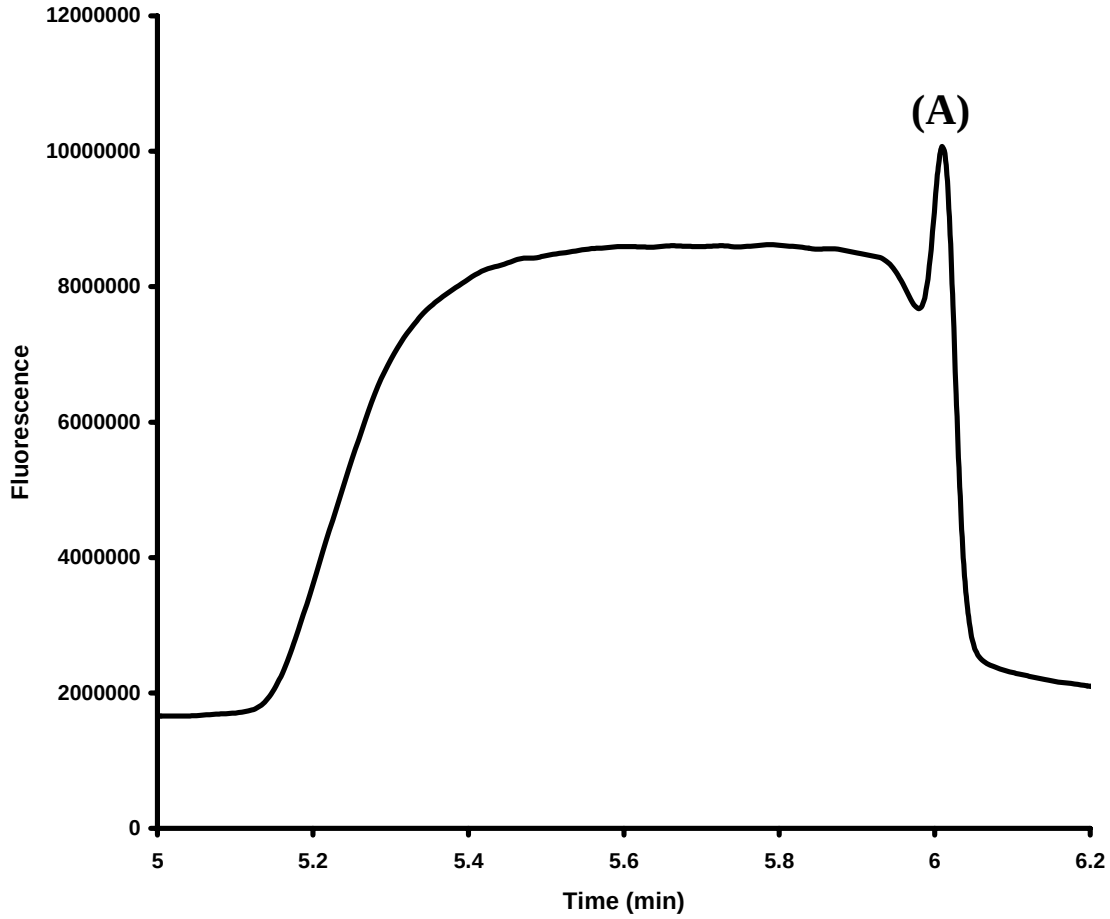


Figure 2.2. Electropherogram of an alkaline phosphatase assay performed in a commercial CE system. A zone of 0.18 nM ALP was injected for 3 s at 0.5 psi into a capillary filled with 0.10 mM AttoPhos and 50 mM DEA at pH 9.5. The applied electric field was 310 V/cm. Capillary length to the detector was 50 cm.

457.9 nm line of an argon ion laser was used; however, the laser was coupled to the instrument via an optical fiber. The laser beam was focused through a plano-convex lens onto the tip of the optical fiber, which was held in a freestanding coupler. This optical fiber was plugged directly into the instrument. A second optical fiber located in the instrument directed the beam onto the capillary. The laser power at the capillary was 3 mW. Several changes were also made in the way the assay was performed. A 3.0 s zone of 0.18 nm ALP was injected at 0.5 psi. The applied electric field remained the same at 310 V/cm. Both the capillary and samples were held at a constant 25° C in order to prevent fluctuations in the reaction rate due to temperature changes. The electropherograms from the automated system appear the same as the electropherograms from the laboratory constructed system. The results may be interpreted the same way. In moving these assays to the automated CE system, RSD values were used as a measure of the success of the technique in the new system. All trials had RSD values of less than 2%, which is the same precision obtained from the laboratory constructed system. It was concluded that the on-column CE enzyme assay works well in a commercial automated CE system and that the use of this system could be extended to enzyme-inhibition assays. The commercial system offered several advantages over the laboratory constructed system as well. The signal-to-noise ratio appears better with the automated system. The system itself is less complicated to operate since all individual parts are enclosed within the instrument. There is no need for alignment or a dark room. Also, all injections are performed by the system. The user may simply program a sequence to run a large number of samples without having to monitor the process continuously or perform manual injections.

2.3.2 On-Column Enzyme-Inhibition Assay of Alkaline Phosphatase and Theophylline

Theophylline is a reversible, noncompetitive inhibitor of ALP. It is a clinically important compound that has been used as a bronchodilator, respiratory stimulant, and anti-inflammatory drug.[12] Theophylline is a very potent inhibitor of ALP and its inhibition effects have been studied extensively. For these reasons, it has been used as a model system for developing on-column enzyme-inhibition assays.

Just as with the laboratory constructed on-column ALP assays, the theophylline inhibition assays were used as a control for this study. Experiments were performed on both the laboratory constructed CE system and the commercial automated CE system for comparison. In the laboratory constructed system,[12] a 4 s zone of 100 μM theophylline was injected at 17.8 kV. A constant potential of 17.8 kV was applied for 20 s. Next, a 3 s zone of 0.18 nm ALP was injected at 17.8 kV. A separation potential of 17.8 kV (310 V/cm) was applied. The analogous experiment in the automated system consisted of a 5 s injection of 100 μM theophylline at 0.5 psi followed by application of a constant potential of 18.6 kV for 30 s. Next, a 3 s zone of 0.18 nm ALP was injected at 0.5 psi. A separation potential of 18.6 kV was reapplied for the duration of the assay. The electropherograms from both systems were interpreted in the same manner and can be seen in Figures 2.3 and 2.4 for comparison. In the electropherogram for the commercial system, there appears to be a second smaller inhibition peak. It is believed to be an impurity in the inhibitor solution and has previously been seen with the laboratory constructed system.

Theophylline was injected first because the ALP-AttoPhos complex has a greater mobility than the inhibitor. In order to achieve enzyme inhibition in any system, the zones of enzyme and inhibitor must electrophoretically mix. Therefore, the injection order depends upon the mobility of the components. Experiments may initially be performed to determine which component has a greater mobility. If an inhibitor is injected first and no inhibition occurs, then a corresponding experiment in which enzyme is injected first must be performed.

Once both components have been injected in the correct order, the zones will migrate through the capillary under applied potential. Product forms continuously until the zones of inhibitor and ES complex electrophoretically mix. When this occurs, product formation decreases and can be seen as a dip in the product plateau on the electropherogram. Once the zones of inhibitor and ES complex move away from each other, the enzyme returns to its original level of activity. This indicates that theophylline is a reversible inhibitor.

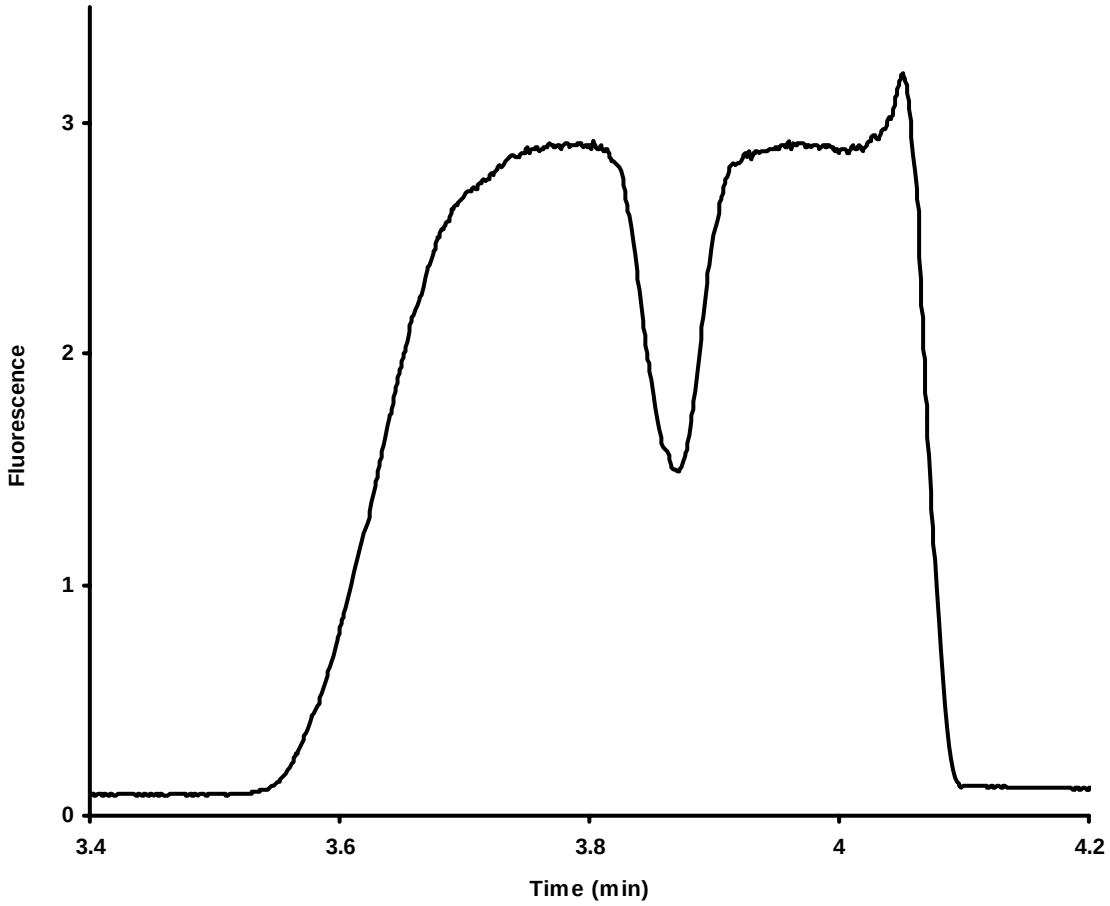


Figure 2.3. Electropherogram of an enzyme-inhibition assay with theophylline in a laboratory constructed CE system. A 4 s zone of 100 μM theophylline was injected at 17.8 kV into a capillary filled with 0.10 mM AttoPhos and 50 mM DEA at pH 9.5. A 3 s zone of 0.18 nM ALP was injected after applying a potential of 17.8 kV for 20 s. The applied electric field was 310 V/cm. The capillary length to the detector was 42.8 cm.[12]

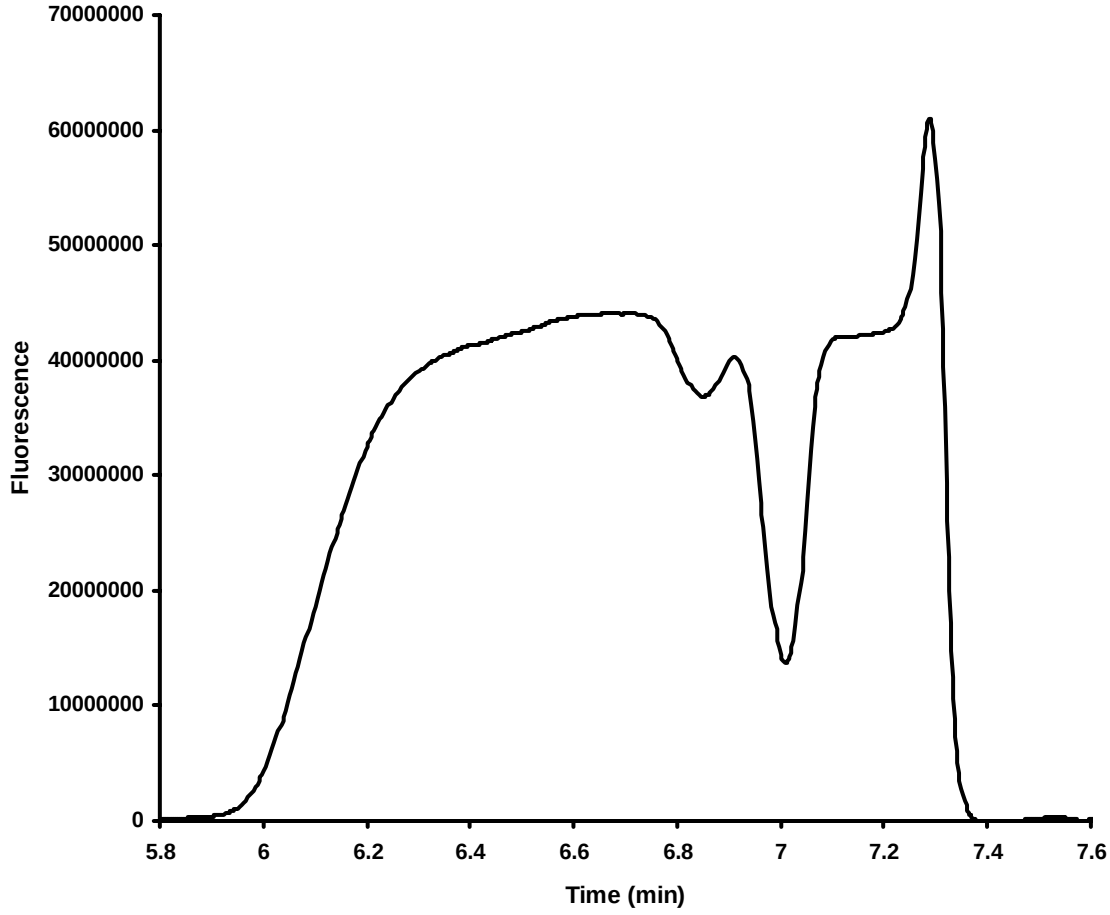


Figure 2.4. Electropherogram of an enzyme-inhibition assay with theophylline in a commercial CE system. A 4 s zone of 100 μM theophylline was injected at 0.5 psi into a capillary filled with 0.10 mM AttoPhos and 50 mM DEA at pH 9.5. A 3 s zone of 0.18 nM ALP at 0.5 psi was injected after applying a potential of 17.8 kV for 30 s. The applied electric field was 310 V/cm. The capillary length to the detector was 50 cm.

The reproducibility of theophylline measurements using the laboratory constructed instrument was 4%.[12] The assay was then transferred to a commercial automated CE system. The reproducibility of the assay on the new system was 5%. The assay showed similar reproducibility with both instruments, therefore the automated CE system was used for the metal chelator inhibition studies.

2.3.3 On-Column Enzyme-Inhibition Assay of Alkaline Phosphatase and EDTA

ALP inhibition by EDTA has been a widely studied and widely debated topic. Results of inhibition experiments vary extensively in the literature. Conyers et al.[41] used human ALP from bone, intestine, and placenta. At EDTA concentrations of 10^{-5} to 10^{-3} M, ALP underwent a loss of activity to the same extent regardless of concentration. At concentrations of 10^{-3} M EDTA and above, bone and intestinal ALP exhibited lower activity with increasing concentration. Placental ALP showed an increase of activity with increasing EDTA concentration. The authors noted that the first effect could be due to an activator in solution being removed. At high concentrations, the EDTA could remove the proposed activators and chelate the Zn^{2+} required by ALP rendering it inactive. Ensinger et al.[42] showed that calf intestinal ALP was completely inactivated by 0.01 to 1 M EDTA. Fortuna et al.[44] found that EDTA irreversibly inhibited bovine fetal ALP. Inactivation studies on green crab ALP showed a time dependent deactivation from 1 to 50 mM EDTA.[40] Activation has been reported for *Gastrothylax crumenifer* and *Cotylophoron orientale* ALP at 1.0 mM EDTA[48] and for *Ascaris suum* ALP at 10 mM EDTA.[49] All of these studies are based on traditional enzyme assay formats. Inhibitor was allowed to incubate with the enzyme for a set time period before measurements were made. One study was performed on immobilized bovine intestinal mucosa ALP.[50] The ALP was immobilized on controlled pore glass (CPG) and placed in a flow-injection system. It was found that EDTA inhibition was reversible by merely passing buffer through the system. The ALP in all of these experiments came from various sources and many were in crude preparations. All used different detection methods and various incubation periods. A direct comparison of the results would be difficult based on these

differences. A clear, concise explanation of the method of EDTA inhibition is still lacking.

The first EDTA inhibition studies in our lab (work by Whisnant)[27]were performed on the laboratory constructed CE system described in section *2.3.1*. In these experiments, EDTA at the chosen concentration was injected first for 55.0 s at 17.8 kV. Then, a constant potential of 17.8 kV was applied for 180 s. Next, a 3.0 s zone of 0.18 nM ALP was injected at 17.8 kV. A separation potential of 17.8 kV was applied. When performing the assay in the commercial system, several experimental conditions were changed. First, a 10 s zone of EDTA was injected at 0.5 psi. Then, a constant potential of 18.6 kV was applied for 36 s. Next, a 3.0 s zone of ALP was injected at 0.5 psi. A separation potential of 18.6 kV was then applied. The separation time of 36 s differs from the 55 s used in the laboratory constructed system assays. The difference in the optimal value for these times is possibly due to the use of pressure injection for 10 s instead of electrokinetic injection of EDTA for 55 s. Electrokinetic injections were used in the laboratory constructed system, while pressure injections were used with the commercial system.

To quantify the extent of inhibition, the ratio of enzyme activity before and after reaction with EDTA was measured over a range of concentrations. This value is known as fractional activity and is calculated as follows[27]:

$$\text{fractional activity} = (I_1 - I_0) / (I_2 - I_0) \qquad (2a)$$

where I_1 is the activity of ALP after interacting with EDTA, I_2 is the activity of ALP before interacting with EDTA, and I_0 is the baseline fluorescence. A graph of fractional activity versus EDTA concentration is shown in Figure 2.5.

The data from the assays performed on the laboratory constructed system, showed that ALP was irreversibly inhibited at EDTA concentrations of 1.0 mM or higher.[27] During the assay, product formation decreased as the zones of enzyme-substrate complex and inhibitor mixed. When the zones separated, the enzyme did not return to its original activity, which indicated that the enzyme had been irreversibly inhibited. The same

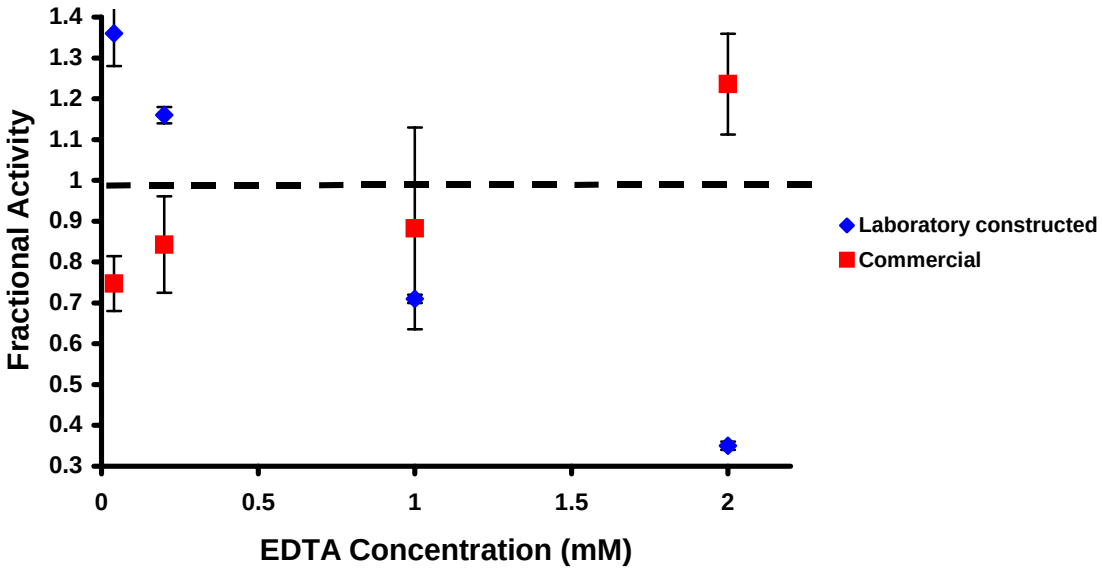


Figure 2.5. Plot of Fractional Activity versus EDTA concentration.

experiment performed with EDTA concentrations of less than 1.0 mM caused activation of the enzyme rather than irreversible inhibition. RSD values for these experiments are 1.4 and 2.9% for 1.0 and 2.0 mM respectively, and 5.8 and 1.7% for 0.04 and 0.2 mM. As the concentration of EDTA increased, the average fractional activity of ALP decreased. The data from the assays performed on the commercial CE system show the opposite pattern. As the concentration of EDTA increased, the average fractional activity of ALP also increased. Concentrations of 0.04, 0.2, and 1 mM EDTA inactivated ALP while 2 mM EDTA caused activation. Electropherograms are shown in Figures 2.6 and 2.7. The two sets of assays were performed at different temperatures. The laboratory constructed system was held at 40 °C while the commercial system was set at 25 °C. This fact could explain some of the differences seen between the two data sets. It is noted that the optimum temperature for ALP is around 38 °C.[32] Performing assays at a less than optimum temperature could possibly change the way the enzyme interacts with the inhibitor or the extent to which it is inhibited. One other possible explanation for the discrepancies is the composition of the solutions. For the first set of experiments, all of the solutions were prepared in ultrapure water (>18 MΩcm, Barnstead E-pure System, Dubuque, IA). Due to a problem with the water system, solutions for the second set of experiments were prepared in distilled, deionized water bought from VWR (West Chester, PA). It has been suggested in the literature that solutions could contain contaminants, often free metal ions, that would cause a decrease or increase in enzyme activity.[31] In theory, metal chelators would first remove these contaminants before affecting the enzyme itself. It could be possible that one of these sets of solutions contained such ions and this effect is being seen here.

2.3.4 On-Column Enzyme-Inhibition Assays of Alkaline Phosphatase by Various Metal Chelators

In order to better understand the mechanism of enzyme inhibition by metal chelators, a range of chelators have been studied using CE enzyme-inhibition assays with LIF detection. While EDTA has been thoroughly studied, many of the results found in the literature are conflicting,[27, 40-42, 44, 48] as were the results in our

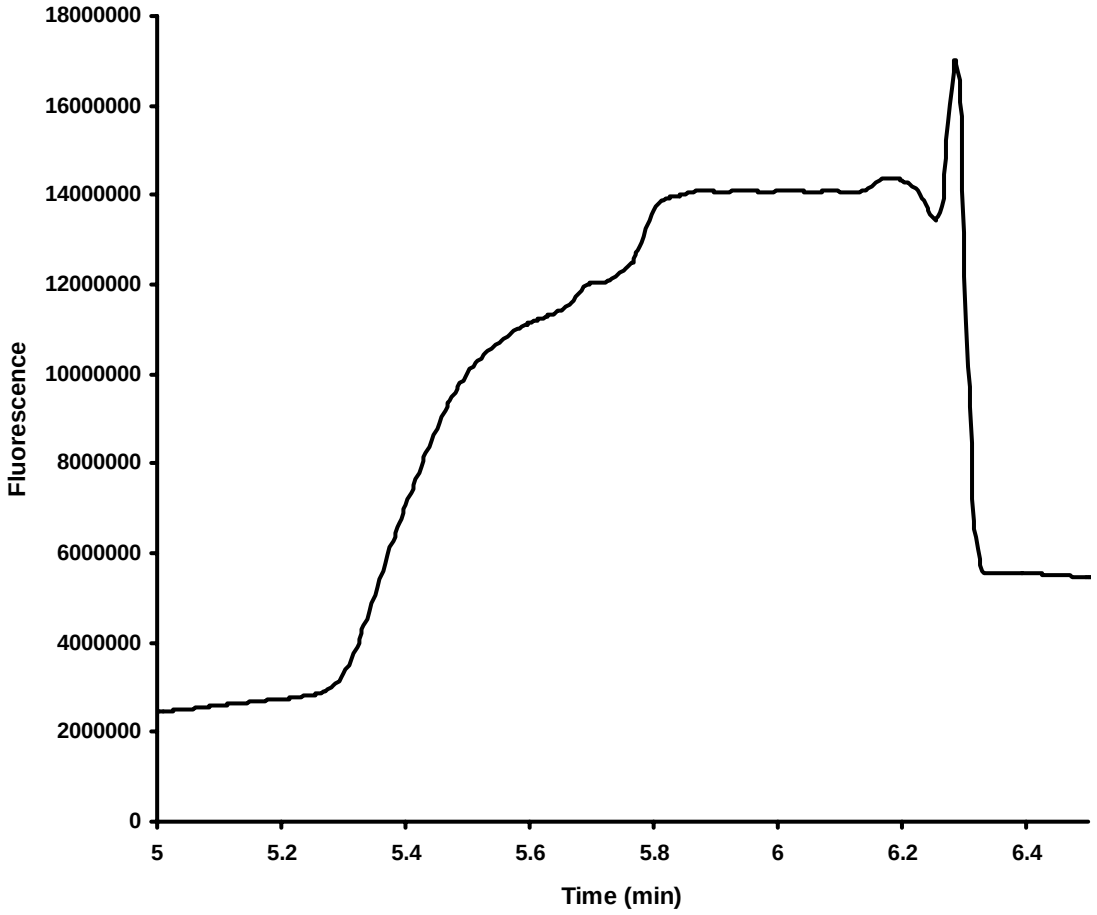


Figure 2.6. Electropherogram of an enzyme-inhibition assay with 0.04 mM EDTA. A 10 s zone of EDTA was injected at 0.5 psi into a capillary filled with 0.10 mM AttoPhos and 50 mM DEA at pH 9.5. A 3 s zone of 0.18 nM ALP at 0.5 psi was injected after applying a potential of 17.8 kV for 36 s. The applied electric field was 310 V/cm. The capillary length to the detector was 50 cm.

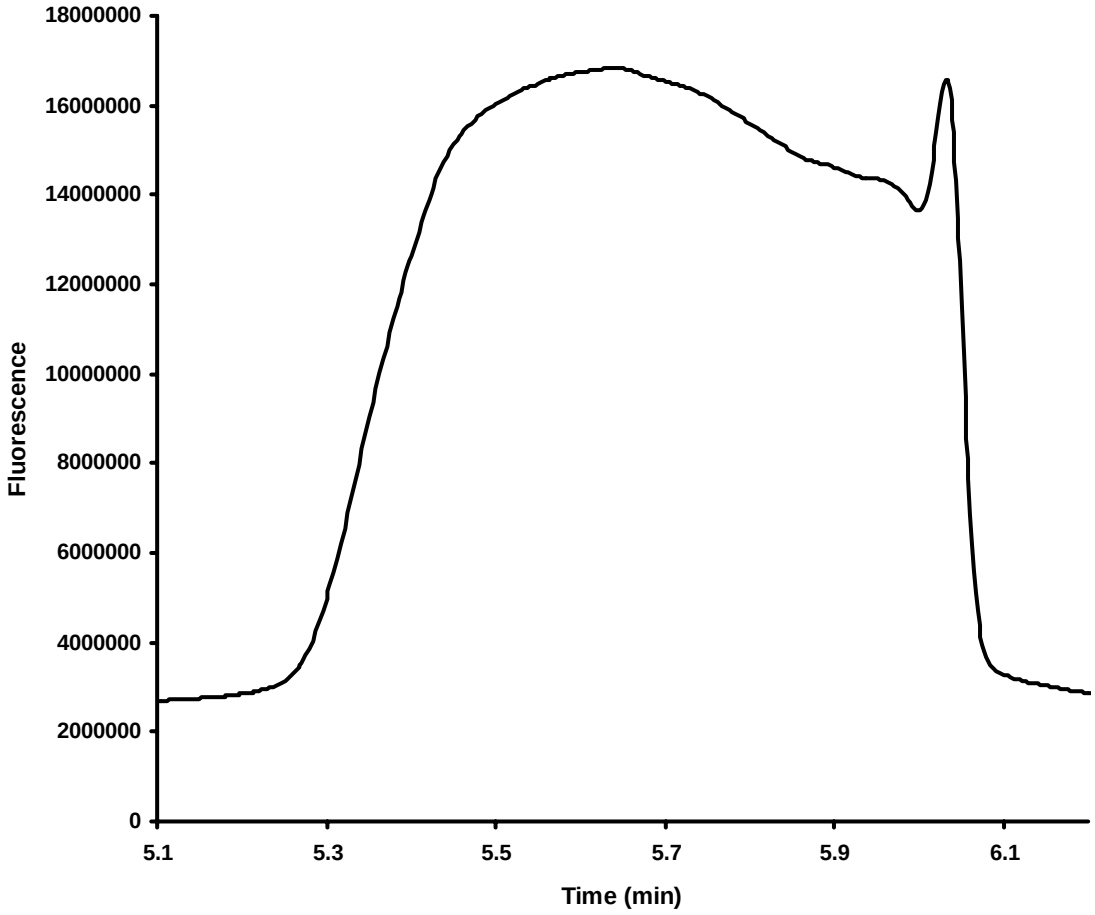


Figure 2.7. Electropherogram of an enzyme-inhibition assay with 2 mM EDTA. A 10 s zone of EDTA was injected at 0.5 psi into a capillary filled with 0.10 mM AttoPhos and 50 mM DEA at pH 9.5. A 3 s zone of 0.18 nM ALP at 0.5 psi was injected after applying a potential of 17.8 kV for 36 s. The applied electric field was 310 V/cm. The capillary length to the detector was 50 cm.

own studies. Many of the chelators included have not been extensively studied nor used with the CE enzyme-inhibition assay format.

One way to evaluate the properties of metal chelators is by looking at the first stability constants for metal-ligand complexes. First stability constants are basically a measure of how tightly the metal is bound by a ligand.[51] In general, the stability constant of a metal complex can be defined as:

$$K = [ML] / [M] [L] \qquad (2b)$$

where K is the stability constant, [M] is the concentration of metal ion, and [L] is the concentration of ligand present. The concentration of M is dependent on the stability constant of the complex and the free concentration of the ligand, which is dependent on pK and pH values. Low stability constants (less than 1) mean that the metal-ligand complex readily dissociates. High stability constants mean that only a small amount, if any, of the metal-ligand complex will dissociate into metal ions and ligands. Based on this idea of stability constants, it is hypothesized that the higher the stability constant of the ligand-Zn^{2+} complex, the greater the extent of inhibition in alkaline phosphatase. First stability constants for the chelators used in the study are shown in Table 1.[51]

The metal chelator [ethylenebis(oxy-ethylenenitrilo)]tetraacetic acid, or EGTA, is structurally similar to EDTA and has a first stability constant close to the value of EDTA (Table 1). Very few enzyme inhibition studies have been performed using EGTA; however, it was found that chondrocytic ALP was irreversible inhibited by 1.0 mM EGTA when incubated for 15 min.[44] No studies have reported using EGTA in a CE enzyme-inhibition assay.

Studies were performed on the commercial CE system. First, a 10 s zone of EGTA was injected at 0.5 psi. A constant potential of 18.6 kV was applied for 36 s. Next, a 3.0 s plug of 0.18 nm ALP was injected at 0.5 psi. A separation potential of 18.6 kV was reapplied. Data for these assays were analyzed in the same manner as the EDTA data. An electropherogram can be seen in Figure 2.8. It was found that EGTA at low concentrations inhibited ALP. A plot of fractional activity versus inhibitor concentration

Table 1. First Stability Constants for Various Inhibitors[51]

Chelator	Zinc	Magnesium
EDTA	16.5	8.7
EGTA	14.5	5.21
NTA	10.67	5.46
1,10-phenanthroline	6.36	1.2
12-Crown-4	**NA**	**NA**
18-Crown-6	**NA**	**NA**

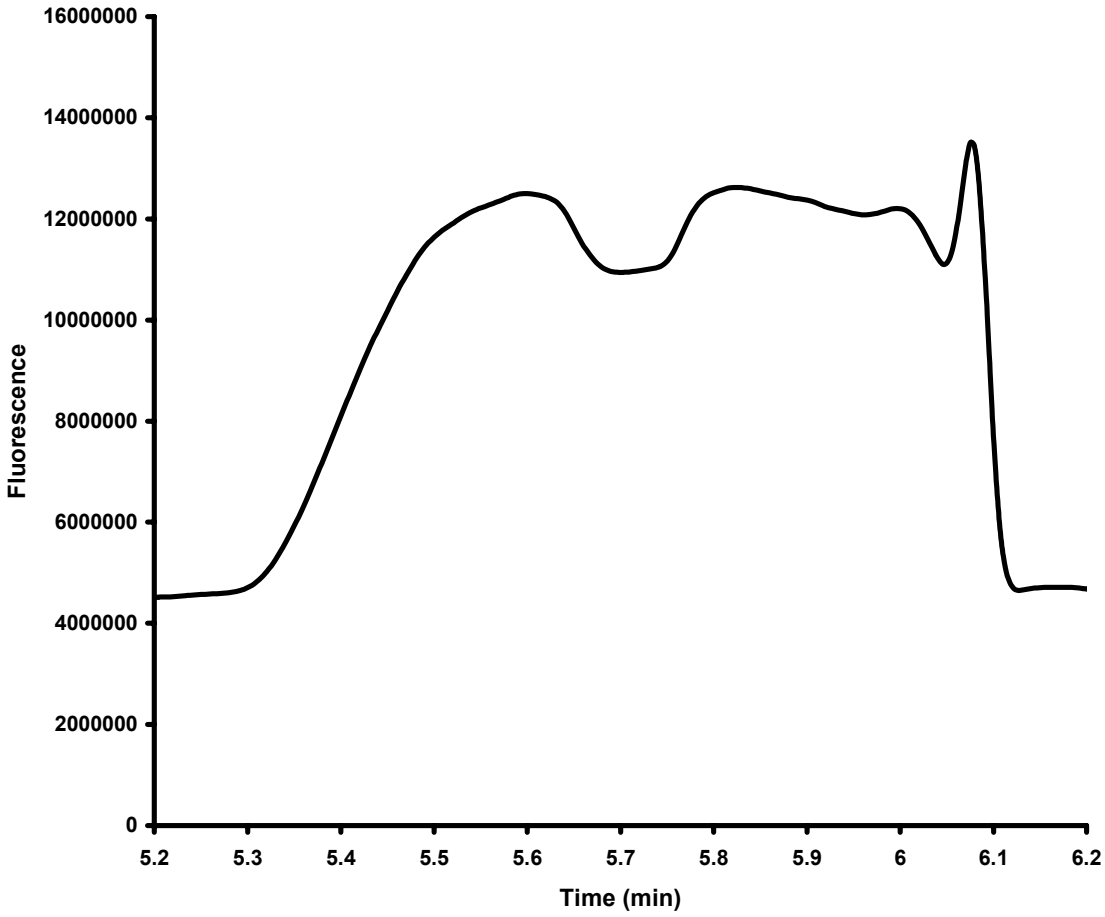


Figure 2.8. Electropherogram of an enzyme-inhibition assay with 2 mM EGTA. A 10 s zone of EGTA was injected at 0.5 psi into a capillary filled with 0.10 mM AttoPhos and 50 mM DEA at pH 9.5. A 3 s zone of 0.18 nM ALP at 0.5 psi was injected after applying a potential of 17.8 kV for 36 s. The applied electric field was 310 V/cm. The capillary length to the detector was 50 cm.

can be seen in Figure 2.9. As the concentration of EGTA increased from 0.08 to 0.3 mM, fractional activity decreased. However, there was a significant increase in fractional activity at a 1.0 mM concentration. As the concentration was increased to 2.0 mM, fractional activity decreased again. One interesting observation is that at the low concentrations, EGTA appeared to be a reversible inhibitor. However at a 2.0 mM concentration, the first two trials showed reversible behavior and the last two show irreversible behavior. Irreversible behavior can be seen in Figure 2.10. Due to the similarities in structure and first stability constants between EGTA and EDTA, it would be expected that these two chelators would affect ALP in the same manner. However, the results followed no apparent pattern. The difference in inhibition type, irreversible for EDTA and reversible for EGTA is interesting.

The chelator 1,10-phenanthroline had the lowest first stability constant of any studied (Table 1). Studies using this chelator have found that inhibition occurs only at relatively high concentrations, with one study quoting that concentrations of up to 1 mM had no effect on placental ALP[41], while another stated that concentrations of less that 0.5 mM had no effect on calf intestinal ALP.[42] The consensus among the studies is that a relatively long incubation period is needed before any inhibition effects are seen. Once again, no studies were found using this chelator with CE enzyme-inhibition assays.

The assay was performed exactly as described for the previous chelators. An electropherogram is presented in Figure 2.11 and a plot of fractional activity versus inhibitor concentration can be seen in Figure 2.9. Once again, reversible inhibition was observed. As seen in the plot, activity increases sharply from 0.08 to 0.3 mM and continues until reaching a concentration of 2.0 mM, where a decrease in activity occurs.

Nitrilotriacetic acid, or NTA, is another commonly used metal chelator.[35] It has rarely been used as an enzyme inhibitor and no reports were found using it in conjunction with CE enzyme-inhibition assays. The assay was performed in the exact manner as described for EGTA. An electropherogram is showen in Figure 2.12. It was once again found that this chelator showed a reversible inactivation and greatly inhibited ALP at relatively low concentrations. A plot of fractional activity versus inhibitor concentration is presented in Figure 2.13.

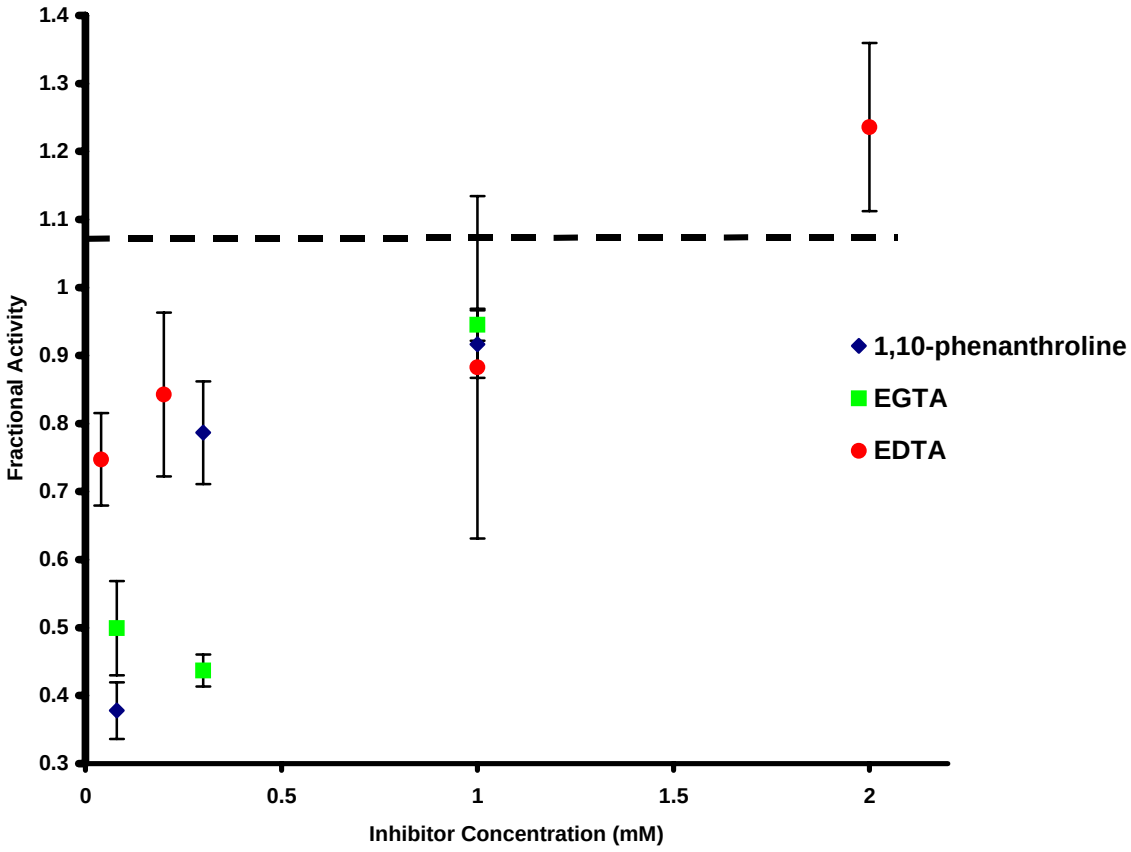


Figure 2.9. Plot of Fractional Activity versus Inhibitor Concentration for EDTA, EGTA, and 1,10-phenanthroline

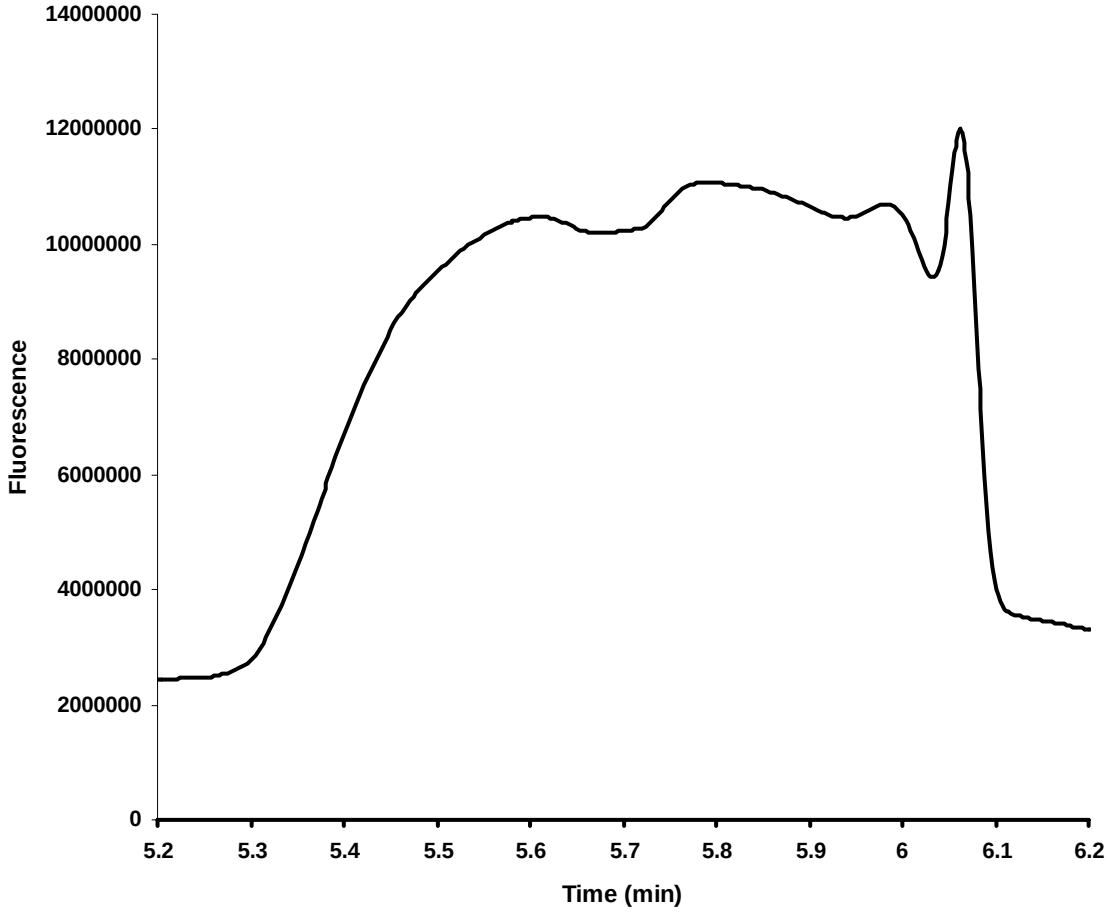


Figure 2.10. Electropherogram showing irreversible behavior of 2 mM EGTA. Experimental conditions are the same as in Figure 2.8.

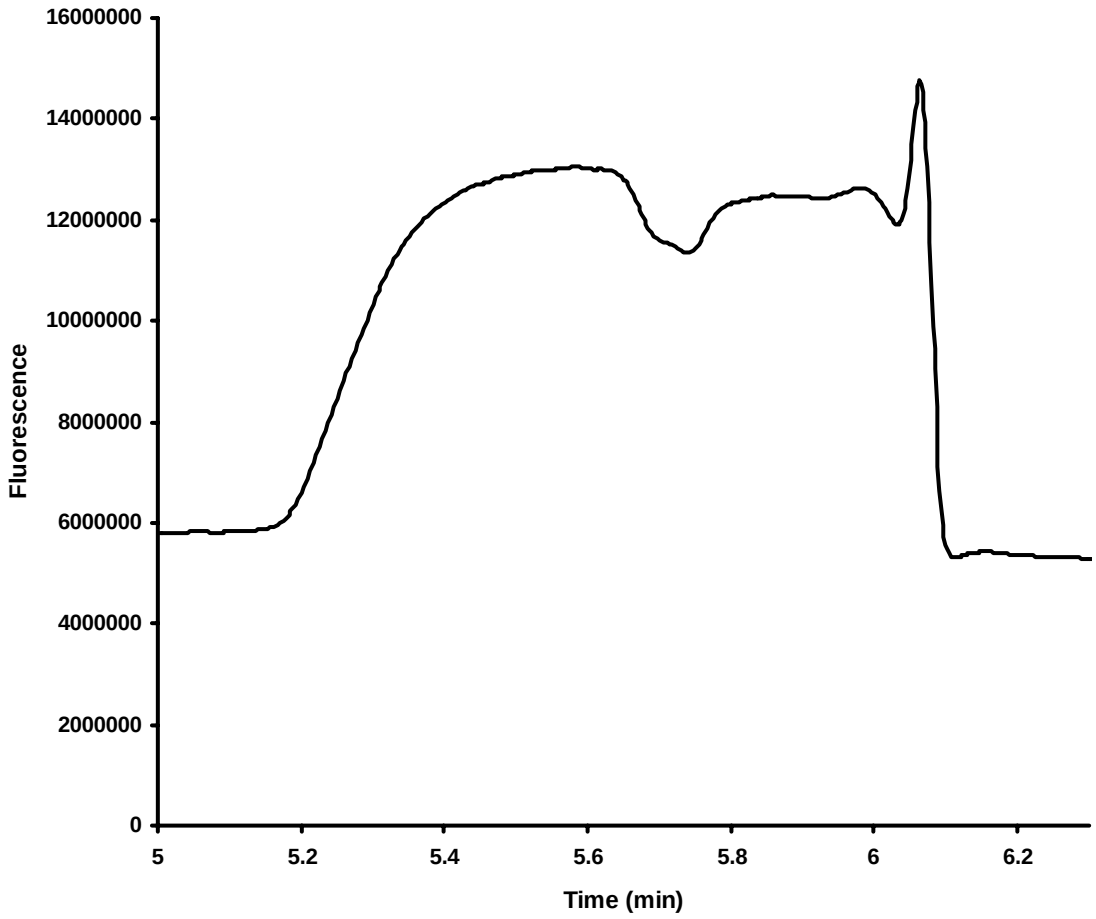


Figure 2.11. Electropherogram of an enzyme-inhibition assay with 1 mM 1,10-phenanthroline. A 10 s zone of 1,10-phenanthroline was injected at 0.5 psi into a capillary filled with 0.10 mM AttoPhos and 50 mM DEA at pH 9.5. A 3 s zone of 0.18 nM ALP at 0.5 psi Was injected after applying a potential of 17.8 kV for 36 s. The applied electric field was 310 V/cm. The capillary length to the detector was 50 cm.

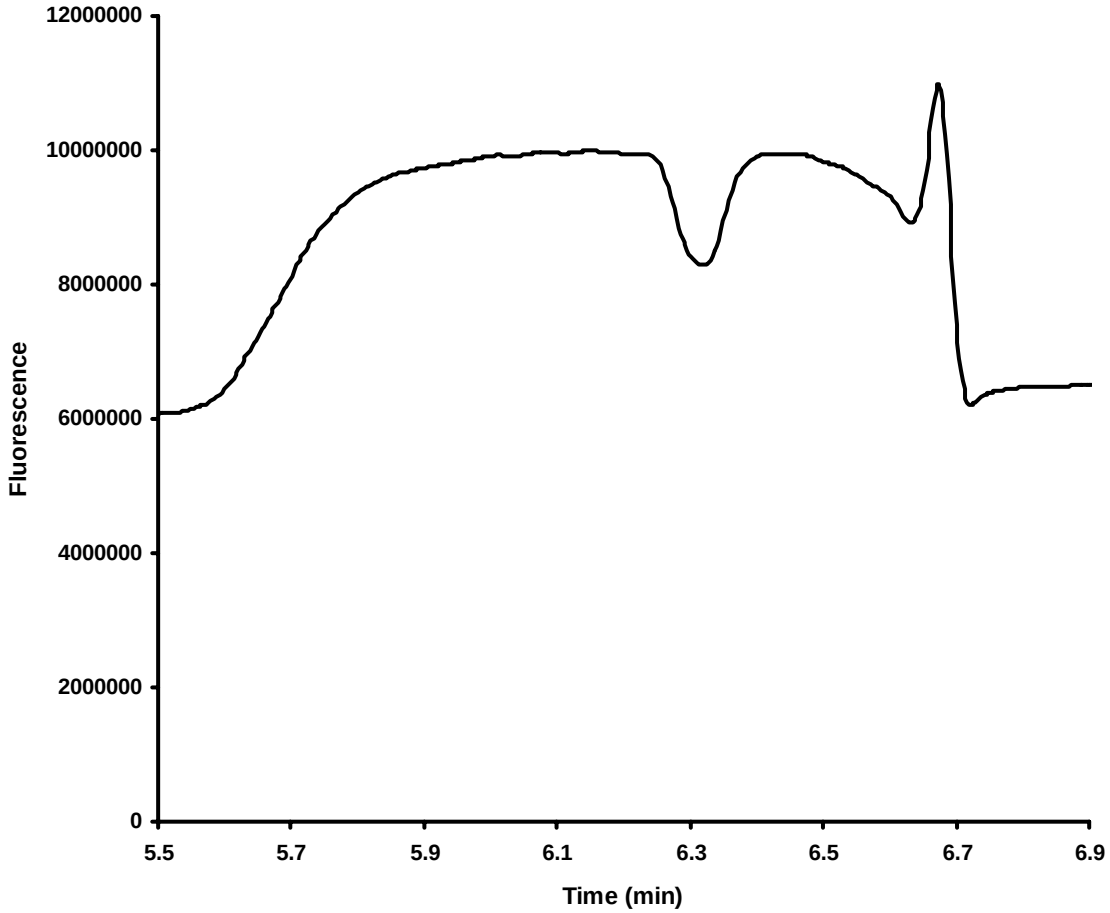


Figure 2.12. Electropherogram of an enzyme-inhibition assay with 0.3 mM NTA . A 10 s zone of NTA was injected at 0.5 psi into a capillary filled with 0.10 mM AttoPhos and 50 mM DEA at pH 9.5. A 3 s zone of 0.18 nM ALP at 0.5 psi was injected after applying a potential of 17.8 kV for 36 s. The applied electric field was 310 V/cm. The capillary length to the detector was 50 cm.

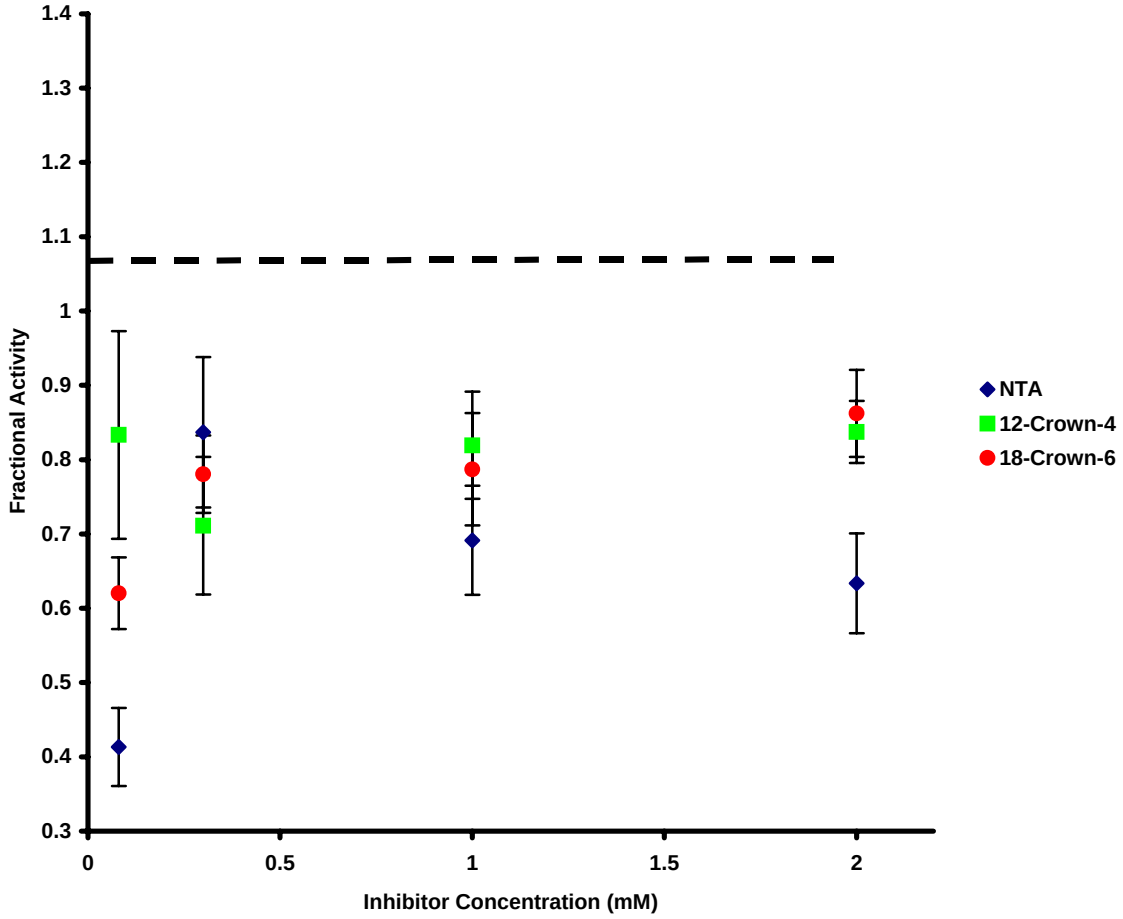


Figure 2.13. Plot of Fractional Activity versus Inhibitor Concentration for NTA, 12-Crown-4, and 18-Crown-6

Fractional activity of ALP was low at a NTA concentration of 0.08 mM. The fractional activity increased at 0.3 mM. Above 0.3 mM, the activity decreased with increasing concentration. Due to the high stability constant for the metal-ligand complex (Table 1), it would be expected that high concentrations of the chelator would inhibit to a greater extent than low concentrations. However, this trend was not observed.

Crown ethers were studied to determine their effect on ALP. Crown ethers, while complexing with metals, are not considered chelators and have not been previously used for inhibition studies. Crown ethers are macrocyclic compounds that possess electron-rich interior cavities and can complex with metals of compatible dimensions through dipole-dipole or ion-dipole interactions.[52] The radius of the cation compared to the effective radius of the cavity of the crown ether is the most important consideration in metal complexing ability.[53] However, there is little experimental data to elucidate the exact mechanism and no reliable stability constants have been published.

Two crown ethers, 12-crown-4 and 18-crown-6 were chosen for these studies. Zn^{2+} has a radius of 0.74 Å.[53] The effective radii of crown ethers are hard to estimate, but accepted values are 0.72 Å for 12-crown-4 and 1.45 Å for 18-crown-6.[53] These assays were performed as previously described for the other inhibitors. A plot of fractional activity versus inhibitor concentration for both compounds can be seen in Figure 2.13.

Both crown ethers showed reversible inhibition behavior. Data for the assay performed using 12-crown-4 showed a decrease in fractional activity from 0.08 to 0.3 mM. However, from 0.3 to 2.0 mM activity increased with increasing concentration. Compared to all the other inhibitors in the study, 12-crown-4 showed the least extent of inhibition overall. This could be explained by the fact that the radius of Zn^{2+} is approximately the same as the effective radius of the interior cavity of the molecule so that complexation may be weak. An electropherogram is shown in Figure 2.14.

The data for the 18-crown-6 assays showed that fractional activity increased with concentration. This crown ether seemed to be more effective at inhibiting ALP overall than the 12-crown-4, which could be due to the larger effective radius of the cavity. The Zn^{2+} "fits" the larger cavity better. An electropherogram can be seen in Figure 2.15.

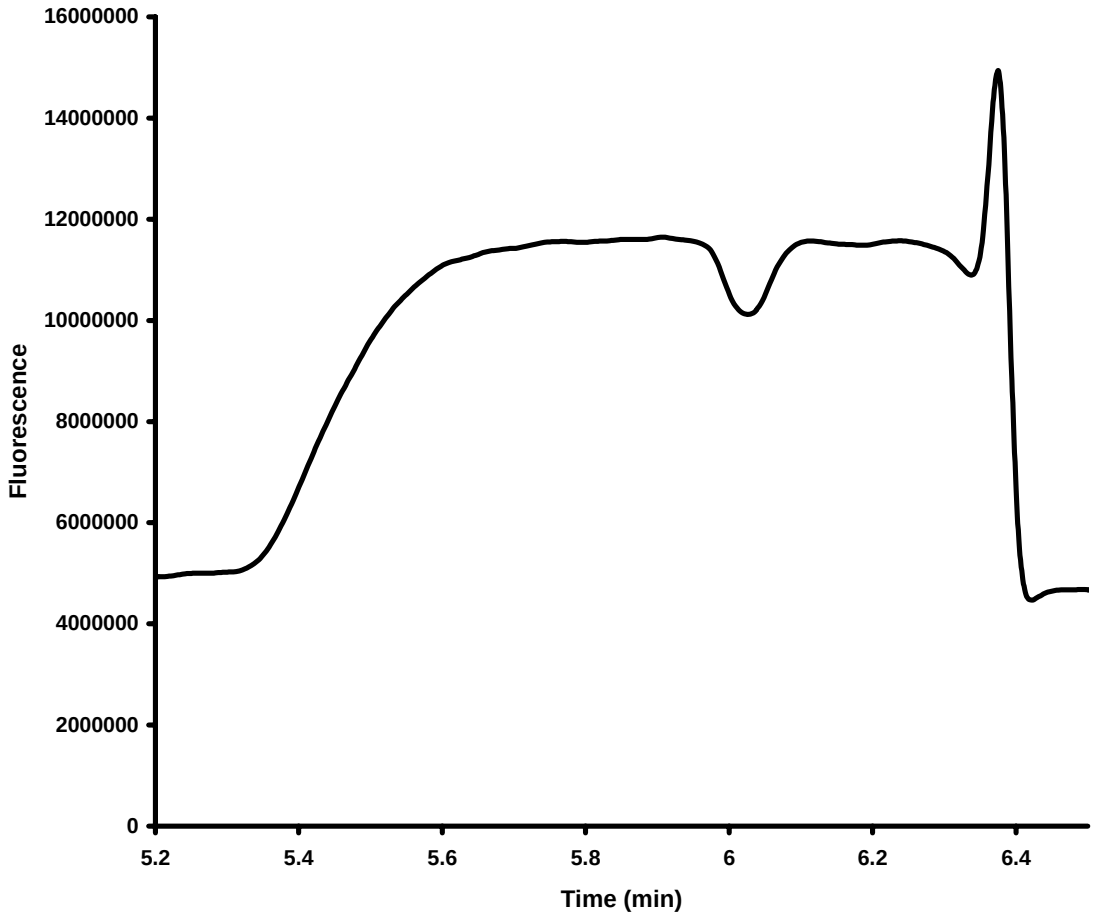


Figure 2.14. Electropherogram of an enzyme-inhibition assay with 0.3 mM 12-Crown-4. A 10 s zone of 12-Crown-4 was injected at 0.5 psi into a capillary filled With 0.10 mM AttoPhos and 50 mM DEA at pH 9.5. A 3 s zone of 0.18 nM ALP at 0.5 psi was injected after applying a potential of 17.8 kV for 36 s. The applied electric field was 310 V/cm. The capillary length to the detector was 50 cm.

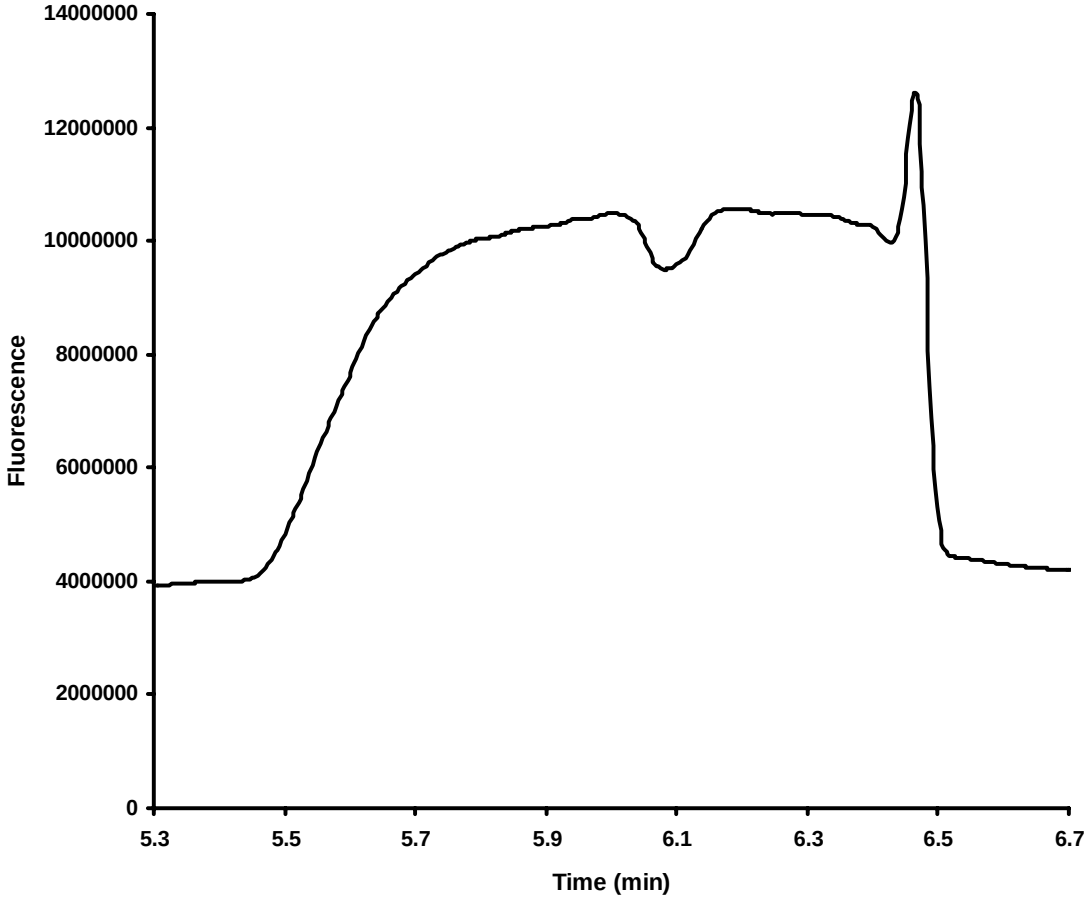


Figure 2.15. Electropherogram of an enzyme-inhibition assay with 1mM 18-Crown-6. A 10 s zone of 18-Crown-6 was injected at 0.5 psi into a capillary filled with 0.10mM AttoPho and 50 mM DEA at pH 9.5. A 3 s zone of 0.18 nM ALP at 0.5 psi was injected after applying a potential of 17.8 kV for 36 s. The applied electric field was 310 V/cm. The capillary length to the detector was 50 cm.

The data for 18-crown-6 followed the same trend as EDTA. As previously discussed, some type of metal contaminants could be present in solution causing an initial decrease in enzyme activity.[35] Due to the larger cavity radius, 18-crown-6 could potentially complex with many metal ions, removing the inhibitor and restoring some of the activity of the enzyme.

The results of the experiments for metal chelators were not consistent between EDTA and the other inhibitors studied. Many references in the literature describe metal chelator inhibition as irreversible; however, most of these studies focus on EDTA and while other chelators have been used, detailed studies have not been performed.[39-42, 44] Another difference between the studies presented here and those found in the literature is the type and time scale of the assays. Most of the previous studies were performed by traditional methods with samples incubated for a fixed time period before measurement. In these CE assays, no pre-incubation of the sample occurs. Ensinger et al.[42] reported that EDTA inhibition was reversible at pH 8.0 and was independent of concentration. However, at pH 9.8, 1 mM EDTA showed irreversible inactivation. Conyers et al.[41] reported that EDTA inhibition was reversible when assays were performed without pre-incubation of the samples. When samples were pre-incubated, EDTA showed a time-dependent, slow inactivation, which was irreversible. Both authors hypothesized that the results could be due to EDTA binding to the Zn^{2+}, and slowly inducing a conformational change in the structure and removing the metal over time. More recent studies by Zhang et al.[39] and Chen et al.[40] also present the same theory of EDTA inhibition. Inhibition appears reversible on a short time scale and irreversible over a longer time period. Both of these studies suggest a three step mechanism. The first is a rapid reversible binding of EDTA and the enzyme. The second step is the rate limiting step in which the enzyme changes from a strained active site to a more conformationally stable inactive state. In the third step, metal ions are removed from the active site to form an EDTA-Zn^{2+} complex. The inhibition is then irreversible. In the studies presented here, no incubation of the samples occurred. Therefore, the time scale for the reactions is very short. EDTA still shows irreversible behavior even with the shorter time scale. The other chelators

presented do show reversible inhibition on the short time scale, and so follow the theory discussed above. Studies with sample incubation should be performed in order to determine if these chelators really follow the inhibition pattern laid out in the theory.

Chapter 3

Conclusions and Future Work

3.1 Conclusions

Alkaline phosphatase inhibition by metal chelators has been studied using capillary electrophoresis. The enzyme-inhibition assays were performed by electrophoretically mixing enzyme and inhibitor zones in a substrate filled capillary. Enzyme inhibition could be seen as a decrease in product formation as detected using LIF. A range of metal chelators was studied and, using this approach, inhibition could easily be identified upon visual inspection of electropherograms and comparison to control electropherograms.

Basic CE enzyme assays were performed using calf intestinal alkaline phosphatase and AttoPhos, a fluorogenic substrate. These assays were first performed in a laboratory constructed CE system[12, 27] as a control and then moved to a commercial, automated CE system. RSD values were used as a measure of reproducibility of the enzyme assay and thus, system reliability. The results obtained with both systems had RSD values of less than 2%. Theophylline, a noncompetitive, reversible inhibitor was also studied as a control. Assays were performed on both the laboratory constructed[12, 27] and commercial CE system with RSD values of 4 and 5%, respectively. Since equivalent data was obtained, the automated CE was used for the CE enzyme-inhibition assays. Both systems give the same type and quality of data; however, there are several advantages to performing the assays on a commercial system. These systems feature user-friendly software systems with data analysis packages, capillary and sample cooling features and autosamplers.[45-47] Most of these instruments have interchangeable detectors and can be coupled to other systems so different detection schemes may be used for the best possible sample analysis. Automated capillary array instruments are also available, which can analyze hundreds of samples simultaneously. These systems will make CE enzyme and enzyme-inhibition assays a very valuable tool for the pharmaceutical and

biotechnology industries. The technique that has been reported would be valuable for large scale screening of compounds for inhibitory effects in order to discover new drug therapies.

EDTA was the first metal chelator studied using CE enzyme-inhibition assays. When performed on a laboratory constructed system, activation of the enzyme was seen at low EDTA concentrations (20 to 400 μM), while irreversible inhibition was observed at high EDTA concentrations (1.0 mM or higher).[27] When performed on the commercial system, the opposite trend was seen. As the concentration of EDTA was increased, fractional activity increased as well. Activation of the enzyme was observed with 2.0 mM EDTA. No other chelator showed activation of the enzyme.

EGTA is a metal chelator with similar structure and binding properties to EDTA. Both are tetraacetic acids, and they have affinity constants for Zn^{2+} of 14.5 and 16.5, respectively. However, a common trend in enzyme inhibition was not seen. EGTA inhibition was reversible except for two trials at 2.0 mM, which showed irreversibility. At concentrations of 0.08 to 0.3 mM, fractional activity decreased. There was a significant jump in enzyme activity at 1.0 mM EGTA, yet as concentration was increased to 2.0 mM activity once again decreased. NTA is another metal chelator similar to EDTA. It is a triacetic acid (EDTA being a tetraacetic acid) with an affinity constant for Zn^{2+} of 10.45. NTA inhibition was also reversible. For NTA, fractional activity increased from 0.08 to 0.3 mM. Above 0.3 mM, the activity decreased with increasing concentration. 1,10-phenanthroline is another metal chelator, with properties different from those of EDTA. It has a cyclic structure, unlike EDTA, with an affinity constant of only 6.36 for Zn^{2+}. With the chelator 1,10-phenanthroline, activity increased sharply from 0.08 to 0.3 mM and continued until reaching a concentration of 2.0 mM, where activity decreased. Reversible inhibition was seen with 1,10-phenanthroline.

Crown ethers, which complex with metals but have but have not been reported in the literature as enzyme inhibition, were also studied. Inhibition for both crown ethers was reversible. For 12-crown-4, there was a decrease in fractional activity from 0.08 to 0.3 mM. However, from 0.3 to 2.0 mM activity increased with increasing concentration of inhibitor. Compared to all the other inhibitors in the study, 12-crown-4 had the least

pronounced inhibition effect. This could be explained by the fact that the effective radius of the inner cavity of 12-crown-4 is comparable to the radius of Zn^{2+} molecule so it is possible that Zn^{2+} is not being removed effectively from the enzyme due to size restraints. Therefore, complexation may be relatively weak. For 18-crown-6, fractional activity increased with concentration, making it the only inhibitor to follow the EDTA pattern. This crown ether seemed to be more effective at inhibiting ALP overall than the 12-crown-4, which could be due to the larger effective radius of the cavity of 18-crown-6.

There was no apparent, overall pattern for inhibition by the metal chelators. A full explanation for the data is not possible at this point. However, there is a possible explanation for the discrepancies between the EDTA data from the laboratory constructed system and the commercial system. The assays in the laboratory constructed system were performed at 40 °C. In the commercial system, the assays were performed at 25 °C. The difference could affect how the enzyme interacts with the inhibitor and the extent of inhibition. There was also a difference in the composition of the solutions. For the first set of experiments, all of the solutions were prepared in ultrapure water.[27] The ultrapure water was not available for the second set of experiments due to water system equipment problems. Therefore, solutions for the second set of experiments were prepared in distilled, deionized water. Solutions could contain a range of contaminants, often free metal ions, that would cause a decrease or increase in enzyme activity.[35] In theory, metal chelators would first remove these contaminants before acting upon the enzyme itself. This effect could be occurring here.

One other interesting discrepancy in the data is that EDTA inhibition appears to be irreversible while the other chelators show reversible behavior. The reversible behavior can be explained by the fact that enzyme inhibition by metal chelators could occur in three steps, the first being a reversible and fast formation of a complex between chelator and ALP. The second step is a slow change from a strained, active complex to a conformationally stable inactive complex. This is the rate-limiting step. The final step is removal of the Zn^{2+} from the enzyme, rendering it irreversibly inactive. This theory has been discussed in the literature previously.[39, 40]

Even though a clear explanation of the data for enzyme inhibition using various metal chelators could not be reached, the utility of these enzyme assays has been expanded through the use of a commercial, automated CE system. While conventional enzyme assays can be performed in a microtitre plate quickly and in parallel, they are limited by the amount of material required. CE is well suited for enzyme assays because only nL size samples are needed. CE enzyme-inhibition assays also give different information about an enzyme-catalyzed reaction as compared to traditional methods. CE allows the activity of the enzyme to be measured before, during, and after interaction with the inhibitor. Also, the type of inhibition or activation may be readily distinguished upon visual inspection of the electropherogram. Other kinetic parameters such as component velocities, time of enzyme and inhibitor interaction, and where in the capillary the interaction occurs can be determined from electropherograms.

3.2 Future Studies

Future studies should include a more in-depth investigation of EDTA inhibition in an effort to resolve the conflicting results in this thesis and the work by Whisnant and Gilman.[27] Microtitre plate studies (traditional enzyme assays) could be performed to gather more information about the kinetics of EDTA enzyme inhibition. Assays should be performed on the laboratory constructed and commercial systems holding the temperature the same for both to see the effects of temperature on inhibition. Repeating the experiments on the laboratory constructed and commercial CE systems using the same water source for making solutions would also determine if contaminants are causing the difference in results between systems. The issue of reversibility should also be studied since most of the metal chelators in the study, with the exception of EDTA, showed reversible inhibition on the short time scale. Incubation experiments should be performed to determine whether or not this changes to irreversible behavior on a longer time scale, as suggested by the theory of Zhang et al.[39] Optimization of the enzyme-inhibition assays on the commercial CE system is also needed. Factors such as capillary and sample temperatures, rinsing procedures, and solution composition can be adjusted for the best possible analysis.

In order to deduce the mechanism of enzyme inhibition by metal chelators, more studies are needed including a wider range of these chelators. N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and a variety of crown ethers are some possibilities. Since activation was seen for some of the trials presented here, activators of alkaline phosphatase should also be studied. Both reactivation, which occurs through the interaction of a known activator with the enzyme after treatment with a metal chelator, and activation on its own should be looked at since this could lend new information to the mechanism of enzyme inhibition. Sodium cyanide and cysteine are some possibilities for future studies as these have been described as possible activators in the literature.[35]

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Appendix

Appendix

Study of Alkaline Phosphatase Inhibition by CE-LIF Utilizing On-Column Reagent Addition

Introduction

Capillary electrophoresis is a technique well suited to performing enzyme and enzyme-inhibition assays due to its relatively simple instrumentation, fast analysis times, and the small amount of enzyme required.[8, 9] Often, enzyme assays are performed by pre-mixing enzyme, substrate, and inhibitor prior to injection into the capillary where separation and detection will occur. When assays are performed in this manner, the reaction begins in the sample vessel rather than on-column, where detection occurs. This could result in the loss of valuable information about initial reaction rates. One way to circumvent this problem is to use on-column reagent addition. On-column reagent addition allows a reagent to be injected directly into the capillary without having to pre-mix the solutions. The entire reaction then takes place within the capillary. On-column reagent addition could also be used as a screening tool for enzyme inhibitors. A mixture of inhibitors can be injected into the capillary and separated prior to reaction with an enzyme, which is added on-column. An electropherogram would show an inhibition peak for each inhibitor present.

On-column reagent addition has played a major role in the development of CE as a versatile separation technique.[54] The first use of on-column reagent addition was postcolumn derivitization for fluorescence detection.[55] The technique has now been used with chemiluminescence, electrochemical, and bioaffinity detection as well as for enzyme assays and electroosmotic flow monitoring.[54] Several designs exist for on-column reagent addition with CE.[54] A coaxial reactor is one of the most common designs and consists of a separation capillary that is inserted into a reaction capillary. The reagent is introduced into the reaction capillary by pressure or applied potential. Free solution

reactors are buffer reservoirs at the detection end of the capillary where the reaction and detection both take place. Sheath flow cuvettes were initially designed to prevent scatter in fluorescence detection but have also been used for the purpose of on-column reagent addition.

A gap reactor is another design for on-column reagent addition and was used in the experiments described here. A gap reactor is a simple and effective design for on-column reagent addition.[54, 56, 57] Reagents are added through a small gap between two capillaries. This gap is typically between 3 to 100 μm. Reagents are typically introduced through the gap by diffusion or differential flow. Flow in the reaction capillary is generated by either an applied potential or pressure difference in the gap reservoir and the end of the capillary. Separated components migrate across the gap from the separation capillary into the reaction capillary. A typical gap reactor can be seen in Figure A.1.

The performance of gap reactors is strongly dependent upon the alignment of the two capillaries and the distance between them. Gap reactors have generally been created by cleaving a single capillary that has been secured to a surface.[54] This requires manual cutting under a microscope, which can make controlling the size of the gap difficult. For the experiments described here, laser ablation was used to create the gap reactors, which allowed more control over the gap size and simplified the process. No manual cutting was required.

Enzyme and enzyme inhibition assays were performed using a gap reactor for on-column reagent addition. Several reversible inhibitors of alkaline phosphatase were studied. Inhibitor solutions can be pre-mixed and separated prior to development of the assay. This technique could be used as a tool to screen mixtures for enzyme inhibitors.

Experimental

Reagents

AttoPhos ([2,2'-bibenzothiazol]-6-hydroxy-benzathiazole phosphate) was purchased from Promega (Madison, WI). Calf intestinal alkaline phosphatase (EC 3.1.3.1) was supplied by ICN Biomedicals (Aurora, OH). DEA (diethanolamine), sodium vanadate, and Theophylline (99%) were obtained from Acros (Pittsburgh, PA).

Figure A.1. Photograph of a 12.9 μm gap created using laser ablation. The gap was created using 400 pulses at 15 Hz and 13.5 mJ/pulse.

All solutions were prepared in ultrapure water (>18 MΩcm, Barnstead E-pure System, Dubuque, IA).

Laser Ablation Instrumentation and Experimental Conditions

A 50 μm i.d. / 200 μm o.d. fused silica capillary supplied by SGE (Austin, TX) was glued to a microscope slide using 5 Minute Epoxy (ITWDevcon; Danvers, MA). A mount was used that was designed to position the capillary at a reproducible distance (~950 μm) from the microscope slide. The microscope slide was then positioned on a translation stage from Newport (Irvine, CA). A plano convex lens with a focal length of 100 mm (Edmund; Barrington, NJ) was used to focus the beam from a laser pointer (λ = 650 nm; Quarton Inc; His-Chih, Taipei Hsien, Taiwan) onto the capillary. The laser pointer was mounted on a translation stage in order to be moved in the same direction as the laser beam. The second harmonic of a Nd:YAG laser (λ = 523 nm; Continuum; Santa Clara, CA) was focused onto the capillary using a fused silica cylindrical lens (Melles Griot; Carlsbad, CA) for ablation. The set up can be seen in Figure A.2. After construction of the gap, a reagent reservoir was formed by gluing a polyethylene vial lid with 5 Minute Epoxy. A reagent hole was added using a hot metal wire to melt the polyethylene. A schematic of a gap reactor may be seen in Figure A.3. The gaps were then examined using a video trinocular head zoom microscope (Edmund), and the image collected using a CCD camera (Panasonic). The image was captured using VIDCAP 32 software (Microsoft) and analyzed using Scion software (Scion Corp.; Fredrick, MD).

CE-LIF Instrumentation and Experimental Conditions

The CE-LIF instrument was constructed in house. The 457.9 nm line of an argon ion laser (Melles Griot; Carlsbad, CA) was focused onto the capillary using a fused-silica plano convex lens (Optosigma; Santa Ana, CA). The laser power at the capillary was 35 mW. Fluorescence was collected at 90° to the laser beam by a 20 X microscope objective (Edmund; Barrington, NJ) and was filtered by both a 560 ± 10 nm bandpass

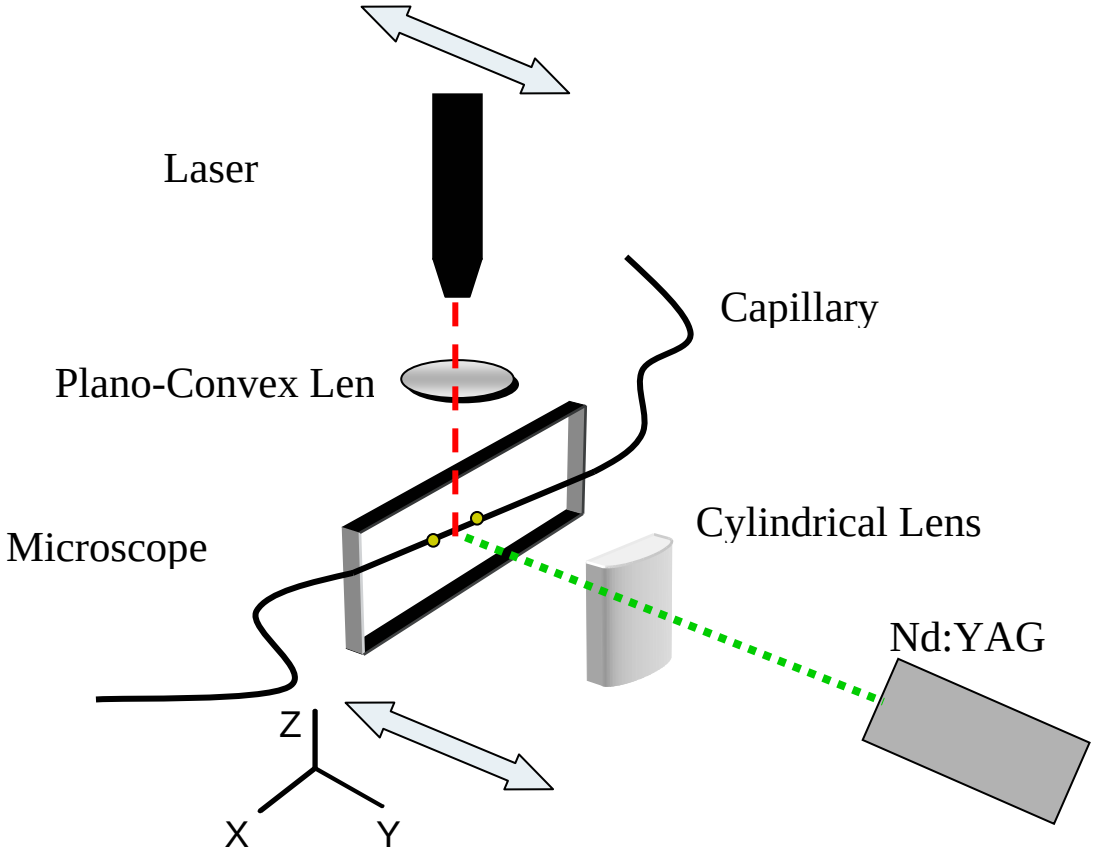

Figure A.2. Setup of the Nd:YAG pulsed laser ablation system.

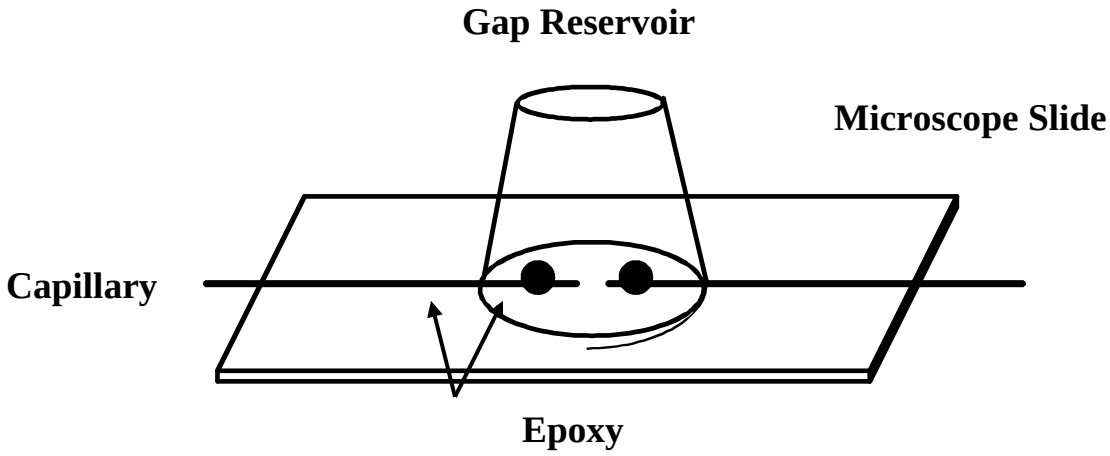

Figure A.3. Schematic of a gap reactor

filter (Oriel; Stratford, CT) and 1 mm diameter aperature (Oriel). Fluorescence was detected using a PMT (Hamamatsu; Bridgewater, NJ) at 750 V. The PMT output was filtered using a low-pass filter at 50 Hz, then sent to an analog-to-digital board (National Instruments; Austin, TX). A LABVIEW program (National Instruments) was used for data acquisition and Excel (Microsoft) and Peakfit (SPSS Inc.; Chicago, IL) were used for data analysis. Fused silica capillaries with a 50 μm i.d. and 220 μm o.d. were used, from which the polyimide coating was removed using a low flame.

The running buffer consisted of 50 mM DEA at pH 9.5 and 0.10 mM AttoPhos, a fluorogenic alkaline phosphatase substrate. The enzyme solution contained 0.18 nM alkaline phosphatase and 50 mM DEA buffer at pH 9.5. Running buffer and enzyme solution were prepared fresh daily. The inhibitor solutions contained 50 mM DEA buffer, 0.10 mM AttoPhos and inhibitor at selected concentrations. The applied electric field for all separations was 310 V/cm. Injections were performed electrokinetically at 17.5 kV.

UV Instrumentation

A Beckman Coulter P/ACE™ MDQ Capillary Electrophoresis System (Fullerton, CA) was used in this study. The instrument was equipped with a UV detector module and all measurements were made at 214 nm. Fused silica capillary with an i.d. of 52 μm and o.d. of 366 μm was used.

Results and Discussion

Enzyme and enzyme inhibition assays were performed using a gap reactor for on-column reagent addition. On-column reagent addition allows enzyme assays to be performed without pre-mixing enzyme, inhibitor, and substrate. When these solutions are pre-mixed, the reaction begins in the sample chamber rather than the column where detection occurs. Important kinetic details, such as initial reaction rates, may be missed. On-column reagent addition may also be used to facilitate separation of inhibitor mixtures. Inhibitors may be pre-mixed and injected onto the column where the components are separated. The enzyme is added on-column where the assay develops.

Each inhibitor would have a representative inhibition peak on the electropherogram. Several clinically important reversible inhibitors of alkaline phosphatase were studied. Theophylline is a reversible, noncompetitive inhibitor that has been used as a bronchodilator, respiratory stimulant, and anti-inflammatory drug.[12] Sodium vanadate is a reversible, competitive inhibitor that has been used for treatment of diabetes.[27]

The basic enzyme assay was performed first. This assay serves as a control for assurance that the gap reactor itself is not interfering with the kinetics of the reaction. The assay was performed by first filling the gap reservoir with running buffer. This essentially creates a system much like a regular CE assay (described in Chapter 2) since no additional reagents are being added. A 3.0 s plug of ALP was injected, followed by an applied separation potential. An electropherogram can be seen in Figure A.4. There is one noticeable difference in the electropherogram from this system and the one from the regular CE enzyme assay without on-column reagent addition. The plateau in Figure A.4 has a noticeable dip, which appears like an inhibition dip. This system was not thermostated and the dip is due to changes in the temperatures of various regions of the capillary, which change the reaction rate as the enzyme moves down the column. An enzyme-inhibition assay was also performed in this manner for another control. A 4.0 s plug of 100 μM theophylline was injected followed by application of a constant potential for 25 s. Then, a 3.0 s plug of ALP was injected and the constant potential reapplied. The inhibition can be seen upon visual inspection of the electropherogram as a well-defined dip in the plateau (Figure A.5). The shape of the plateau remains the same as that for the regular assay so it was determined that the gap reactor does not affect the kinetics of the reaction.

The on-column enzyme-inhibition assay was performed by first filling the gap reservoir (~1 mL) with 0.18 nM ALP. A 4.0 s zone of 100 μM theophylline was injected. The potential was reapplied. When the potential was first applied, ALP from the reservoir diffused in from the gap as a steady stream and mixed with running buffer, which contained the fluorogenic substrate. This caused a rise in the fluorescence level of the background. When the potential was turned off in order to inject the theophylline,

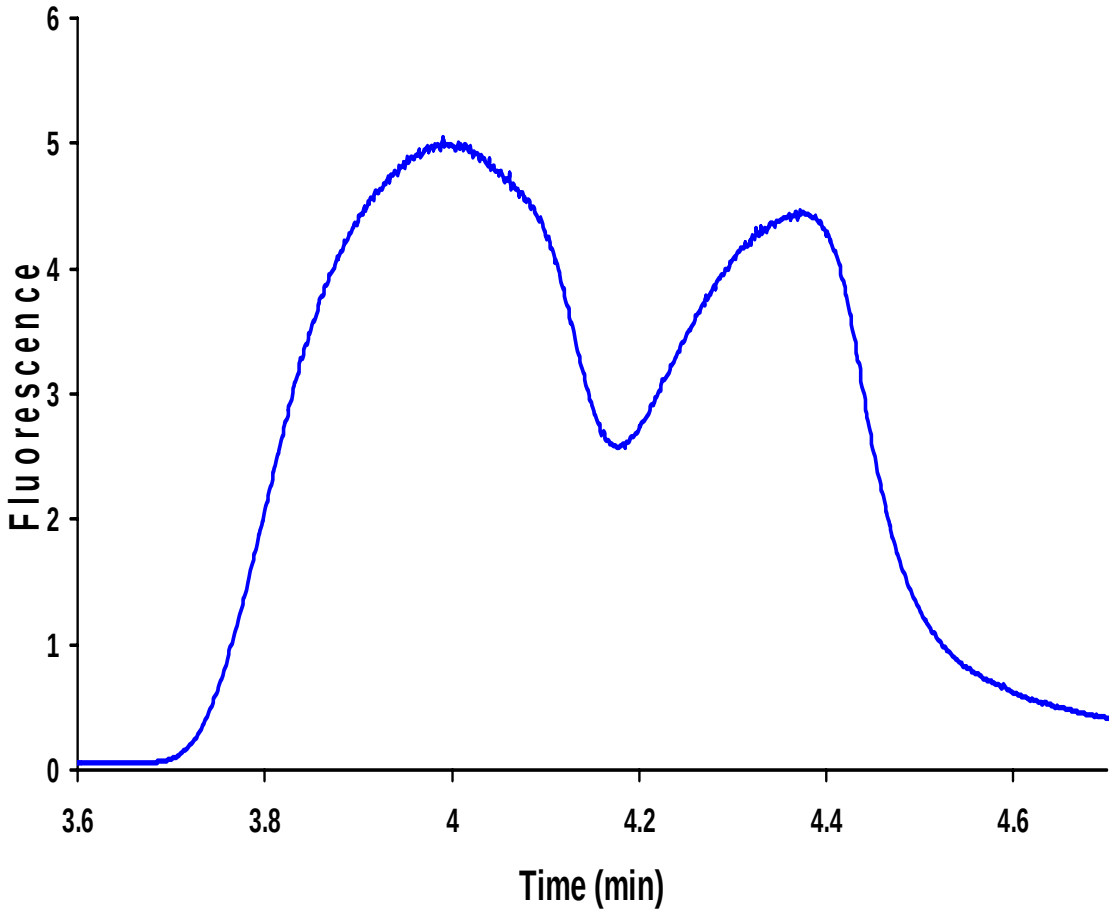

Figure A.4. A control electropherogram of ALP. The gap reservoir contained 50 mM DEA and 0.10 mM AttoPhos at pH 9.5. A 3 s zone of 0.18 nM ALP was injected at 17.5 kV.

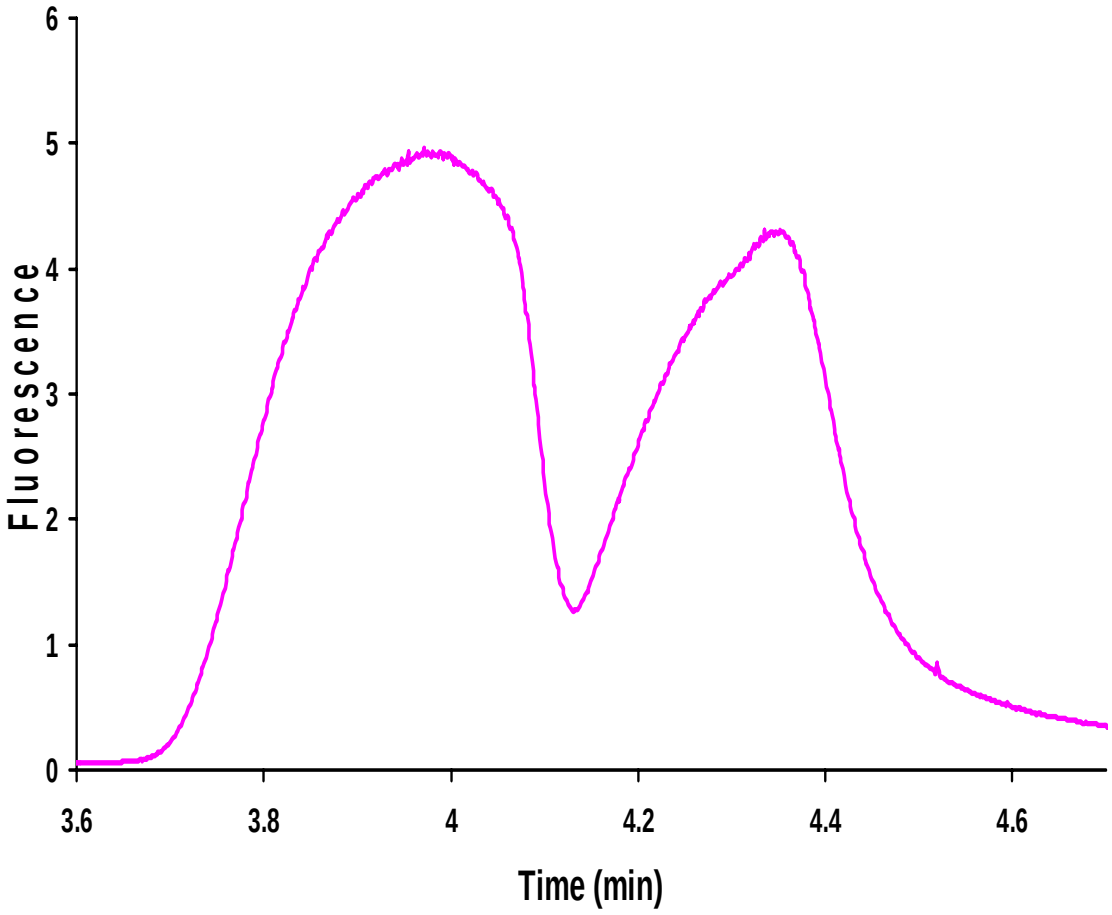

Figure A.5. A control electropherogram of ALP inhibition with theophylline. The gap reservoir contained 50 mM DEA and 0.10 mM AttoPhos at pH 9.5. A 4 s zone of 100 μM theophylline was injected at 17.5 kV. A 3 s zone of 0.18 nM ALP injected at 17.5 kV followed.

ALP from the reservoir interacted with the substrate at zero potential, and a plug of product was formed. When the potential was reapplied, this small product plug moved down the capillary, along with the inhibition peak, to the detector. The potential could also be left off for a set time period (1 to 3 min) to allow product to buildup inside the capillary. An electropherogram with theophylline inhibition can be seen in Figure A.6. When the potential was first applied, a steady stream of ALP reacted with the substrate to form product and caused a rise in the background fluorescence (point A in Figure A.6). When the potential was turned off for the theophylline injection, ALP reacted with substrate at zero potential, and a small plug of product was formed (point B). The potential was then reapplied for 25 s in order for the theophylline zone to interact with the ALP. The potential was then turned off for a set time period to allow zero field incubation. ALP from the reservoir once again bled in and formed another small product plug (point C). The potential was then reapplied and the components were swept to the detector. The inhibition peak can be seen as a negative dip in the high fluorescence background (point D). The same assay was also performed using 75 μM sodium vanadate and can be seen in Figure A.7.

One reason on-column reagent addition is desired for CE is to improve the ability to separate mixtures. For enzyme-inhibition assays, mixtures of compounds could be separated and screened for inhibitory effects using this technique. Therefore, an assay was performed using a mixture of 75 μM sodium vanadate and 100 μM theophylline. However, no separation occurred. If the electropherograms for each of the compounds are compared, it can be seen that each inhibitor dip is in the same place on the electropherogram. The mobilities of the inhibitors must be the same. In order to change the mobility of one of the inhibitors, the pH conditions of the assay were changed. UV data was collected on a commercial CE system. Mesityl oxide was used a neutral marker. The pH was originally 9.5, which is the reported optimum for alkaline phosphatase activity. The mesityl oxide peak can be seen at around 300 s. The theophylline and sodium vanadate peaks come off after the neutral marker indicating that both are negatively charged. The assays were repeated using buffer at pHs ranging from

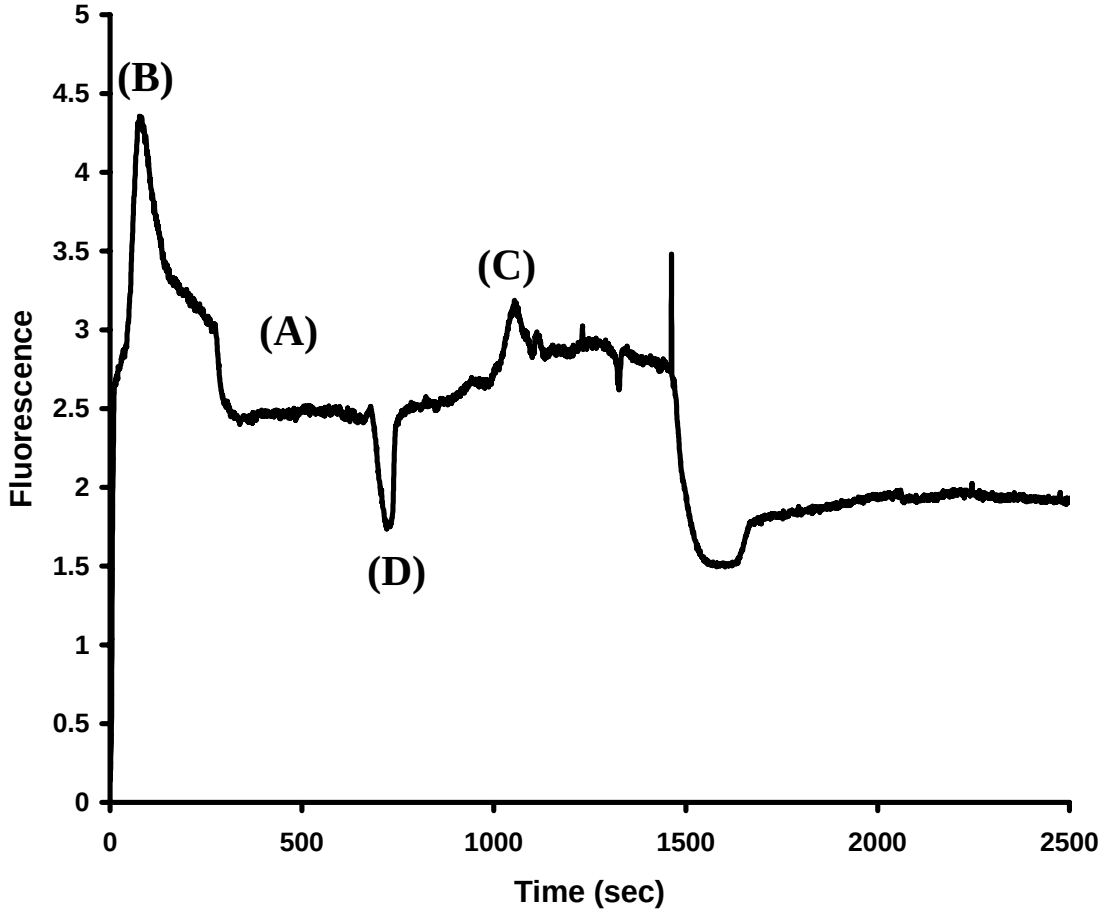

Figure A.6. Electropherogram of ALP inhibition by theophylline using on-column reagent addition. Gap reservoir contained 0.18 nM ALP. A 4 s zone of theophylline was injected at 17.5 kV. (A) High level of background fluorescence (B) Small product plug (C) Small product plug (D) Inhibition peak

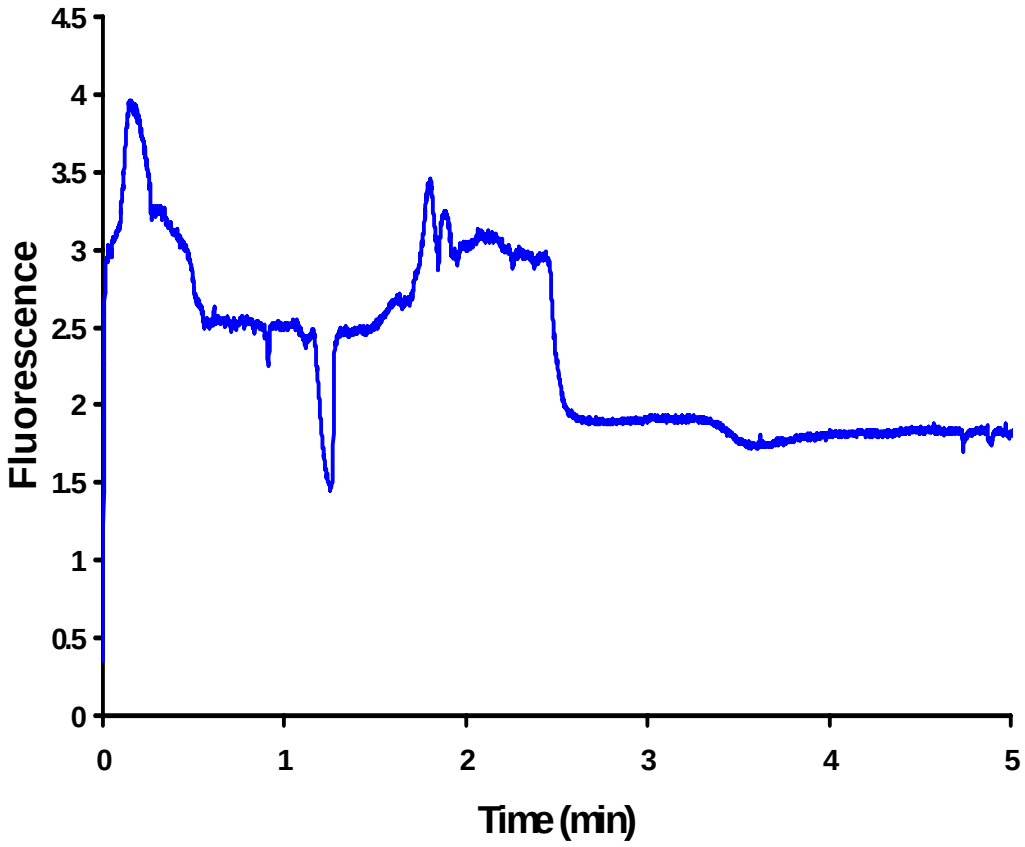

Figure A.7. Electropherogram of ALP inhibition with sodium vanadate. Gap reservoir contained 0.18 nM ALP. A 4 s zone of sodium vanadate was injected at 17.5 kV.

8.0 to 9.0. However, separation never occurred at any pH. Buffers with a pH less than 8.0 were not considered in order to maintain a high level of enzyme activity (optimum pH 9.5).

Conclusions and Future Work

Conclusions

On-column reagent addition was used successfully in conjunction with enzyme-inhibition assays. Theophylline, a reversible, noncompetitive ALP inhibitor, and sodium vanadate, a reversible, competitive ALP inhibitor, were both shown to cause inhibition of ALP. It would be desirable to use CE with on-column reagent addition to separate mixtures of compounds and screen for inhibitory effects in order to design drug therapies. A mixture of theophylline and sodium vanadate was used for demonstration, however separation of the two was never observed. To change the mobility of one of the compounds, various pH buffers were used with the assay and UV data collected. Separation was never achieved at any pH from 8.0 to 9.0.

Future Work

Separation of inhibitors is desired for this study, therefore optimization of separation conditions should continue. Thermostatting of the current system should also be performed in order to avoid temperature fluctuations in regions of the capillary which affect the reaction rate and can show up as negative peaks in the electropherogram. A temperature controlled assay could also be studied. The sample could be pre-mixed at a low temperature in order to slow down the kinetics and prevent the reaction from occurring until it is injected onto the column, where the solution temperature increases and the reaction occurs. This is one way to prevent the reaction from happening until in the column, which is essentially what on-column reagent addition accomplishes. A microchip format is one other possible way to study enzyme assays using this idea. Different channels of the chip would contain the separate components (enzyme, inhibitor, and substrate) until small plugs of each were mixed in a separate channel.

Vita

Courtney Ann Neel was born in July 1979 in Wise, VA. She was raised in Clintwood, VA where she attended both Clintwood Elementary School and Clintwood High School. She was the salutatorian of her graduating class in 1997. She then attended the University of Virginia's College at Wise for four years. She graduated cum laude in 2001 with a B.A. in chemistry. She received a M.S. in analytical chemistry from the University of Tennessee in 2005.