Development of a phosphotriesterase enzyme assay for determination of organophosphate pesticides

Yan Wang

University of Nevada, Las Vegas

Follow this and additional works at: https://digitalscholarship.unlv.edu/rtds

Repository Citation
https://digitalscholarship.unlv.edu/rtds/860

This Thesis is brought to you for free and open access by Digital Scholarship@UNLV. It has been accepted for inclusion in UNLV Retrospective Theses & Dissertations by an authorized administrator of Digital Scholarship@UNLV. For more information, please contact digitalscholarship@unlv.edu.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
NOTE TO USERS

The original manuscript received by UMI contains indistinct, slanted and or light print. All efforts were made to acquire the highest quality manuscript from the author or school. Microfilmed as received.

This reproduction is the best copy available

UMI
DEVELOPMENT OF A PHOSPHOTRIESTERASE ENZYME ASSAY FOR DETERMINATION OF ORGANOPHOSPHATE PESTICIDES

by

Yan Wang

Bachelor of Science
East China University of Science and Technology
1983

A thesis submitted in partial fulfillment of the requirement for the degree of

Master of Science

in

Chemistry

Department of Chemistry
University of Nevada, Las Vegas
May 1998
The Thesis prepared by

Yan Wang

Entitled

Development of a Phosphotriesterase Enzyme Assay for Determination of Organophosphate Pesticides

is approved in partial fulfillment of the requirements for the degree of

Master of Science

Examination Committee Chair

Dean of the Graduate College

Graduate College Faculty Representative
ABSTRACT

Development of A Phosphotriesterase Enzyme Assay for Determination of Organophosphate Pesticides

by

Yan Wang

Dr. Kim R. Rogers, Examination Committee Chair
Research Chemist
U. S. EPA, National Exposure Research Laboratory-Las Vegas

Phosphotriesterase catalyzes the hydrolysis of a wide range of organophosphate insecticides such as paraoxon and parathion and nerve toxins such as soman and sarin. This project resulted in the development of novel phosphotriesterase enzyme assays to measure paraoxon and other representatives of the organophosphate pesticides. The assay is based on a substrate-dependent change in pH at the local vicinity of the enzyme. This enzyme, which was labeled with fluorescein isothiocyanate (FITC), was immobilized on polymethylmethacrylate beads and the pH change is monitored through the pH-sensitive quantum yield of FITC. Analytes were measured using a KinExA fluorescence analyzer. This assay can detect paraoxon concentration down to 11 \( \mu \text{M} \) with a dynamic range of 11 \( \mu \text{M} \) to 600 \( \mu \text{M} \). In addition to the paraoxon, this assay was also used to measure 9 other insecticides and to determine the concentrations of coumaphos in the biofilter treated cattle dip waste samples.
TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................ iii
LIST OF FIGURES .......................................................................................................................... vii
LIST OF TABLES ................................................................................................................................ viii
ACKNOWLEDGMENTS .................................................................................................................. x

CHAPTER 1 INTRODUCTION ........................................................................................................... 1
  1.1 The Properties and Toxicities of Organophosphate Compounds ........................................... 2
  1.2 Organophosphate Compound Biochemical Mode of Action ................................................... 6
  1.3 The Methods of Determination of Organophosphate Compounds ....................................... 8
  1.4 Phosphotriesterase .................................................................................................................. 11
  1.5 Phosphotriesterase Enzyme Assay ....................................................................................... 13
    1.5.1 Optical Assay .............................................................................................................. 14
    1.5.2 The KinExA Fluorescence Assay .................................................................................... 14

CHAPTER 2 THEORY AND INSTRUMENTATION ........................................................................... 17
  2.1 Protein Structure and Reactivity ............................................................................................. 17
  2.2 FITC Properties and Its Reactivity ........................................................................................ 19
  2.3 Enzyme Kinetics .................................................................................................................... 22
  2.4 Enzyme Immobilization ........................................................................................................ 24
  2.5 The Alkalinity and Buffer Capacity of the Environmental Water Matrix ............................ 26
  2.6 KinExA Instrumentation ........................................................................................................ 28

CHAPTER 3 EXPERIMENTAL METHODS ..................................................................................... 31
  3.1 Materials .................................................................................................................................. 31
  3.2 PTE Enzyme Activity Assay .................................................................................................... 32
  3.3 FITC Conjugation with PTE and the Conjugate Properties ................................................... 33
    3.3.1 FITC Labeling PTE ........................................................................................................ 34
    3.3.2 Determination of Relative FITC Concentration of Conjugate and Molar Ratio of FITC to
         PTE Enzyme ...................................................................................................................... 34
    3.3.3 Determination of K_m Values of PTE and FITC-labeled PTE ........................................ 36
  3.4 Immobilized PTE .................................................................................................................... 37
    3.4.1 Immobilization FITC-labeled PTE to PMMA beads .................................................... 37
    3.4.2 pH Profile ...................................................................................................................... 38

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4.3</td>
<td>Thermostability of the Immobilized PTE</td>
<td>39</td>
</tr>
<tr>
<td>3.5</td>
<td>KinExA Assay</td>
<td>40</td>
</tr>
<tr>
<td>3.5.1</td>
<td>KinExA Assay Procedure</td>
<td>40</td>
</tr>
<tr>
<td>3.5.2</td>
<td>Data Analysis</td>
<td>41</td>
</tr>
<tr>
<td>3.6</td>
<td>The Effect of Methanol on KinExA Assay</td>
<td>43</td>
</tr>
<tr>
<td>3.7</td>
<td>Absorbance Assay to Determination the Paraoxon Concentration</td>
<td>43</td>
</tr>
<tr>
<td>3.8</td>
<td>Determination of Coumaphos Concentrations in Biofilter Treated Cattle</td>
<td>44</td>
</tr>
<tr>
<td>3.8.1</td>
<td>KinExA Assay for Determination of Coumaphos Concentrations in Biofilter Treated Cattle Dip Waste Samples</td>
<td>44</td>
</tr>
<tr>
<td>3.8.2</td>
<td>HPLC Method for Determination of Coumaphos Concentrations in Biofilter Treated Cattle Waste Samples</td>
<td>45</td>
</tr>
<tr>
<td>CHAPTER 4</td>
<td>RESULTS AND DISCUSSION</td>
<td>47</td>
</tr>
<tr>
<td>4.1</td>
<td>FITC-Labeled PTE Physical Property and Characterization</td>
<td>47</td>
</tr>
<tr>
<td>4.1.1</td>
<td>The Physical Property of FITC-Labeled PTE Conjugate</td>
<td>47</td>
</tr>
<tr>
<td>4.1.2</td>
<td>F/P Molar Ratio</td>
<td>47</td>
</tr>
<tr>
<td>4.1.3</td>
<td>The pH Profiles of PTE, FITC-Labeled PTE and Immobilized FITC-Labeled PTE</td>
<td>50</td>
</tr>
<tr>
<td>4.2</td>
<td>KinExA Assay Results</td>
<td>52</td>
</tr>
<tr>
<td>4.2.1</td>
<td>The Effect of Initial Buffer pH</td>
<td>53</td>
</tr>
<tr>
<td>4.2.2</td>
<td>The Effect of Buffer Concentration</td>
<td>55</td>
</tr>
<tr>
<td>4.2.3</td>
<td>The Effect of Methanol on KinExA Assay</td>
<td>56</td>
</tr>
<tr>
<td>4.2.4</td>
<td>The Effect of F/P Molar Ratio on KinExA assay</td>
<td>58</td>
</tr>
<tr>
<td>4.2.5</td>
<td>KinExA Assay Accuracy</td>
<td>59</td>
</tr>
<tr>
<td>4.2.6</td>
<td>KinExA Assay for Organophosphate Compounds</td>
<td>60</td>
</tr>
<tr>
<td>4.3</td>
<td>Absorbance Assay and KinExA Assay</td>
<td>63</td>
</tr>
<tr>
<td>4.4</td>
<td>The Effect of Environmental Water Matrix on KinExA Assay</td>
<td>65</td>
</tr>
<tr>
<td>4.5</td>
<td>Stability of the Immobilized Enzyme</td>
<td>67</td>
</tr>
<tr>
<td>4.6</td>
<td>KinExA Assay for Determination of Coumaphos Concentrations in Biofilter Treated Cattle Dip Waste Samples</td>
<td>68</td>
</tr>
<tr>
<td>CHAPTER 5</td>
<td>CONCLUSIONS AND FUTURE WORK</td>
<td>71</td>
</tr>
<tr>
<td>APPENDIX I</td>
<td>Micro BCA Protein Assay</td>
<td>73</td>
</tr>
<tr>
<td>APPENDIX II</td>
<td>The Absorbance Scan Spectra of FITC and FITC-Labeled-PTE</td>
<td>75</td>
</tr>
<tr>
<td>APPENDIX III</td>
<td>Lineweaver-Burk Reciprocal Plot</td>
<td>76</td>
</tr>
<tr>
<td>APPENDIX IV</td>
<td>p-Nitrophenol Extinction Coefficient at Different pH</td>
<td>77</td>
</tr>
<tr>
<td>APPENDIX V</td>
<td>FITC-Labeled PTE Thermostability</td>
<td>79</td>
</tr>
</tbody>
</table>

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
LIST OF FIGURES

Figure 1.1 Model for the enzyme hydrolysis of phosphotriesters ........................................... 13
Figure 2.1 FITC structure .................................................................................................. 20
Figure 2.2 FITC react with amine-containing protein to produce
a thiourea linkage ........................................................................................................ 21
Figure 2.3 The molecule of FITC ionizes with pH ........................................................... 22
Figure 2.4 Fluorescence intensity changes with the pH .................................................. 22
Figure 2.5 KinExA flow cell diagram ............................................................................... 29
Figure 2.6 KinExA flow system ........................................................................................ 30
Figure 3.1 Schematic for the PTE based assay for paraoxon and organophosphate compounds ......................................................................................................................... 41
Figure 3.2 Representative tracing of FITC-labeled enzyme or heat-denatured
FITC-labeled enzyme in the presence or absence of 100 µM paraoxon... 42
Figure 4.1 FITC standard absorption curve at 10 mM PBS buffer,
pH 7.4, λ=492 nm ........................................................................................................ 48
Figure 4.2 Protein concentration standard curve at λ=562 nm................................. 49
Figure 4.3 The effect of pH on the PTE enzyme activity ............................................. 51
Figure 4.4 The effect of pH on the KinExA assay .......................................................... 54
Figure 4.5 The effect of buffer concentration on the KinExA assay.......................... 55
Figure 4.6 The effect of methanol on the KinExA assay for measurement
of paraoxon concentration......................................................................................... 57
Figure 4.7 The effect of F/P molar ratio on the KinExA assay................................. 59
Figure 4.8a KinExA assay standard curves for organophosphate pesticides
in 100 µM HEPES-MES, 100 mM NaCl buffer, pH 8.0................................. 60

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 4.8b KinExA assay standard curves for organophosphate pesticides
in 100 μM HEPES-MES, 100 mM NaCl buffer, pH 8.0......................... 61
Figure 4.9 KinExA assay standard curves for organophosphate pesticides
in 1 mM HEPES-MES, 100 mM NaCl buffer, pH 8.0............................. 62
Figure 4.10 Comparing of the KinExA assay and the absorbance assay .......... 64
Figure 4.11 The standard curves of paraoxon in different buffer systems
and environmental matrices................................................................. 66
Figure 4.12 The stability of FITC-labeled PTE immobilized to PMMA
beads as measured using the KinExA assay........................................ 68
Figure 4.13 The results of KinExA assay, HPLC method and reported
values for biofilter treated cattle dip vat samples................................. 70
Figure 4.14 Comparison of coumaphos concentrations in biofilter-treated
cattle dip waste samples determined by HPLC and KinExA assay....... 70
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>The structures and LD$_{50}$ of organophosphate compounds</td>
<td>4</td>
</tr>
<tr>
<td>Table 2.1</td>
<td>Kinetic constants for the hydrolysis of organophosphorus insecticides</td>
<td>24</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>F/P molar ratio after FITC coupled with PTE at different pH</td>
<td>49</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>The apparent kinetic parameters and the specific activities of PTE and its conjugate, as well F/P molar ratio</td>
<td>50</td>
</tr>
<tr>
<td>Table 4.3</td>
<td>The Effect of Methanol on KinExA Assay</td>
<td>58</td>
</tr>
<tr>
<td>Table 4.4</td>
<td>The detection limit of KinExA assay for organophosphate pesticides</td>
<td>63</td>
</tr>
<tr>
<td>Table 4.5</td>
<td>The alkalinities and buffer capacities of the buffer solutions and environmental water matrices</td>
<td>65</td>
</tr>
<tr>
<td>Table 4.6</td>
<td>Immobilized FITC-labeled PMMA Beads Thermostability</td>
<td>68</td>
</tr>
<tr>
<td>Table 4.7</td>
<td>The results of KinExA assay and HPLC method for the biofilter-treated cattle dip waste samples</td>
<td>69</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

First of all I would like to thank my advisor Dr. Kim R. Rogers for instructing and inspiring me throughout the course of this project. The research atmosphere which he provided was very stimulating. His constant guidance, encouragement and support were invaluable to my research and education.

Next I would like to thank Dr. Brian J. Johnson and Dr. Spencer Steinberg for their instruction, support and encouragement. Without their support this would not have been possible. I also wish to thank my other committee member Dr. David James for his valuable suggestions.

I would like to thank Dr. Ashok Mulchandani (Chemical and Environmental Engineering Department, University of California, Riverside) for providing the key material of phosphotriesterase enzyme. I would also like to thank Dr. Walter Mulbry (Soil Microbial System laboratory, United States Department of Agriculture) for providing the biofilter-treated coumaphos-containing cattle dip waste samples.

Finally, a thank you goes to my family and my friends. They gave me much help and encouragement. I would like to especially thank my husband and my son for their sacrificing so much for me.

This research was supported by the U. S. Environmental Protection Agency and UNLV Chemistry Department.
CHAPTER 1

INTRODUCTION

Organophosphate compounds are the largest class of insecticides and represent the major proportion of the agricultural pesticides utilized in today's agricultural industry [Benning et al., 1994]. Over 30 million kilograms of organophosphorus pesticides are used annually in the United States [Donarski, 1989], and it is estimated that more than 89 million acre-treatments of organophosphates are applied each year within the United States [Smith, 1987]. They are widely used to protect crops and plants from harm caused by insects and other organisms. For example, organophosphate compounds are routinely applied to crops such as cotton, rice, potato, corn, wheat and soybeans [McInnes, 1996; Smith, 1987], and to turf grass [Cisar and Synder, 1996]. These compounds are also applied to livestock to control the population of the ticks which transmit the disease known as cattle fever [Grice et al., 1996]. Approximately 400,000 liters of cattle dip wastes containing approximately 1500 mg/l of coumaphos are generated yearly along the Mexican border [Mulbry, 1996].

Although many of these organophosphate compounds exhibit relatively rapid decomposition and low accumulation in the biological food chain, they have a wide range of biological effects [Farran and Pablo, 1988]. The expanded use of pesticides increases the risks of environmental contamination of groundwater, food and fiber products, and
water resources [Lai et al., 1995]. Korns and co-workers [ACS, 1987] pointed out that that pesticide contamination of groundwater may occur as a consequence of the application of pesticides under field conditions for the control of active pests and the improper disposal of waste waters generated by farmers, commercial applicators, and industry. Reports in the literature have expressed concern over exposure of non-target organisms such as birds [McInnes et al., 1996] and fish [Hai et al., 1997], as well as the potential for human exposure from sources such as fresh fruits and vegetables [Smart, 1987] and processed foods [Saul et al., 1995]. Organophosphate is also the leading cause of pesticide poisoning cases in the United States [U.S. EPA, 1976]. As a consequence of these issues, there is strong interest in the development of rapid, low cost methods to monitor and screen field contamination by organophosphate compounds.

1.1 The Properties and Toxicities of Organophosphate Compounds

Pesticides are chemical substances used for controlling, preventing, destroying, repelling, or mitigating any pest. Insecticides, fungicides, and herbicides are the three major classes of pesticides. They are designed to combat the attacks of various pests on agricultural and horticultural crops.

Many organophosphate compounds now used as the insecticides were developed during World War II, in Germany, as a by product in the research and study of nerve gas related compounds sarin, soman and tabun [Cremlyn, 1978]. The first organophosphate insecticide compound used in agriculture was TEPP (tetraethyl pyrophosphate) in 1946. Further research and development resulted in the second phosphate insecticide (Parathion) to be introduced into agriculture. Since then, the organophosphate compounds were
rapidly developed as insecticides and the commercial use of organophosphates expanded markedly during the 1950's. Use of these compounds was accelerated after the organochlorine insecticides were banned or restricted. Organophosphate insecticides are generally much more toxic to vertebrates than are the organochlorine insecticides, however they are more unstable in the environment, or less-persistent. In contrast, organochlorine insecticides, such as DDT are persistent in the environment, and bioconcentrate in food chains, and can severely affect whole populations or species of wildlife. In principally, organophosphate compounds should not have this problem.

Normally "organophosphate" is used as a generic term to include all of the insecticides containing phosphorus [Ware, 1983]. The following shows the general chemical structure of organophosphate pesticides [Buchel, 1983]:

\[
\begin{array}{c}
R \\
\text{O(S)} \\
\text{P} \\
R' \\
Z
\end{array}
\]

where R, R' may be alkoxy, alkyl or amino residues. The Z represents the anion of an organic or inorganic acid such as fluorine, cyanate, thiocyanate, or other acidic residues (enol residues, mercapto, etc.). Most of these compounds are soluble to some extent in organic solvents, but they are usually very low soluble for water.

The mechanism of biocide action of these compounds is due to their inhibition of acetylcholinesterase (neurotoxicity) in insects. The inhibition of acetylcholinesterase by organophosphorus compounds is well known [McInnes et al., 1996; Cremisini et al., 1995; Roda et al., 1994; Leon-Gonzalez and Townshend, 1990; Smart, 1987; Smith 1987; Cremlyn, 1978;]. This is also the primary cause of toxicity in non-target organisms,
including humans. Because of their chemical structure and mode of action, they are related to the “nerve gases.” The acute toxicity for these compounds can be described by their LD$_{50}$ values. That is the single dose required to kill 50% of the animals under test and is expressed as mg/kg of the body weight of the animal. The smaller the LD$_{50}$ value, the more toxic the chemical so that the toxicities of chemicals can be graded by the LD$_{50}$ values as follows [Smith, 1987]:

<table>
<thead>
<tr>
<th>LD$_{50}$ (mg/kg)</th>
<th>I. Supertoxic</th>
<th>II. Extremely toxic</th>
<th>III. Very toxic</th>
<th>IV. Moderately toxic</th>
<th>V. Slightly toxic</th>
<th>VI. Practically nontoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 5</td>
<td>5-50</td>
<td>50-500</td>
<td>500-5,000</td>
<td>5,000-15,000</td>
<td>&gt;15,000</td>
</tr>
</tbody>
</table>

Table 1.1 shows some organophosphate compound structures and their LD$_{50}$ which is based upon OSHA’s (Occupational Safety and Health Act) assessment.

Table 1.1 The structures and LD$_{50}$ of organophosphate compounds

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>LD$_{50}$ (oral rat or mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>paraoxon</td>
<td>C$_2$H$_5$O$_3$POO$_3$PhNO$_2$</td>
<td>2 mg/kg</td>
</tr>
<tr>
<td>ethyl parathion</td>
<td>C$_2$H$_5$OSPOO$_3$PhNO$_2$</td>
<td>4 mg/kg</td>
</tr>
<tr>
<td>coumaphos</td>
<td>C$_2$H$_5$SPOO$_3$PhC$_6$H$_5$Cl</td>
<td>16 mg/kg</td>
</tr>
<tr>
<td>Pesticide</td>
<td>Chemical Structure</td>
<td>Concentration (mg/kg)</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>Fensulfothion</td>
<td><img src="" alt="Fensulfothion Structure" /></td>
<td>5 mg/kg</td>
</tr>
<tr>
<td>Diazinon</td>
<td><img src="" alt="Diazinon Structure" /></td>
<td>66 mg/kg</td>
</tr>
<tr>
<td>Dursban</td>
<td><img src="" alt="Dursban Structure" /></td>
<td>82 mg/kg</td>
</tr>
<tr>
<td>Methyl parathion</td>
<td><img src="" alt="Methyl Parathion Structure" /></td>
<td>6 mg/kg</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td><img src="" alt="Dichlorvos Structure" /></td>
<td>56 mg/kg</td>
</tr>
<tr>
<td>Mevinphos</td>
<td><img src="" alt="Mevinphos Structure" /></td>
<td>3 mg/kg</td>
</tr>
<tr>
<td>Crotoxyphos</td>
<td><img src="" alt="Crotoxyphos Structure" /></td>
<td>53 mg/kg</td>
</tr>
<tr>
<td>Soman</td>
<td><img src="" alt="Soman Structure" /></td>
<td>~ 0.01 mg/kg</td>
</tr>
<tr>
<td>Sarin</td>
<td><img src="" alt="Sarin Structure" /></td>
<td>~ 0.01 mg/kg</td>
</tr>
</tbody>
</table>

* Dumas et al., 1990.
1.2 Organophosphate Compound Biochemical Mode of Action

Acetylcholinesterase (AChE, Enzyme Commission Number 3.1.1.7) is a hydrolase enzyme and exists at high concentration in nervous tissue. It is an essential component of the nervous system of both insects and mammals. The basic mechanism of toxic action of all of the organophosphate compounds is considered to be inhibition of this enzyme [Cremlyn, 1978]. Acetylcholinesterase catalyses the hydrolysis of acetylcholine to choline and acetic acid:

\[
\text{acetylcholinesterase} \quad (\text{CH}_3\text{N}^+\text{CH}_2\text{CH}_2\text{OCOCH}_3) \quad \overset{\text{H}_2\text{O}}{\underset{\text{acetylcholine}}{\rightleftharpoons}} \quad (\text{CH}_3\text{N}^+\text{CH}_2\text{CH}_2\text{OH} + \text{CH}_3\text{CO}_2\text{H} \quad \text{choline})
\]

Acetylcholine (ACh) is neurotransmitter secreted by cholinergic postganglionic neurons, which allows for transmission of nerve impulses across the synapse. The liberated acetylcholine must not persist in the synapse too long, otherwise, there would be a continuous chain of nerve impulse that cause loss of muscular coordination, convulsions and ultimately death.

Reaction 1. Acetylcholinesterase (ECH\textsubscript{2}OH) and the organophosphate compound reaction:

\[
\text{organophosphate enzyme} \quad (\text{RO})_2\text{P} - \text{X} + \text{ECH}_2\text{OH} \quad \overset{(1.1)}{\rightleftharpoons} \quad (\text{RO})_2\text{P} \cdot \text{ECH}_2\text{OH} \quad \overset{(1.2)}{\rightleftharpoons} \quad (\text{RO})_2\text{P} \cdot \text{OCH}_2\text{E} \quad \overset{(1.3)}{\longrightarrow} \quad (\text{RO})_2\text{P} \text{OH} + \text{ECH}_2\text{OH}
\]

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Paraoxon and other organophosphate insecticides mimic the natural substrate acetylcholine by binding to the enzyme’s active site. Initially a complex is formed between the enzyme and the phosphate (Rxn 1.1) which subsequently gives the phosphorylated enzyme (Rxn 1.2), the latter is very slowly hydrolysed to regenerate the free active enzyme (Rxn 1.3).

**Reaction 2. Acetylcholinesterase normal reaction:**

\[
\begin{align*}
(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OCCH}_3 + \text{ECH}_2\text{OH} & \xrightarrow{(2.1)} (\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OCCH}_3 \cdot \text{ECH}_2\text{OH} \\
\text{H}_2\text{O} & \xrightarrow{(2.3)} \text{CH}_3\text{COOH} \\
\text{CH}_3\text{COOCH}_2\text{E} + \text{HOCH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3 & \xrightarrow{(2.2)}
\end{align*}
\]

In the case of the normal physiological reaction between acetylcholinesterase and acetylcholine (Rxn 2.1), the acetylated enzyme is very rapidly hydrolysed by water (Rxn 2.3), so that the active enzyme is quickly regenerated. The inactive phosphorylated enzyme is only very slowly hydrolysed to the active enzyme because the P-O bond is stronger than C-O bond [Cremlyn, 1978]. Thus, the organophosphate blocks efficient hydrolysis of acetylcholine to choline. Consequently, there is an accumulation of acetylcholine at the synapse which results in continuous nerve firing and eventual failure of nerve impulse propagation. Respiratory paralysis is generally the immediate cause of death after poisoning with these compounds.
1.3 The Methods of Determination of Organophosphate Compounds

The increasing use of pesticides has generated interest for development of rapid and economical new methods for environmental monitoring. Gas chromatography (GC), high-performance liquid chromatography (HPLC) and GC-coupled with mass spectrometry and capillary electrophoresis are the best developed available techniques [Barcelo, 1991; Sherma, 1989, Shi and Stein 1996]. Although these methods can measure a wide variety of organophosphate compounds in different environmental matrices and allow discrimination between different compounds that belong to the same class, they are expensive, time consuming, and require highly trained technicians. Also, because these early laboratory-based methods often require turnaround times of several weeks, they are not amenable to remediation and bioremediation process monitoring where rapid analysis times are essential.

There is an increasing requirement to develop fast and cost-effective methods to monitor and screen field contamination by compounds such as organophosphate pesticides. Field monitoring assays are capable of continuous and *in situ* operation under defined conditions for a given period of time. Field screening assays are designed for operator-assisted discrete sampling, the test should define a specific analyte or class of analytes above or below a given level of concern. Many efforts have been made in development of relatively inexpensive enzymatic and immunological methods. Biosensors and immunoassays can offer advantages for reducing time and cost of analysis.

A biosensor is an analytical device composed of a biological recognition element in intimate contact with a physical transducer [Rogers and Williams, 1995]. Recognition
elements can be enzymes, antibodies, or microorganisms. These systems tend to be very selective for the target analytes. The physical transducer could be an electrochemical, optical, or thermal device. Enzyme-based and antibody-based biosensors have been reported for detection of compounds ranging from polyaromatic hydrocarbons to pesticides [Marty et al., 1995]. The enzyme-based biosensors usually detect their substrates or inhibitors, which could be several specific compounds or an entire class of compounds. The principle for enzyme-based biosensors relies on the catalytic conversion of a non-detectable substrate into an optically or electrochemically detectable product. The analytes also can be extended by the coupling of a second enzyme reaction converting of a non-detectable primary product into a detectable second product.

Biosensors for the detection of organophosphate compounds are mostly based on AChE. When AChE encounters an organophosphate compound, the compound acts as a competitive inhibitor as previously described. The complex then forms an inactive phosphorylated enzyme. Insecticides are measured by comparing the initial velocity of the reaction catalysed by this enzyme before and after an incubation step with insecticides:

\[
\text{Acetylcholine} + \text{H}_2\text{O} \xrightarrow{\text{AChE}} \text{Acetic acid} + \text{Choline}
\]

Several biosensors using different transducers have been developed. pH electrodes that have been modified with an AChE layer, or combination of a pH electrode with an enzyme membrane have been used to detect pesticides in drinking water [Stein and Schwedt, 1993]. Fiber-optic methods that use immobilized fluorescein isothiocyanate labeled AChE [Rogers et al., 1991] have also been reported. Light addressable potentiometric methods, which use a biotinilated nitrocellulose membrane to immobilize
AChE through a streptavidin bridge [Guilbault and Mascini (eds.), 1993], have also been developed for detection of anticholinesterases. In addition, an amperometric biosensor for the determination of paraoxon has also been developed [Cremisini et al, 1995]. For this method, the product choline was coupled with choline oxidase reaction (Reaction 3) [Roda et al., 1994] and the system was linked with an H$_2$O$_2$ probe.

\[
\text{Choline} \xrightarrow{\text{choline oxidase}} \text{betaine} + \text{H}_2\text{O}_2
\]

Because enzymes can be immobilized on various water-insoluble carriers and packed into columns, they present great opportunities for pesticide determination. AChE has been immobilized on a maleic anhydride-butanediol divinyl ether copolymer, on controlled porosity glass [Leon-Gonzalez and Townshend, 1990], and on methacrylate beads [Roda et al., 1994] for the detection of AChE inhibitors.

Immunoassays are also recognized as promising methods for screening environmental contaminants. The enzyme-linked immunosorbent assay (ELISA) is the most commonly used assay in pesticides [Meulenberg et al., 1995]. In this assay, the antibody for a target analyte is coated onto a solid phase, and enzyme-labeled and sample antigen compete for a restricted number of binding sites. After the reaction, the free phase is removed, and the amount of bound enzyme-labeled antigen, which is inversely proportional to the analyte, is determined spectrophotometrically after adding appropriate substrate and chromogen such as p-nitrophenyl phosphate for alkaline phosphatase label. The assay has been reported for the determination of paraoxon, parathion and other organophosphorus pesticides. [Meulenberg, 1995].

All of these methods are either based on enzyme activity inhibition [Cremisine et
al, 1995; Marty et al., 1995; Roda et al., 1994; Rogers et al., 1991; Shi and Stein 1996; Takruni et al., 1993;] or selective immunoassay [Meulenberg, 1995; Marty et al., 1995]. Assays based on the inhibition of cholinesterase are quite sensitive and amenable to field sample screening applications. These assays, however, are somewhat non-specific in that they also are sensitive to carbamate pesticides and other anticholinesterase compounds. In addition, many pesticides must be converted to their oxidative metabolites to show maximum sensitivity. After exposure to the organophosphate compounds, the enzyme is typically irreversibly inhibited. As a result, the mechanism of these assays is not well suited for development of continuous or in situ monitoring applications. Immunoassays have also been developed to detect a number of organophosphate compounds. ELISAs for organophosphates are quite sensitive to specific compounds such as parathion and fenitrothion; however, like most immunoassays, they require multiple incubations of up to several hours each and generate contaminated plates, tubes, etc. In this respect enzyme assays, which could measure organophosphates as substrates, would be well suited for process control applications.

1.4 Phosphotriesterase

Phosphotriesterases (PTE, EC 3.8.1) [Dave, 1993] are enzymes that catalyze the hydrolysis of a wide spectrum of organophosphate pesticides such as paraoxon and parathion in addition to nerve toxins such as soman and sarin (structures in table 1.1) [Benning et al., 1994; Donarski et al., 1989; Dumas et al., 1989; Caldwell et al., 1991a, b; Lai et al. 1995; Yang et al., 1995]. Reaction 4 shows the phosphotriesterase catalyzed hydrolysis of paraoxon. The enzyme will not hydrolyze phosphomonoesters or -diesters
Reaction 4. Phosphotriesterase hydrolyzes the paraoxon to diethylphosphate and $p$-nitrophenol.

\[
\text{CH}_3\text{CH}_2\text{O}-\text{P}-\text{O}\text{C}_6\text{H}_4\text{NO}_2 + \text{H}_2\text{O} \xrightarrow{\text{PTE}} \text{CH}_3\text{CH}_2\text{O}-\text{P}-\text{OH} + \text{HO}\text{-C}_6\text{H}_4\text{NO}_2
\]

diethyl $p$-nitrophenylphosphate  

diethylphosphate  

$P$-nitrophenol

[Donarski et al., 1989]. Although the enzyme has demonstrated the ability to hydrolyze a variety of phosphotriesters, a "natural" substrate for the enzyme has remained unidentified. This enzyme activity has been identified in bacteria, yeast, fungi, and mammals. The enzyme was first detected in the soil microbe *Pseudomonas diminuta*, where parathion hydrolysis was observed, later it was found to be associated with a protein encoded by a plasmid-born gene [Dumas et al., 1989]. Plasmids are discrete, autonomously replicating pieces of DNA that are distinct from the chromosomal DNA of the organism. This enzyme also has been detected in another soil microbe, *Flavobacterium sp.*, and a gene of identical sequence was found in a slightly smaller plasmid. The genes (opd, organophosphate degradation) encoding the phosphotriesterase have been expressed in *Escherichia coli* [ACS, 1987].

The first report that the enzyme was purified to homogeneity was in 1989. Since then, phosphotriesterase has been fully characterized. The enzyme is a monomeric, spherical protein having a molecular weight of 39,000. The isoelectric point is 8.3 [Dumas et al, 1989]. The mechanism of phosphotriesterase hydrolysis involves the base-catalyzed attack of water at the phosphorus center and a net stereochemical inversion (Figure 1.1). The active site of the enzyme is hydrophobically bound to the substrate and does not
accept negative charges associated with the substrate [Donarski et al., 1989]. Figure 1.1 shows a model for the enzyme hydrolysis phosphotriesters proposed by Dumas et al. [Dumas et al., 1989].

Figure 1.1 Model for the enzymatic hydrolysis of phosphotriesters. [Dumas et al., 1989].

The analysis of recently obtained crystalline packing of the apoenzyme showed that the quaternary structure of the enzyme is a dimeric rather than monomeric [Vanhook et al., 1996]. It contains 365 amino acid residues and needs a binuclear metal center for activity. In addition to the native form of the enzyme which binds zinc ions, replacement with Ni^{2+}, Cd^{2+}, Co^{2+} and Mn^{2+} are all catalytically active forms of the enzyme. Removal of the bound metal ions from PTE results in the loss of enzyme activity [Benning et al., 1995]. The hydrolysis rate of organophosphate by this enzyme is diffusion limited for those substrates with leaving groups having pK_a values of less than 7 [Caldwell et al., 1991a].

1.5 Phosphotriesterase Enzyme Assay

For screening, remediation or process control applications, a large number of samples must be processed rapidly. Thus a rapid method with adequate sensitivity is
required. Phosphotriesterase-based assays respond to organophosphate compounds as enzyme substrates rather than inhibitors or antigens. Because the enzymes of these phosphotriesterase-based assays are reversible and the assays require only the analyte of interest, these assays provide are potentially useful for process control applications.

1.5.1 Optical Assays

Laboratory-based phosphotriesterase optical assays measure the increase in absorbance resulting from the product. For example, paraoxon is catalytically hydrolyzed to diethylphosphate and p-nitrophenol. The hydrolysis product of p-nitrophenol is measured by monitoring its absorbance at 400 nm. In addition to paraoxon, this type of optical assay has been used to measure other insecticides, nerve agents and synthetic compounds [Dumas et al, 1989; 1990; Lai et al, 1995; Caldwell et al, 1991; Donarski et al, 1989]. The major limitation of the optical assay, when used to measure pesticide substrates, however, is that the $\lambda_{max}$'s and molar absorptivity coefficients are different. As a consequence, these colormetric assays may not be well suited for development in field screening and monitoring applications.

1.5.2 The KinExA Fluorescence Assay

Because phosphotriesterase has broad substrate specificity and high catalytic rate, the enzyme has generated significant interest in its ability to detoxify organophosphate pesticides and mammalian acetylcholinesterase nerve agents. It has a great potential to be developed as a tool for determination of organophosphates in the environment, particularly for process control applications.
The objective of this project is to develop a universal assay for all pesticide substrates using phosphotriesterase, by measuring released protons rather than the absorbance caused by the organic product. In the PTE catalyzed reaction, protons are released due to production of a relatively strong acid. To measure enzyme catalysis, the fluorescent pH indicator, fluorescein isothiocyanate (FITC) is covalently attached to the enzyme. The fluorescence yield of this indicator is pH-dependent. A pH decrease will result in fluorescence quenching. We have developed a phosphotriesterase enzyme assay to measure paraoxon, as a representative organophosphate pesticide, that is based on change in the fluorescence observed when using FITC-labeled PTE immobilized to polymethylmethacrylate (PMMA) beads. During this study, the labeled enzyme was characterized with respect to kinetics, also, the assay was characterized for different buffers and buffer capacity and initial pH values. Due to the low solubilities of organophosphates in the aqueous solution, methanol was added to the solution to enhance substrate solubility. As a result, methanol effect on the assay was also studied. This assay was also applied to the detection and measurement of several organophosphate compounds in 0.1 mM or 1.0 mM HEPES-MES, 100 mM NaCl, pH 8.0 buffer systems and various natural water samples.

At the US-Mexico border, there is a Tick Eradication Program for cattle imported from Mexico or likely exposure to ticks from Mexico. This program involved the dipping of cattle in a coumaphos solution. The cattle dipping operation produced highly concentrated coumaphos wastes. The Eradication Program currently employs 42 vats each containing about 15,000 liters of coumaphos at a level of about 2000 mg liters\(^{-1}\) [Mulbry
et al., 1998] and no other significant toxic components. Those waste from this operation is
an excellent candidate for disposal by biodegradation. Recently, a laboratory-scale
experiment and field trial were conducted using biofilters for the degradation of the
coumaphos in cattle dip wastes by Dr. Mulbry et al.. The KinExA assay was used to
determine the concentrations of these different batch samples from the biofilter
experiment. The results were also compared to a conventional HPLC method for the
determination of coumaphos.
CHAPTER 2

THEORY AND INSTRUMENTATION

Enzymes are catalysts that enhance the rates of biochemical reactions. All enzymes are proteins. Protein molecules can be modified to contain a chemical or biological tracer to allow detectability. This type of protein complexes can be designed to retain their ability to bind its natural target, while the tracer portion can provide the means to find and measure the location, quantity and in some cases the function of the target protein.

In this project, enzyme PTE was labeled by FITC and then immobilized on the PMMA beads. The organophosphate assay is based on PTE catalyzing the hydrolysis reaction which releases protons and pH changes, previously mentioned in Chapter 1. As a result of the pH change, the fluorescence intensity of the linked fluorescein is changed and KinExA fluorescence analyzer monitors the fluorescence signal. The reaction of the functional groups of the enzyme PTE and the tracer FITC provides the foundation of the analytical method.

2.1 Protein Structure and Reactivity

Each protein contains one or more specific regions or sites on its surface that directly participates in its biological activity. Proteins are mostly composed by 20 common amino acids. They are linked together by peptide bonds, CO-NH, as follows:
The structure of the R group (side chain) may be hydrophilic or hydrophobic, acidic, basic or neutral. The properties and the biological functions of a protein are largely determined by its three-dimensional structure. The three-dimensional structure is determined by its amino acid sequence and is stabilized by noncovalent interactions between the peptide bonds and the R groups in the polypeptide chain. The side chains do not participate in peptide formation and are free to interact and react with their environment. For those amino acids containing relative nonpolar and hydrophobic side chains, they are poorly soluble in water, and usually occur in the interior of a protein out of contact with the aqueous solvent and form the hydrophobic core of protein. Polar or charged hydrophilic side chains are usually locate at or near the surface where they can be hydrated with the surrounding aqueous environment.

The most significant amino acids for modification and conjugation purposes are the ones containing ionizable side chains: aspartic acid, glutamic acid, lysine, arginine, cysteine, histidine, and tyrosine and the N-terminal α-amino and the C-terminal α-carboxylate groups. Ionizable groups can exist in one of two forms: protonated or unprotonated. The ionic state of the amino acids depends on pH. If the pH values are larger than their pKₐ values, the side groups will be in unprotonated. The Henderson-Hasselbach equation (Eq. 2.1) explains the relationship of pH and pKₐ to the relative ratios of protonated (acid) and
unprotonated (base) forms of an ionizable group.

\[
\text{pH} = \text{pK}_a + \log \frac{[\text{base}]}{[\text{acid}]} \quad \text{Eq. 2.1}
\]

where \( \text{pK}_a \) is the weak acid -log dissociation constant and \([\text{acid}]\) and \([\text{base}]\) are the concentrations of the undissociated weak acid and its conjugate base. This equation indicates that when the ratio of \([\text{base}] / [\text{acid}]\) is nearly one, the protonated and unprotonated forms of the protein are the same, \( \text{pH} \) will equal \( \text{pK}_a \). When the \( \text{pH} \) value is one unit above \( \text{pK}_a \), the ionizable group will be 91% unprotonated.

Nucleophilic attack at an atomic center of electron deficiency or positive charge is the basis for many of the coupling reactions that occur during chemical modifications. A nucleophile is any atom containing an unshared pair of electrons or an excess of electrons able to participate in covalent bond formation. Lysine, argine, and histidine have ionizable amine side chains that are often exposed on the surface of proteins and can be derivatized with ease. At \( \text{pH} \) values greater than the \( \text{pK}_a \), the amines are unprotonated and are much more powerful nucleophiles than in the protonated form. As a result, their side chains can be alkylated with activated alkyl group quite easily.

Isothiocyanates, one kind of the alkylating reagents, are of intermediate reactivity in the "amine reagents" and have quite high stability in water [Haugland, 1996]. Among the isothiocyanates, FITC is probably the most commonly used fluorescent derivatization reagent to modify the amines. In this project, FITC as a tracer was coupled to the PTE to allow detection and quantification of organophosphate pesticides.

2.2 FITC Properties and Its Reactivity
FITC is probably the most popular fluorescent labeling agent for its high fluorescence efficiency, chemical stability in the excited state, good absorption characteristics and protein combining capacity. This dye was first synthesized by Riggs and his co-workers by treating amino-fluorescein with thiophosgene instead of phosgene [Kawamura, 1977]. The dye has bluish-green fluorescence, a molecular weight of 389, an absorbance maximum at 494 nm and an emission wavelength of 520 nm [Haugland, 1994]. Its fluorescent character is created by the presence of a multi-ring aromatic structure more specifically the planar nature of its upper, fused three-ring system (Figure 2.1).

![FITC structure](image)

Figure 2.1 FITC (fluorescein-5-isothiocyanate) structure. Molecular weight is 389, Excitation at 492 nm, Emission at 520 nm.

Isothiocyanate compounds are almost entirely selective for modifying ε- and N-terminal amines in proteins or primary amines in other molecules [Jobbagy and Kiraly, 1966]. It appears that ε-amino groups of lysine residues and terminal amino groups of protein chains are the major sites of reaction with FITC [Goldman, 1968]. In the coupling reaction, the thiocarbonyl group of a FITC reacts with amino group of a protein to form a covalent bond, a stable thiourea linkage (Figure 2.2). The reaction usually is proceed in the alkaline condition.
Figure 2.2 FITC react with amine-containing protein to produce a thiourea linkage.

A protein combined with many dye molecules has a relative high in negative charge and is easily adsorbed nonspecifically by positive charged tissue sections. For this reason, compounds with a molar combining ratio (F/P, molar ratio of FITC to protein) of 1 to 2 are desirable. Usually, the proteins can have F/P molar ratio 1 to 15. If the derivatization of a protein is done at too high a level, fluorescein-fluorescein can result in self-quenching through space charged coupling.

FITC is generally stable in the solid state. The fluorescence decreases rapidly in solution. The pH variation can cause a change in the ionizing balance of the molecules as Figure 2.3. Under ideal conditions, FITC quantum yield can be very high. Below pH 7, however, fluorescein’s quantum yield is significantly quenched (Figure 2.4). Fluorescent quenching of the fluorescence is greater than 50% when conjugated to the proteins [Haugland, 1994]. Even so, the fluorophore usually maintains excellent detectability in assay system [Hermanson, 1996]. The fluorescence assay of this project is based on the
Figure 2.3 The molecule of FITC ionizes with pH

fact that the fluorescence yield of FITC is pH-dependent, and a pH decrease will result in fluorescence quenching. PTE catalyzed a hydrolysis reaction that will produce protons and change ionization state of the FITC. Because FITC is bound to the PTE and the enzyme has weak base and acid properties which can serve as a buffer, the pH of the FITC local environment is also affected by the enzyme's ionizable groups.

Figure 2.4 Fluorescence intensity changes with the pH.

2.3 Enzyme Kinetics
Michaelis and Menten are regarded as the founders of modern enzymology. They proposed a mechanism for the enzyme reaction:

\[ E + S = ES \rightarrow E + P \]

where:
- \( E \): enzyme
- \( S \): substrate
- \( ES \): enzyme-substrate complex
- \( P \): product

The equation:

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

Eq. 2.2

called the Michaelis-Menten equation gives the initial velocity \( v \) relative to maximum velocity \( V_{\text{max}} \) at a given substrate concentration \( S \). \( K_m \) is called the Michaelis constant (i.e. the \( K_m \) is the substrate concentration at which the reaction velocity is half-maximal). \( K_m \) establishes an approximate value for the intracellular level of the substrate. It also indicates the relative "suitability" of alternate substrates of a particular enzyme. That is, the substrate with the lowest \( K_m \) (\( = \frac{[E][S]}{[ES]} \)) value has the highest apparent affinity for the enzyme. Or, if an enzyme has a small value of \( K_m \), it achieves maximal catalytic efficiency at low substrate concentrations.

For the Michaelis-Menten model, an enzyme's catalytic efficiency is measured by turnover number \( k_{\text{cat}} \) (catalytic number) over \( K_m \) value (i.e. \( k_{\text{cat}}/K_m \)). Enzyme based assays are typically designed so that the analytes (substrates) are considerably lower than \( K_m \).
When \([S] \ll K_m\), the enzyme reaction rate is linearly proportional to the substrate concentration. So, the assay’s dynamic range and detection limits are primarily determined by the enzyme’s \(K_m\) value. In the case of PTE, the previously described optical assay has been used to measure paraoxon as well as a number of other insecticides, nerve agents and synthetic compounds [Dumas et al, 1989; 1990; Lai et al, 1995; Caldwell et al, 1991; Donarski et al, 1989]. Table 2.1 cites the some reported the data [Dumas et al, 1989]. It can be seen that paraoxon is the best substrate for the enzyme which has the lowest \(K_m\) value. However, other compounds also show the quite low \(K_m\) values and serve as the properly substrate for the enzyme and can be measured.

Table 2.1 Kinetic constants for the hydrolysis of organophosphorus insecticides

<table>
<thead>
<tr>
<th>Common name</th>
<th>(\lambda_{max}) (nm)</th>
<th>(\Delta) (OD/mM)</th>
<th>(K_m) (mM)</th>
<th>(V_{max})</th>
<th>(V_{max}/K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraoxon</td>
<td>400</td>
<td>17</td>
<td>0.09</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Dursban</td>
<td>276</td>
<td>5.3</td>
<td>0.11</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>Parathion</td>
<td>400</td>
<td>17</td>
<td>0.24</td>
<td>30</td>
<td>11.25</td>
</tr>
<tr>
<td>Coumaphos</td>
<td>348</td>
<td>9.1</td>
<td>0.39</td>
<td>29</td>
<td>6.70</td>
</tr>
<tr>
<td>Diazinon</td>
<td>228</td>
<td>3.3</td>
<td>0.45</td>
<td>8.4</td>
<td>1.68</td>
</tr>
<tr>
<td>Fensulfothion</td>
<td>284</td>
<td>8.0</td>
<td>0.46</td>
<td>3.2</td>
<td>0.63</td>
</tr>
<tr>
<td>Cyanophos</td>
<td>274</td>
<td>18</td>
<td>2.1</td>
<td>7.5</td>
<td>0.32</td>
</tr>
<tr>
<td>methyl-parathion</td>
<td>400</td>
<td>17</td>
<td>0.84</td>
<td>2.4</td>
<td>0.26</td>
</tr>
</tbody>
</table>

2.4 Enzyme Immobilization

The specific conformation and an active center of an enzyme interacting with the substrate are regarded as essential features of the catalytic activity of enzyme. Some
enzymes are highly specific for the identity of their substrates, others can bind a wide range of structurally related substrates and catalyze a variety of related types of reactions. The geometry of an enzyme's functional site is clearly only one feature of the protein's conformation. Other structural features serve to stabilize the functional site, and provide sites of interaction of the protein with other molecules, or may serve other functions. Retention of the enzyme's native structure after immobilization is very important. Since the tertiary structure of enzyme protein is maintained by relatively weak noncovalent binding forces, such as hydrogen binding forces, hydrophobic and ionic forces, immobilization should be carried out under mild conditions. If the orientation of amino acid residues at the active center, or the native structure are altered, the catalytic activity may be lost or enzymatic properties may be changed.

The immobilization of an enzyme permits its reuse of the biological molecules and simplifies the analytical procedure. Various methods have been described for enzyme immobilization. Basic methods for the binding of enzymes to water-insoluble carriers include: physical adsorption, ionic binding, covalent binding, cross-link binding (intermolecular cross-linkings of enzymes by means of bifunctional or multifunctional reagents), or entrapping method (incorporating enzymes into the lattice of a semipermeable gel or enclosing the enzymes in a semipermeable polymer membrane). Among these methods, the adsorption method for the immobilization of an enzyme is based on the physical adsorption of enzyme protein on the surface of water-insoluble carrier and is accomplished primarily through van der Waals forces, hydrophobic forces. The immobilization of proteins through ionic bonding (e.g., as on an ion exchange resin)
are also simple and effective methods. The reaction conditions for this type of binding procedures are mild, and often cause little or no conformational change of the enzyme, or disruption of its active center. However, these methods have the disadvantages that the enzyme linkages are highly dependent on pH, solvent and temperature. The adsorbed enzyme may be lost from the carrier during utilization due to weak binding forces. The reaction conditions for the entrapment method are mild too. Consequently, the method has almost the same disadvantages as the adsorption such as the enzyme leakage from the carrier.

Covalent bonding is based on the binding of enzymes and water-insoluble carriers by covalent bonds. The binding forces between the enzyme and carrier are strong. The enzyme-carrier complex is stable and lost of the enzyme is very unlikely. The disadvantages are that the binding reaction is complicated and time-consuming. The conformational structure of the enzyme could be changed or the active center be altered. Thus, the enzyme could lose activity or change the substrate specificity. The cross-link method is simple and chemical binding is stable. But the reaction conditions are relatively severe, difficult to control and require a large amount of enzyme.

2.5 The Alkalinity and Buffer Capacity of the Environmental Water Matrix

Natural waters contain a number of weak acid-bases components such as carbon dioxide, phosphate, silicate, ammonia, sulfide, or borate that are from a variety of sources. In most natural waters, carbon dioxide far exceeds the other weak acids. This component originates from dissolution of carbonate rock, atmospheric CO₂ transfer, and respiration of aquatic organisms [Morel and Hering, 1993]. These acids and bases regulate the pH and
the composition of natural waters.

The alkalinity of the water is defined as the resulting net concentration of strong base in excess of strong acid. The alkalinity of natural water can be measured by titration to the CO₂ equivalence. Because in most natural waters carbonate is much more abundant than other weak acids and bases, the alkalinity formula can usually be expressed as [Stumm and Morgan, 1996]:

\[ \text{Alk} = [\text{HCO}_3^-] + 2[\text{CO}_3^{2-}] + [\text{OH}^-] - [\text{H}^+] \]  

Eq. 2.3

Consequently, alkalinity and the partial pressure of CO₂ in the atmosphere can be said to determine the pH value.

Buffers are substances which, by their presence in solutions increase the amount of acid or alkali that must be added to cause unit change in pH. The buffer capacity provides a measure of the ability of the system to resist pH changes upon addition of strong acid or base. The formula that describes buffer capacity is as follows:

\[ \beta = \frac{dC_B}{d\text{pH}} = -\frac{dC_A}{d\text{pH}} \]  

Eq. 2.4

where \(dC_A\) and \(dC_B\) are the numbers of mol liter\(^{-1}\) of strong acid or strong base required to produce a change in pH of \(d\text{pH}\).

Due to the dominance of carbonate in natural waters, and because surface water is usually equilibrated with the atmosphere where CO₂ is at a constant partial pressure (\(p_{\text{CO}_2}=10^{3.5}\) atm, assumed at 25°C). The concentrations of the individual carbonate species can be expressed as:

\[ [\text{H}_2\text{CO}_3] = K_H p_{\text{CO}_2} \]  

Eq. 2.5
\[
\begin{align*}
[HCO_3^-] &= [H_2CO_3^-][H^+] K_1 \\
[CO_3^{2-}] &= [H_2CO_3^-][H^+] K_1 K_2 \\
[OH^-] &= [H^+] K_w
\end{align*}
\]
Eq. 2.6
Eq. 2.7
Eq. 2.8

where \(K_h, K_1, K_2\) and \(K_w\) are equilibrium constants. The water buffer capacity can be expressed as:
\[
\beta = \frac{\delta c_b}{\delta pH} = \frac{\delta[HCO_3^-]}{\delta pH} + \frac{2\delta[CO_3^{2-}]}{\delta pH} + \frac{\delta[OH^-]}{\delta pH} - \frac{\delta[H^+]}{\delta pH}
\]
Eq. 2.9

After computing, the derivatives,
\[
\beta = \frac{2.3 K_1 K_h P_{CO_2}}{[H^+]} + \frac{4.6 K_1 K_2 K_h P_{CO_2}}{[H^+]^2} + \frac{2.3 K_w}{[H^+]} - 2.3[H^-]
\]
Eq. 2.10

The relationship of alkalinity and buffer capacity is as follows:
\[
\beta = 2.3 ([HCO_3^-] + 2[CO_3^{2-}] + [OH^-] - [H^-])
\]
\[
= 2.3 \cdot \text{Alk}
\]
Eq. 2.11

Because in the Equation 2.10, \(K_h, K_1, K_2\) and \(K_w\) are constants, buffer capacity is a function of pH for a water in equilibrium with the atmosphere.

2.6 KinExA Instrumentation

The KinExA instrument is a general purpose, automated instrument designed for measuring the interaction of specific binding ligands. It uses fluorescence for detection. The heart of the KinExA instrument is a capillary flow cell (Figure 2.5). The flow cell is embedded in a lens that is backed by a reflector.

The assay system uses particles as a solid phase material. Typically, the beads are 98 µm diameter PMMA beads. In this experiment, the PMMA beads diameter are 108
μm. The fluorescently labeled particles, which are optically transparent microparticles are suspended in buffer and drawn into a capillary flow cell where they are trapped against the screen. All samples and buffer are drawn through the particles. The fluorescent signal is monitored through the flow cell and the fluorescence intensities are reported on the monitor in real time.

Figure 2.6 is KinExA flow system diagram. The KinExA flow system is operated under negative pressure. An in-line vacuum degasser is built in to reduce the bubbles in the flow cell. The beads are drawn into the flow cell first. Prior to and during the beads loading, the bead reservoir is stirred by a motor, which ensures the homogeneity of the bead solution. Then the sample or buffer is drawn through the packed beads. The beads are removed using back-flush to the waste container after the assay. The pinch valves and selector valve regulate the path of the beads or sample flow. The flow system also incorporates an optional injection pump (pump 2) that can be used to inject a reagent into
a stream of sample. All of the pumps and valves are operated through the KinExA
hardware interface and PC-based software using a Microsoft Windows™ environment.
The system can be programmed to cycle automatically through the five samples, each
cycle using a new bead pack. For the KinExA fluorescence enzyme assay, each cycle was
506 seconds and fluorescence signal as a voltage value was recorded every second and
used for analysis.

Figure 2.6 KinExA flow system.
3.1 Materials

Phosphotriesterase (PTE) was supplied by Dr. Ashok Mulchandani, Chemical and Environmental Engineering Department, University of California, Riverside. The isolation and purification procedures for the production of recombinant PTE from Escherichia coli have been described in detail by Mulchandani et al. [Mulchandani et al., in press]. The specific activity of the enzyme used in this experiment was 5500 or 5300 units/mg protein.

Fluorescein isothiocyanate (FITC), N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES), 2-N-(morpholino) ethanesulfonic acid (MES), 2-(N-cyclohexylamino)-ethanesulfonic acid (CHES) and p-nitrophenol were obtained from Sigma Chemical Co. (St. Louis, Mo.). Diethyl p-nitro-phenolphosphate (paraoxon), O,O-dimethyl O-p-nitrophenyl phosphorothioate (methyl parathion), O,O-diethyl O-p-nitrophenyl phosphorothioate (ethyl parathion), O,O-diethyl-[3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl] (coumaphos), 2,2-dichlorovinyl dimethylphosphate (dichlorvos), 2-bomethoxy-1-methlyvinyl dimethyl phosphate (mevinphos, phosdrin™), α-methylbenzyl-3-hydroxycrotonate dimethyl phosphate (crotoxyphos), O,O-diethyl O-(2-isopropyl-4-methyl-6-pyridimyl) phosphoro-thioate (diazion), O,O-diethyl O- (3,5,6-trichloro-2-pyridyl) phosphorothioate (dursban, chlorpyrifos), and O,O-diethyl [p-(methylsulfinyl)
phenyl] phosphorothioate (fensulfothion) were purchased from Chem Service (West Chester, PA); the purities of all chemicals were greater than 98%. Polymethylmethacrylate (PMMA) beads (diameter 100 μm) were purchased from Bang’s Laboratories (Carmel, IN). Slide-A-lyzer™ dialysis cassettes and micro bicinchoninic acid (BCA) protein assay reagent kit were purchased from Pierce (Rockford, IL). Alkalinity Titrator Kit was from LaMotte Co. (Chestertown, MD). All other compounds and solvents used were of reagent grade.

River water samples were obtained from the Lake Mead in Southern Nevada and Virgin River in Southern Utah. Well water sample (Well HM-I and HM-L) was obtained from Salmon site, Louisiana. The water was stored at 4°C until use. Biofilter treated coumaphos-containing cattle dip vat samples were provided by Dr. Mulbry, Soil Microbial Systems Laboratory, United States Department of Agriculture. For sampling information, see the articles [Mulbry et al, 1998, 1996]. The samples were stored at 4°C until use.

3.2 PTE Enzyme Activity Assay

Enzymatic activity was routinely measured by monitoring absorbance at 400 nm using 1.0 mM paraoxon. The substrate was hydrolyzed to diethylphosphate and p-nitrophenol (ε=17,000 M⁻¹ cm⁻¹, at wavelength of 400 nm) [Cadwell and et al., 1991a] in 50 mM CHES buffer, pH 9.0. absorbance was measured using a Varian model DMS 300 UV visible spectrophotometer at room temperature (about 25°C). One unit of activity is defined as the hydrolysis of 1 μmol of paraoxon/min. The activity measurement procedure is as follows: two spectrophotometer cuvettes were prepared, each one contained 1.00 ml
reaction mixture which was 1.0 mM paraoxon in 50 mM CHES buffer, pH 9.0; the absorbance was then set to zero, the enzyme 10.00 μl (0.32-0.42 μg/ml) was added to one of the cuvettes, the solution was mixed and the difference in absorbance between the cuvettes was monitored and recorded for two minutes. A spreadsheet (MS-Excel) was used to plot the absorbance against time (min.), and the absorbance change per minute was determined and then used to calculate the enzyme activity and specific activity as Equation 3.1 and 3.2.

Protein concentration was determined by the bicinchoninic acid (BCA) assay (Pierce) with bovine albumin as a standard (see Appendix I) using a Spectronic 20D spectrophotometer (Milton Roy Company).

Enzyme PTE activity (U):

\[ U(\mu\text{mol} / \text{ml} \cdot \text{min.}) = \frac{\Delta A / \text{min.}}{17,000 (A / M \cdot cm) \times 1(cm) \times 1000} \]  \hspace{1cm} \text{Eq. 3.1}

Enzyme PTE specific activity (SA):

\[ SA(\mu\text{mole} / \text{mg} \cdot \text{min.}) = \frac{U(\mu\text{mol} / \text{ml} \cdot \text{min.})}{\text{protein concentration} (\text{mg} / \text{ml})} \]  \hspace{1cm} \text{Eq. 3.2}

### 3.3 FITC Conjugation with PTE and the Conjugate Properties

In the PTE-catalyzed paraoxon reactions and other organophosphate compounds, protons are released due to production of relatively strong acids which would cause the fluorescence intensity to decrease. FITC was conjugated to the PTE enzyme, then the enzyme was immobilized to the PMMA beads. When using FITC-labeled PTE immobilized to PMMA beads to catalyze the hydrolysis of paraoxon and other...
organophosphate compounds, the fluorescence intensity change was monitored through a KinExA fluorescence analyzer.

### 3.3.1 FITC Labeling of PTE

Prior to the coupling reaction of FITC with PTE, FITC (1.0 mg/ml) was prepared in dimethyl formamide (DMF). PTE (200 μl) was added to a microcentrifuge tube containing 750 μl of 50 mM bicarbonate, 100 mM NaCl buffer, pH 9.5. Then FITC (50.0 μl) was added dropwise to this microcentrifuge tube. The FITC to enzyme final molar ratio is around 1:5. The enzyme solution was gently swirled as the FITC was added. For protection from light, the microcentrifuge tube was wrapped in aluminum foil then incubated for 1.5 hr. at room temperature (about 25°C) in a incubator shaker (Model 136400, Boekel Industries Inc.) with a gentle rock.

For removal of unconjugated FITC, the enzyme solution (1.0 ml) was transferred to a dialysis cassette (3 ml, 10,000 MW cut off) and dialyzed against 1L 0.01 M phosphate-buffered saline (100 mM NaCl), pH 7.4 (PBS) which was kept in a 4°C cool room. The PBS solution was continually stirred by a magnetic stirrer. The buffer was changed 3 times over a 12 hr. period. The absorbance of the FITC in the dialysate was determined using a Varian model DMS 300 UV visible spectrophotometer. The FITC-labeled PTE was then aliquotted (100 μl) into 1.5 ml microcentrifuge tubes and stored at -20°C.

### 3.3.2 Determination of Relative FITC Concentrations of Conjugates and Molar Ratio of FITC to PTE enzyme (F/P molar ratio)
Determination of absolute FITC content binding to PTE enzyme is difficult because of the uncertainty concerning absorption and fluorescence characteristics of dyes attached to large protein molecules. When fluorescein is conjugated to protein, the absorption spectra (as with most fluorescent dyes) has a different pattern than the free dye, showing a shift in the wavelength of its maximum absorption. The extinction coefficient also changes slightly. Jobbagy and co-workers have thoroughly investigated and characterized the conjugates of protein with FITC [Jobbagy, 1965] and determined that the extinction coefficient of FITC-protein conjugates is nearly identical to that of the free dye. The relative molar ratio of fluorescein in the conjugates is readily obtained by relating absorption or fluorescence of conjugate to protein concentration.

In this project, the molar ratios of various batches of FITC-labeled PTE were determined by reading absorbance of the conjugate solutions at the absorption maximum of the free FITC. Standard concentrations of free FITC were prepared by dissolving an exact amount of FITC in 10 mM PBS, pH 7.4, and measuring absorbance of the standards in a DMS 300 UV-visible spectrophotometer. From the standard curve, the extinction coefficient was determined and then used to determine the amount of relative FITC concentration of FITC-labeled PTE conjugate as follows:

\[
\text{Conc. of FITC in conjugate (}\mu\text{g/ml)} = \frac{\Delta A}{\varepsilon \cdot b}
\]

Eq. 3.3

where

\(\Delta A\) : absorbance of FITC-labeled PTE conjugate.

\(\varepsilon\) : Free FITC extinction coefficient, \(\mu g^{-1} \cdot cm^{-1}\).

\(b\) : light path length, cm.
The protein concentration was determined by the same method used to determine the unconjugated enzyme. The F/P molar ratio was calculated according to the following equation:

\[
\frac{F}{P} \text{ molar ratio} = \frac{\left(\mu g \text{ of FITC}\right) \times 10^{-3}}{\mu g \text{ of PTE} + 39,000}
\]  
Eq. 3.4

3.3.3 Determination of \(K_m\) values of PTE and FITC-Labeled PTE

For measuring the Michaelis-Menten constant \(K_m\) of the PTE and FITC-labeled PTE, reaction mixtures were prepared containing paraoxon at concentrations ranging from 10 µM to 400 µM in 50 mM CHES buffer, pH 9.0. In each assay, reaction mixture (1.0 ml) was added to a disposable plastic cuvette. The reaction was then started by adding 10.0 µl PTE solution (containing 1.7 U or 0.85 U) or 10.0 µl FITC-labeled PTE (containing 1.4 U), and mixed. The absorbance change was monitored at 400 nm with a DMS 300 spectrophotometer and data were recorded every 0.1 minute for more than 2 minutes. A spreadsheet program written using MS-Excel was used to determine the initial absorbance change per minute by using the recording data (10 points). The least-squares method of the Delta-graph program was used to determine the initial rates and applied to the following equations to determine the value of the kinetic constant by a plot of \(1/V\) vs. \(1/[S]\) that is described by Segel [Segel, 1976]:

\[
\frac{1}{V} = \frac{K_m}{V_{\text{max}}} \frac{1}{[S]} + \frac{1}{V_{\text{max}}}
\]  
Eq. 3.5

where
V: initial velocity
S: substrate concentration
$K_M$: Michaelis-Menten constant
$V_{max}$: maximum velocity

3.4 Immobilized PTE

The non-covalent adsorption method is based on the physical adsorption of enzyme protein on the surface of water-insoluble carrier. Protein adsorption to plastic surfaces is mainly attributed to non-specific hydrophobic interactions and independent of the net charge of the protein [Tijssen, 1985]. The method often causes little or no conformational change of the enzyme protein, or destruction of its active center, as was mentioned earlier. It is simple and effective. It works generally well and is the most frequently used the method to immobilize the protein to the solid phase.

3.4.1 Immobilization FITC-labeled PTE to PMMA Beads

The commercially PMMA (polymethylmethacrylate) beads are made by polymerizing pure methyl methacrylate, $\text{CH}_2\text{C(CH}_3\text{)CO}_2\text{CH}_3$, using persulfate as the polymerization initiator. PMMA beads have been successfully used as a solid phase material for KinExA instrument and as a solid phase for protein immobilization [Rogers et al., 1996]. In this project, PMMA beads were used as the immobilization material for the FITC-labeled PTE. The binding method is a physical adsorption method.

For immobilization of FITC-labeled PTE to the PMMA beads, the first step was to add 100 $\mu$L of FITC-labeled PTE (containing about 1100 units/ml or 57 $\mu$g protein) to 200
mg PMMA beads which were suspended in 900 µl of 10 mM PBS, pH 7.4 in a microcentrifuge tube. The mixture was gently rocked for 3 hr. at room temperature (about 25°C). Occasionally, the beads were stirred by a Vortex mixer. Second, the beads were removed from the uncoupled enzyme by centrifugation (2000×g, 2 min.). The supernatant was discarded and the beads were washed by centrifugation 3 more times, then 1.0 ml running buffer (specified concentration HEPES-MES, 100 mM NaCl) was added to the beads. Last, the beads were stored in 1.0 ml running buffer at 4°C until use.

3.4.2 pH Profile

The absorbance assay was used to measure PTE (0.42 µg/ml protein) and FITC-labeled PTE (0.71 µg/ml protein) activity (initial velocity) at different initial pH values. The product, formation of p-nitrophenol which was produced by hydrolysis of paraoxon, was determined in the pH range from 6.0-9.5. The paraoxon (1.0 mM) was prepared in a system of three buffers, each at 50 mM. The effective pH range for these buffers is as follows: MES, pH 6.0, pH 6.5; HEPES, pH 7.0, pH 7.5, pH 8.0; CHES, pH 9.0, pH 9.5. Because the extinction coefficient for p-nitrophenol varies as a function of pH, extinction coefficients at 400 nm were determined at standard concentrations. From the absorbance, the apparent extinction coefficients were obtained from Beer's law:

\[ \varepsilon_{\text{app}} = \frac{A}{bc} \quad \text{Eq. 3.6} \]

Equation 3.7 (see Appendix II) also can be used to obtain the apparent extinction coefficients:
\[ \varepsilon'_{\text{app}} = \varepsilon \left(10^{pK_a - \rho H} + 1\right)^{-1} \]  
Eq. 3.7

where

\( \varepsilon'_{\text{app}} \): calculation the apparent extinction coefficient (M\(^{-1}\) cm\(^{-1}\))

\( \varepsilon = 17,000 \text{ M}^{-1} \cdot \text{cm}^{-1} \)

\( pK_a = 7.16 \) (dissociation constant from Merck Index)

For measuring immobilized enzyme activity at different initial pH values, the beads were suspended in 0.1 mM buffer (1 batch of prepared beads (200 mg) described previously added to 19 ml of 0.1 mM buffer). The bead suspension (1.0 ml) was then added to a test tube. After the beads settled down to the bottom of the tubes, the supernatant was pipetted out. The beads were washed three times using a specified buffer and incubated for 1 hr. to equilibrate them in the buffer. The reaction was initiated by adding 2.85 ml of the specified pH reaction mixture as previously described to start the hydrolysis. The solution was stirred for about 25 s., after which the beads settled, and the absorbance was read every minute for 10 min. using Spectronic 20D (Milton Roy) spectrophotometer.

3.4.3 Thermostability of the Immobilized PTE

The effect of temperature on the immobilized PTE was also determined. PTE immobilized to beads were exposed to different temperatures, using a water bath for specific time periods. After cooling the particles and adding new buffer, the KinExA assay was run as described in the next section. The paraoxon concentration was 100 \( \mu \text{M} \) in HEPES (100 \( \mu \text{M} \)), NaCl (100 mM) pH 8.0.
3.5 KinExA Assay

3.5.1 KinExA Assay Procedure

The KinExA instrument was used to determine paraoxon and other organophosphate concentrations. First, PTE coated beads were prepared as previously specified (1 batch of prepared beads added to 19 ml of specified buffer) and placed into the instrument bead reservoir. The buffer and five samples (aspirated from microcentrifuge tubes) were also placed into the KinExA system.

Organophosphate stock solutions prepared in methanol (100 mM, except coumaphos 25 mM) were diluted to specified concentrations into specified concentration of HEPES-MES buffer containing 100 mM NaCl. The final concentration of methanol in the sample was about 1%. For some organophosphate compounds such as fensulfothion, crotoxyphos, methyl parathion, ethyl parathion, diazinon, dursban, 10% methanol (20% for coumaphos) was used to increase their limited solubilities.

Second, immobilized FITC-labeled PTE particles (750 μl) were drawn into the flow cell from the stirred bead suspension. The particles were retained on the nylon screen and 600 μl of buffer was drawn through the cell to equilibrate the beads in the running buffer.

Third, specified buffer (750 μl) with various concentrations of paraoxon was pumped through the packed beads. The buffer flow was then stopped for 5 min. then continued with 1.0 ml of the same buffer. The instrument controlled by a computer program automatically started the next cycle. Each cycle was about 11 min. The fluorescent signal was monitored throughout the programmed cycle and mV values were
recorded to a data file each second.

3.5.2 Data Analysis

The capillary flow cell served as a reaction vessel and a detection unit. When paraoxon passed through the PTE immobilized beads, the hydrolysis reaction occurred. PTE catalyzed the hydrolysis of paraoxon which produced protons, and the fluorescence

![Diagram of the PTE based assay for paraoxon and organophosphate compounds. FITC-labeled PTE is immobilized to the surface of PMMA beads (100 μm diameter) which are trapped on a nylon screen in a capillary. The catalytic activity of the enzyme which releases a proton during substrate hydrolysis can be continuously monitored by a filter type fluorometer through the fluorescence of the FITC label.](image)

Figure 3.1 Schematic for the PTE based assay for paraoxon and organophosphate compounds. FITC-labeled PTE is immobilized to the surface of PMMA beads (100 μm diameter) which are trapped on a nylon screen in a capillary. The catalytic activity of the enzyme which releases a proton during substrate hydrolysis can be continuously monitored by a filter type fluorometer through the fluorescence of the FITC label.
Figure 3.2 Representative tracing of FITC-labeled enzyme or heat-denatured FITC-labeled enzyme (80 °C for 1 hr.) in the presence or absence of 100 μM paraoxon. Signal response upon (A) addition of substrate; (B) interruption of buffer flow through the bead pack; and (C) re-initiation of buffer flow. The buffer was 0.1 mM HEPES and MES, 100 mM NaCl, pH 8.0.

Intensity decreased due to a decrease in the local pH. Figure 3.1 shows a schematic for the PTE based KinExA assay for paraoxon and other organophosphate compounds.

Once the assay was finished, the fluorescence signal data of voltage values vs. time were transferred to the Microsoft Excel program. It was observed that the interruption of the buffer flow resulted in a more sensitive response to substrate hydrolysis. This was probably due to a larger change in pH in the local quenching of the FITC probe. To correct the difference of initial fluorescence intensity and fluctuations of the background signal during the processing of samples, for each sample, the value is obtained by taking the average of 6 signals just prior to the continuation of the buffer flow dividing by the average of 6 signals just prior to the interruption of the buffer flow (see Eq.3.8 and Figure.
3.2. The final value (relative fluorescence signal) for each sample in a set is taken as a difference by one subtraction each value at each concentration. The relationship of relative fluorescence signal and each concentration of organophosphate compounds were determined as follows:

\[ FS = 1.000 - \frac{S_c}{S_b} \]  

\text{Eq. 3.8} 

where

FS: reported relative fluorescence signal 

S_b: average of 6 signals taken at one second intervals just prior to the interruption of the buffer flow, see Figure 3.2.

S_c: average of 6 signals taken at one second intervals just prior to the continuation of the buffer flow, see Figure 3.2.

3.6 The Methanol Effect to KinExA Assay

To determine the effect of methanol on the KinExA assay, different concentrations of methanol ranging from 5% to 30% were added to the 100 \( \mu \text{M} \) HEPES-MES, 100 mM NaCl buffer, pH 8.0. Calibration curves with paraoxon concentrations ranging from 0.500 \( \mu \text{M} \) to 600 \( \mu \text{M} \) in 10% methanol were also run the KinExA.

3.7 Absorbance Assay to Determination the Paraoxon Concentration

The KinExA assay was compared to the absorbance assay. For the absorbance assay, different concentrations of paraoxon were prepared in 50 mM CHES buffer, pH 9.0. Unlabeled PTE (0.14 U) was used to catalyze hydrolysis of the paraoxon. The
procedure was same as enzyme assay. When the reaction reached steady state (ΔA stable, after 10 minutes reaction), the absorbance change was read or the solution was diluted to certain concentration and the absorbance change was read.

3.8 Determination of Coumaphos Concentrations in Biofilter Treated Cattle Dip Samples

KinExA assay was used to determine the coumaphos concentrations in biofilter treated cattle dip vat samples. Seven samples obtained in different days were analyzed. The KinExA assay results were compared to HPLC determinations.

3.8.1 KinExA Assay for Determination of Coumaphos Concentrations in Biofilter Treated Cattle Dip Waste Samples

To eliminate the effect of the original high concentration of base (sodium hydroxide and phosphate fertilizer were used to control pH) in the samples, the samples were extracted by chloroform. First, the sample was vigorously shaken to suspend the insoluble particulates, then 5.0 ml sample was pipetted out from each sample, and added to 5.0 ml chloroform contained in a glass tube with a Teflon cap. The tubes were inverted occasionally for 30 minutes. Second, the samples were centrifuged for 5 min. (2000 r. p. m.) by using a IEC centra-8R centrifuge (Internal Equipment Company, USA) in order to completely separate the solvent layer and aqueous layer. Third, Pasteur pipettes were used to transfer the supernatant (aqueous) to the waste container, then the chloroform solvent layer was pipetted to another tube. For each sample, 1.0 ml of the chloroform solvent solution was aliquotted, then N₂ gas was used to blow dry the samples.
Because coumaphos has very low aqueous solubility, before running the KinExA assay, the samples were dissolved in adequate amount of the methanol, then diluted in 20% methanol, 0.1 mM HEPES-MES, 100 mM NaCl buffer, pH 8.0. The final concentration of methanol was between 20% to 24%.

To observe the chloroform extraction efficiency, 1.2 mg/ml of the coumaphos standard in the water was prepared. The same volume of the chloroform was then used to extract the standard using the same procedure as for extraction of the biofilter treated cattle waste samples.

3.8.2 HPLC Method for Determination of Coumaphos Concentrations in Biofilter Treated Cattle Dip Waste Samples

Prior to high performance liquid chromatography (HPLC) analysis, the samples were vigorously shaken to suspend the insoluble particulates, then 1.0 ml each sample was aliquotted and added to a tube containing 4.0 ml methanol tube. Then a Vortex mixer was used to shake each sample for 1 min. After centrifuging the samples for 5 min. (2000 rpm) (using a IEC centra-8R centrifuge, Internal Equipment Company, USA), the supernatants were transferred to other tubes. Because some samples had high concentrations, they were further diluted into 80% methanol (methanol and water) before running the HPLC.

A standard curve was generated by preparing standard coumaphos in 80% methanol. The concentration range of the coumaphos standard was from 1.00 to 25.0 μM. An Alltech C-18 reverse phase (5 μm particles) column was used for all separation. Coumaphos was detected with a Lambda-Max Model 481 LC UV detector. The wavelength was set at 280 nm. The gradient was as follows:
<table>
<thead>
<tr>
<th>Time(min.)</th>
<th>water %</th>
<th>acetonitrile %</th>
<th>flow rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70.0 %</td>
<td>30.0 %</td>
<td>1.0 ml/min.</td>
</tr>
<tr>
<td>2.0</td>
<td>70.0 %</td>
<td>30.0 %</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>30.0 %</td>
<td>70.0 %</td>
<td></td>
</tr>
<tr>
<td>22.0</td>
<td>30.0 %</td>
<td>70.0 %</td>
<td></td>
</tr>
<tr>
<td>25.0</td>
<td>70.0 %</td>
<td>30.0 %</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 4

RESULTS AND DISCUSSION

4.1 FITC-Labeled PTE Physical Property and Characterization

4.1.1 The Physical Property of FITC-labeled PTE Conjugate

The absorption spectra of FITC and FITC-labeled PTE at pH 7.4 (PBS buffer) are shown in Appendix II. Jobbagy and co-workers have investigated and characterized the conjugates of protein with FITC [Jobbagy, 1965]. The maximum absorption wavelength of FITC-labeled PTE conjugates shifts to a slightly longer wavelength (495 nm) compared to the free FITC, which has a maximum absorption wavelength at 492 nm. Although a shift of 3 nm wavelength was observed in the absorption peak, this change had no significant effect on the extinction coefficient as compared to the free dye. Relative fluorescein content of conjugates is readily obtained by relating absorption or fluorescence of conjugate to protein concentration. We assumed that the extinction coefficient of fluorescein-protein was nearly identical with that of free fluorescein.

4.1.2 F/P Molar Ratio

The relative FITC concentration of FITC-labeled PTE conjugate was determined from the standard calibration curve at 492 nm (Figure 4.1). Its extinction coefficient
is 79,000 M$^{-1}$-cm$^{-1}$. The protein concentration of FITC-labeled PTE was determined using the BCA assay. The standard curve is shown in Figure 4.2. The absorption was read at 562 nm. Because the suggested wavelength (562 nm) for measuring the protein was substantially longer than the fluorescein absorption maximum, it avoids the contribution from fluorescein to the optical absorbance of the conjugates.

Table 4.1 shows the experiment results of F/P molar ratio. The F/P molar ratio of FITC coupled with PTE varied with the pH value of the conjugation buffer. The fact that the pH value of the reaction mixture can have an effect on the labeling efficiency was pointed out early by Lewis and McKinney [Lewis, 1963; McKinney, 1964]. It was reported that the labeling efficiency is significantly improved with increasing pH in the pH range 6.0-10.0, reaching a maximum around pH 9.5.
Figure 4.2 Protein Concentration Standard Curve at $\lambda = 562$nm

Table 4.1 F/P molar ratio after FITC coupled with PTE at different pH.

<table>
<thead>
<tr>
<th>FITC and PTE coupling buffer</th>
<th>F/P molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM PBS, pH 7.4</td>
<td>0.07</td>
</tr>
<tr>
<td>50 mM carbonate buffered saline, pH 9.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

The molar ratio (FITC/enzyme) at which FITC conjugated with PTE in 50 mM carbonate buffered saline, pH 9.5 was 0.7. Although the enzyme contains 9 lysine residues and a terminal amine [Dumas, 1989], the $\epsilon$-amine of lysine has a slightly higher $pK_a=9.3-9.5$ than $\alpha$-amines $pK_a=7.6-8.0$. The protein was only labeled with an average of slightly
less than one fluorescein per enzyme molecule. The low labeling efficiency may be due to
the fact that the phosphotriesterase has a relative high isoelectric point of 8.3. The
Michaelis constants (Table 4.2) of the enzyme and labeled enzyme determined from
Lineweaver-Burk reciprocal plot (Appendix III) show that the labeling process had little
effect on the apparent $K_m$, which reflects the affinity of the enzyme for the substrate. The
labeling process, however, resulted in about 62% loss of enzyme activity. Since the
activity for the enzyme population was measured, how labeling at specific locations
ccontributed to the loss in enzyme activity is uncertain.

Table 4.2 The apparent kinetic parameters and the specific activities of PTE and
its conjugate, as well F/P molar ratio conjugated at 50 mM carbonate, 100 mM
NaCl buffer, pH 9.5.

| Enzyme            | $K_{app}$ (μM) | Specific Activity (μmol/min·mg) | F/P  
|-------------------|----------------|-------------------------------|------
| PTE               | 60             | 5300                          | NA   |
| FITC-labeled PTE  | 53             | 1900                          | 0.7  |

* reported values range from 43 μM [Caldwell, 1991] to 90 μM [Dumas, 1989].

b molar ratio of FITC/PTE.

c not applicable.

4.1.3 The pH Profiles of PTE, FITC-Labeled PTE and Immobilized FITC-Labeled
PTE

The pH activity profiles demonstrating the ability of the soluble enzyme PTE,
FITC-labeled PTE and immobilized FITC-labeled PTE to catalyze the hydrolysis of
paraoxon are shown in Figure 4.3. The absorbance was measured when $p$-nitrophenol was
released by paraoxon hydrolysis. The experimental apparent extinction coefficients of the
$p$-nitrophenol at different pH values were close to the calculated values (see Appendix IV
Figure 4.3 The effect of pH on the PTE enzyme activity. The paraoxon concentrations were 1.0 mM in 50 mM buffer.

For comparing the enzyme activity using the same scale at different pH values, the percentage of maximal enzyme activity is reported for the soluble enzyme PTE, FITC-labeled PTE and immobilized FITC-labeled enzyme. PTE and FITC-labeled PTE show a similar pH profile. Labeling FITC does not affect the enzyme’s pH profile for the catalysis of paraoxon. The enzyme activity of free PTE increased from pH 6.0 to 9.5. This result is similar to what has been reported for the free enzyme [Caldwell, 1991]. After the enzyme was immobilized to the PMMA beads, the activity of immobilized enzyme was less affected by the initial pH of the buffer, although it reaches the highest activity around pH
In general, after enzyme was immobilized to the support or carrier, the changes of enzymatic properties might be considered to be caused by the following two factors. One possible explanation involves changes in the enzyme itself, and the other involves physical and chemical properties of the beads used for immobilization. Because immobilization of the enzyme to the PMMA beads was a physical adsorption process, it could not involve the modification of amino acid residues in the active center of the enzyme. However, the negative charges on the beads may cause a charge related effect on the enzyme.

4.2 KinExA Assay Results

With respect to the previously described KinExA enzyme assay, PTE catalyzed the hydrolysis reaction and resulted in a drop in the pH (in the local environment of the FITC-labeled PTE), thus causing in the fluorescence quenching of FITC. This rapid drop in fluorescence was not observed in the absence of substrate or in the presence of substrate with heat-denatured enzyme (1 hr. at 80 °C) (Figure 3.2). The control experiment for this assay was conducted by heating treatment of the immobilized enzyme. In this experiment, the paraoxon concentration was 100 μM containing 100 μM HEPES buffer, pH 8.0 or just buffer without paraoxon. Appendix V shows the KinExA assay raw data after exposure of the immobilized enzyme to different temperatures for specified times. For comparing these raw data, the starting fluorescence intensity tracing for each heat-treated bead pack was adjusted. The capability of the enzyme to catalyze the hydrolysis reaction decreased with increasing the water bath temperature and exposure time. However, the enzyme still maintained its quite high activity after exposure to 60 °C in water bath for 1.5 hr.. Activity was completely lost after exposure to 80°C in water bath for 1.5 hr. and the
assay did not show the typical fluorescence intensity drop.

4.2.1 The Effect of Initial Buffer pH

To determine the effect of initial pH on this assay, the three initial buffer pH values 6.0, 7.0, 8.0 (0.1 mM HEPES-MES, 100 mM NaCl) were used. Paraoxon concentrations ranged from 0.500 μM to 600 μM (Figure 4.4). Because the paraoxon and other substrate stock solutions were prepared in methanol, then diluted to the assay buffer, the final concentration of methanol in samples was about 1% unless otherwise specified. The low concentration of added methanol had no effect on the assay (demonstrated in Figure 4.6 and Table 4.3). The effect of initial pH value on the assay is shown in the Figure 4.4. An initial pH value of 6.0 yielded the lowest assay response and pH 8.0 showed the highest response and dynamic range. In the low range of paraoxon concentrations, differences in the assay response were small. When the paraoxon concentration was in the high range, the pH 8.0 buffer showed the highest sensitivity. The lowest dynamic range response for an initial pH value of 6.0 can be explained using Figure 2.4. As shown in Figure 2.4, when pH decreases from 8.0 to 6.0, the fluorescence intensity of FITC dramatically decreases. The fluorescence intensity is nearly maximum at pH 8.0 and decreases to very low values at pH 6.0. So, when using an initial pH value of 6.0 for the KinExA assay and at high range concentrations of paraoxon, the protons produced in this reaction are expected to further lower the pH, but the fluorescence intensity change per change in pH unit are expected to be very small compared to using a higher pH value (pH 8.0). As a result, the assay response for an initial pH value of 6.0 would be expected to be smaller when compared to the other two curves in Figure 4.4. In addition, a pH value of 8.0 is the most
Figure 4.4. The effect of pH on the KinExA assay. The initial buffer concentrations were 100 μM HEPES-MES, 100 mM NaCl. The initial pH values are as specified: pH 6.0, pH 7.0, pH 8.0. The paraoxon was prepared in the specified pH buffer solution. The relative fluorescence signal at 0.1 μM represents the assay with no paraoxon condition. This is also the case for the other figures. The error bars represent the standard errors of the mean (n=3).

The favorable condition for immobilized PTE to catalyze the hydrolysis of paraoxon, as shown by the pH dependence of the immobilized FITC-labeled enzyme (Figure 4.3). The initial pH of 8.0 was selected as an optimum value.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 4.5 The effect of buffer concentration on the KinExA assay. The paraoxon was prepared at specified concentration in 0.1 mM, 1 mM and 10 mM HEPES-MES, 100 mM NaCl, pH 8.0 buffer solution. The error bars represent the standard error of the mean (n=3).

4.2.2 The Effect of Buffer Concentration

Because for this assay, measured fluorescence intensity changes depend on the production of protons, the effect of buffer concentration was investigated. Various concentrations (0.1 mM, 1 mM and 10 mM) of a HEPES-MES buffer system (each containing 100 mM NaCl) at an initial pH value of 8.0 were used as running buffers. Paraoxon concentrations of 0.500 μM to 600 μM were used with each of the specified
buffers. These results show the assay is sensitive to the buffer concentration (Figure 4.5). This result is expected because the buffer will consume some protons. From this figure, it can be seen that using 0.1 mM buffer gives the highest sensitivity, while the 10 mM buffer shows the least sensitivity. As might be expected, at the highest buffer concentration, paraoxon concentrations larger than 100 µM are required to yield a signal. For the 0.1 mM buffer, the assay dynamic range for determination of paraoxon is 11 µM to 600 µM. The differences in sensitivity were most pronounced from 40 µM to 400 µM. This working range is similar to that reported for an enzyme-electrode biosensor using the same enzyme [Mulchandani, in press].

It should be noted that for Figure 4.6-4.8, the KinExA assay shows a greater relative fluorescence signal response in the high range of paraoxon concentrations (400 to 600 µM). This is due to the fact that the later experiments were conducted using an improved KinExA instrument and using different batch of enzyme. In this project, two batches of PTE enzyme were used (first batch of PTE was 5500 U/mg-min., second PTE was 5300 U/mg-min.) and experiments run using two KinExA instruments. The previously described experimental results were conducted using the first batch of enzyme and run using the earlier model of the instrument. Nevertheless, the results are comparable and the conclusions are not changed by these minor modifications.

4.2.3 Methanol Effect on KinExA Assay

Organophosphate pesticides have limited solubilities in aqueous systems and thus require an organic solvent to solublize higher concentrations. To improve the solubilities
Figure 4.6 The effect of methanol on the KinExA assay for measurement of paraoxon. The paraoxon was added to 100 µM HEPES-MES, 100 mM NaCl, pH 8.0 buffer with or without 10% methanol.

of the organophosphate pesticides, 10% methanol was used for kinetic analysis [Dumas, 1989]. There is no loss of activity for the free enzyme at these low concentrations of methanol [Donarski, 1989]. It has also been reported that 10% to 20% methanol is used for enzymatic hydrolysis in bioreactors [Caldwell, 1991b] using this enzyme. Table 4.3 shows as methanol concentrations increased by 5% increments in the buffer (1 mM HEPES-MES, 100 mM NaCl, pH 8.0) without addition of any substrate (paraoxon), the fluorescence signal measured using the KinExA instrument shows almost no any change. Although it has been reported that 10% methanol in the elution buffer results in
approximately a 10-fold increase in enzyme leakage from a bioreactor [Caldwell, 1991b], immobilized enzyme does not appear to be released from the PMMA beads under these conditions. The results of the KinExA enzyme assay for measurement of paraoxon in 10% methanol and in methanol free buffer systems are identical (Figure 4.6). Figure 4.6 shows calibration curves for paraoxon concentrations ranging from 0.500 μM to 600 μM in 100 μM HEPES-MES, 100 mM NaCl, pH 8.0 buffer.

Table 4.3 The Effect of Methanol on KinExA Assay. The assay used 1.0 mM HEPES-MES, 100 mM NaCl buffer, pH 8.0 and contained specified methanol concentrations without substrate.

<table>
<thead>
<tr>
<th>methanol concentration (%)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>relative fluorescence signal 1.000-(Sc/Sb)</td>
<td>-0.03</td>
<td>-0.02</td>
<td>-0.02</td>
<td>-0.02</td>
<td>-0.04</td>
<td>-0.05</td>
</tr>
</tbody>
</table>

4.2.4 F/P Molar Ratio Effect to KinExA Assay

Early experiments showed that, when FITC was coupled with PTE at different pH values, the F/P molar ratio (0.07, 0.7) varied significantly. The initial fluorescence intensities for the KinExA assay are also very different when using these conjugates (FITC-labeled PTE) immobilized to the PMMA beads. However, the shapes of the KinExA standard curves for measuring the paraoxon are similar and appear only to be shifted along the y axis as shown in Figure 4.7. This indicates that the FITC is simply an indicator of the local pH, and the fluorescence intensity ratio rather than the initial fluorescence intensity for the most part can be used to measure the paraoxon concentration. The assay did however show a stronger response at the highest concentration of paraoxon when using a lower molar ratio of the conjugate. Nevertheless,
to reduce the signal noise of the KinExA instrument, the higher F/P molar ratio conjugate enzyme was used.

4.2.5 KinExA Assay Accuracy

The KinExA assay precision was determined by consecutively running the assay 10 times at a 50 μM concentration of paraoxon in 10% methanol, 100 μM HEPES-MES, 100 mM NaCl buffer, pH 8.0. The mean of the relative fluorescence values was 0.162

Figure 4.7 The effect of F/P molar ratio on the KinExA assay. The initial buffer concentrations were 100 μM HEPES-MES, 100 mM NaCl, pH 8.0.
Figure 4.8a KinExA assay standard curves for organophosphate pesticides in 100 μM HEPES-MES, 100 mM NaCl buffer, pH 8.0.

(standard deviation was 0.0047). The percent coefficient of variation ($\frac{SD}{Mean} \times 100$) of the assay was within the 2.92%.

4.2.6 KinExA Assay for Organophosphate Compounds

The KinExA assay was also conducted on a number of representative organophosphate pesticides, including coumaphos, mevinphos, dichlorvos, fensulfothion,
crotoxyphos, methyl parathion, ethyl parathion, diazinon, dursban. Because of limited the solubilities (in buffer) of coumaphos and some other compounds (fensulfothion, crotoxyphos, methyl parathion, ethyl parathion, diazinon, dursban), 10% methanol was added to the buffer (20% methanol for coumaphos). A total of 10 compounds were evaluated in the 100 \( \mu \text{M} \) buffer. In order to provide a clearer presentation for the data, the more soluble compounds are arranged in Figure 4.8a and others are presented in another in Figure 4.8b.
Figure 4.9 KinExA assay standard curves for organophosphate pesticides in 1 mM HEPES-MES, 100 mM NaCl buffer, pH 8.0.

As expected from the $K_m$ values reported, for the enzymatic hydrolysis of various organophosphate pesticides (Dumas, et. al., 1989, table 2.1.), paraoxon and coumaphos show the most sensitive fluorescence assay response followed by dursban, ethyl parathion, diazinon and mevinphos, methyl parathion, dichlorvos and fensulfothion. Table 4.5 summarizes these results. The detection limits for these compounds were determined using 3 times the standard deviation (for the lowest measured substrate concentration above zero, see Appendix VI and VII). It is unknown why coumaphos and methyl parathion,
which have a relatively high $K_m$ values, show a higher than expected sensitivities. Figure 4.9 shows the determination of some of the organophosphate pesticides in 1 mM buffer solution. The detection limit order is same as for the 0.1 mM buffer, the detection limit concentrations, however, are several times higher.

Table 4.4 The detection limit of KinExA assay for organophosphate pesticides.

<table>
<thead>
<tr>
<th>compound</th>
<th>LOD in 0.1 mM, pH 8.0 buffer (μM)</th>
<th>LOD in 1 mM, pH 8.0 buffer (μM)</th>
<th>reported $K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>paraoxon</td>
<td>11</td>
<td>20</td>
<td>0.09</td>
</tr>
<tr>
<td>ethyl parathion</td>
<td>12$^a$</td>
<td></td>
<td>0.24</td>
</tr>
<tr>
<td>methyl parathion</td>
<td>20$^a$</td>
<td>40$^a$</td>
<td>0.84</td>
</tr>
<tr>
<td>dursban</td>
<td>10$^a$</td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td>diazinon</td>
<td>15$^a$</td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td>mevinphos</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dichlorvos</td>
<td>25</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>fensulfothion</td>
<td>40$^a$</td>
<td></td>
<td>0.63</td>
</tr>
<tr>
<td>crototxyphos</td>
<td>50$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coumaphos</td>
<td>8$^b$</td>
<td></td>
<td>0.39</td>
</tr>
</tbody>
</table>

$^a$ buffer containing 10% methanol.

$^b$ buffer containing 20% methanol.

4.3 Absorbance Assay and KinExA Assay

Figure 4.10 shows KinExA assay and absorbance assay response to the different concentrations of paraoxon. In the case of the absorbance assay, when the concentrations of the hydrolyzed product ($p$-nitrophenol) became to high for the absorbance assay to adhere to Beers law, the samples were diluted as necessary. The relationship of diluted
Figure 4.10 Comparison of the KinExA assay and the absorbance assay. The KinExA assay was conducted in 100 μM HEPES-MES, 100 mM NaCl buffer, pH 8.0. The absorbance assay was conducted using 50 mM CHES buffer, pH 9.0.

solutions and absorbances was still linear. The relative absorbance response was calculated by multiplying the absorbance change by a dilution factor. Except at the highest paraoxon concentration, the two curves showed similar responses. One advantage for the KinExA assay, however, is that for different compounds, the absorbance assay requires the use of different wavelengths to measure the hydrolyzed products and different extinction coefficients to determine their concentrations whereas, for the KinExA assay only one excitation and one emission wavelength needed to be monitored. In this respect, the KinExA assay is a more universal assay for measuring the all of the PTE substrates.
4.4 The Effect of Environmental Water Matrix on KinExA Assay

To investigate the potential for use of the KinExA assay to measure organophosphates in environmental settings, impacts of various environmental water matrices were tested with the KinExA enzyme assay. The alkalinitities and buffer capacities of environmental water samples and prepared buffer solutions are shown in Table 4.6. The alkalinitities were determined by following the instructions for a commercially available titration test kit. The titrator is calibrated in terms of total alkalinity expressed as parts per million (ppm) calcium carbonate (CaCO₃).

Table 4.5 The alkalinitities and buffer capacities of the buffer solutions and environmental water matrices.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Alkalinity (ppm)</th>
<th>Alkalinity (mM)</th>
<th>Buffer capacity * (mM)/pH</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Mead water</td>
<td>132</td>
<td>2.64</td>
<td>5.28</td>
<td>8.34</td>
</tr>
<tr>
<td>Virgin River water</td>
<td>161</td>
<td>3.22</td>
<td>7.41</td>
<td>8.37</td>
</tr>
<tr>
<td>Well water HM-I</td>
<td>61</td>
<td>1.22</td>
<td>2.81</td>
<td>8.09</td>
</tr>
<tr>
<td>Well water HM-L</td>
<td>137</td>
<td>2.74</td>
<td>6.30</td>
<td>8.27</td>
</tr>
<tr>
<td>100 µM HEPES-MES</td>
<td>8.0</td>
<td>0.16</td>
<td></td>
<td>7.98</td>
</tr>
<tr>
<td>1 mM HEPES-MES</td>
<td>92</td>
<td>1.84</td>
<td></td>
<td>7.99</td>
</tr>
</tbody>
</table>

* buffer capacity = 2.3 x alkalinity, it was assumed that the surface water was equilibrated with atmosphere.

Figure 4.11 shows the paraoxon response in the different environmental water media. Before running the KinExA assay, the environmental aqueous media were adjusted to the pH 8.0 using hydrochloric acid or sodium hydroxide. The pH adjusted water
samples were then used to prepare solutions of paraoxon at various concentrations. Lake Mead water and Virgin River water showed the highest alkalinities. The assays for environmental samples yielded responses similar to those for 1 mM HEPES-MES buffer. Although the well water from HM-L showed a relatively high alkalinity (close to the Lake Mead water), it still yielded a higher signal response than expected. This response was
somewhat between that observed for the 0.1 mM and 1.0 mM HEPES-MES buffers. The reason for this unexpected sensitivity is not known. Because differences in assay response sensitivity can not be solely explained by measured alkalinity (as measured using the kit method); the use of this assay for measurement of organophosphate pesticides in environmental samples (without extraction or dilution) requires that a calibration curve be run using the sample matrix.

4.5 Stability of the Immobilized Enzyme

The stability of the enzyme immobilized to the beads was investigated after storage at 4°C for up to 55 days (Figure 4.12). In order to account for small variations in the fluorescence degradation of the FITC label, normalization initial fluorescence intensities from batch to batch were made by comparing the fluorescence signal (Sc/Sb) at 50 μM paraoxon to the fluorescence signal (Sc/Sb)_0 at zero concentration of paraoxon after running the KinExA. The experimental result showed that the immobilized FITC-labeled PTE is very stable. Although the initial fluorescence intensity varied from batch to batch, it did not affect to the measurement of paraoxon. As shown in Figure 4.12b, even when the beads were stored in 10% methanol buffer solution for 29 days, there was only slight attenuation of activity. This enzyme is also stable to thermal denaturation. Results indicate that after the FITC-labeled PTE beads were exposed to temperatures of 60°C in a water bath for 1.5 hr., it still maintained substantial activity. The activity is lost completely only after exposure to a temperature of 80°C in a water bath for 1.5 hr. (Table 4.7).
Figure 4.12 The stability of FITC-labeled PTE immobilized to PMMA beads as measured using the KinExA assay. Figure a shows the immobilized enzyme stored in 100 μM HEPES-MES, 100 mM NaCl buffer, pH 8.0 at 4°C. Figure b shows the immobilized enzyme stored in 100 μM HEPES-MES, 100 mM NaCl buffer, containing 10% methanol, pH 8.0 at 4°C.

<table>
<thead>
<tr>
<th>Water bath Process</th>
<th>none</th>
<th>60°C, 45 min.</th>
<th>60°C, 1.5 hr.</th>
<th>80°C, 1.5 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>paraoxon (50 μM)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>relative fluorescence signal 1.000-(Sc/Sb)</td>
<td>0.230</td>
<td>0.246</td>
<td>0.217</td>
<td>0.000</td>
</tr>
</tbody>
</table>

4.6 KinExA Assay for Determination of Coumaphos Concentrations in Biofilter Treated Cattle Dip Waste Samples

The KinExA assay was used to determine the coumaphos concentrations in biofilter-treated cattle dip waste samples. The samples were extracted into chloroform. The extraction efficiency was investigated by preparing a 1.2 mg/ml coumaphos standard in water followed by the same extraction procedure as for extraction of biofilter-treated cattle dip waste samples. Each sample was analyzed two times. The coumaphos
concentrations in the samples were quantified using standard curve as shown in Figure 4.8b. The results of KinExA assay were also compared to a HPLC method. Table 4.8 and Figure 4.13 show the experimental results. The reported values for the samples were determined by Dr. Mulbry (Soil Microbial Systems Laboratory, United States Department of agriculture) using a HPLC method. A HPLC method similar to that used by Dr. Mulbry was also run as part of this study. Due to the 80% extraction efficiency determined using a coumaphos standard, the coumaphos concentrations determined by the KinExA assay were expected to be lower than the reported values (Table 4.9). Taking this extraction efficiency into consideration, the results determined by KinExA assay were almost same as HPLC method used for this study. Figure 4.14 shows a good correlation between the HPLC and KinExA assays, for determination of coumaphos concentrations in biofilter-treated cattle dip waste samples. Consequently, this technique could be potentially developed into a rapid and convenient method for bioremediation process control monitoring for these applications.

Table 4.7 The results of KinExA assay and HPLC method for the biofilter-treated cattle dip waste samples.

<table>
<thead>
<tr>
<th>sample</th>
<th>reported value(ppm)</th>
<th>HPLC (ppm) / percentage of reported data</th>
<th>KinExA (ppm) (0.1 mM buffer, pH 8.0)/ percentage of reported data</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-24-97</td>
<td>1165</td>
<td>1196 / 103%</td>
<td>917 / 79%</td>
</tr>
<tr>
<td>11-26-97</td>
<td>570</td>
<td>638 / 112%</td>
<td>449 / 79%</td>
</tr>
<tr>
<td>11-28-97</td>
<td>404</td>
<td>461 / 114%</td>
<td>340 / 84%</td>
</tr>
<tr>
<td>12-1-97</td>
<td>183</td>
<td>213 / 116%</td>
<td>165 / 90%</td>
</tr>
<tr>
<td>12-3-97</td>
<td>76</td>
<td>81 / 107%</td>
<td>65 / 86%</td>
</tr>
<tr>
<td>12-5-97</td>
<td>29</td>
<td>35 / 121%</td>
<td>32 / 110%</td>
</tr>
<tr>
<td>12-8-97</td>
<td>≈2.5</td>
<td>≈2.07 / 83%</td>
<td>≈2.5 / 100%</td>
</tr>
<tr>
<td>Standard</td>
<td>*1200</td>
<td></td>
<td>960 / 80%</td>
</tr>
</tbody>
</table>

*prepared before experiment.
Figure 4.13. The results of KinExA assay, HPLC method and reported values for biofilter-treated cattle dip waste samples.

Figure 4.14 Comparison of coumaphos concentrations in biofilter-treated cattle dip waste samples determined by HPLC and KinExA assay.
CHAPTER 5

CONCLUSIONS AND FUTURE WORK

This project developed and characterized a novel assay to measure paraoxon and representative organophosphate pesticides such as methyl parathion, ethyl parathion, diazinon, dursban, fensulfothion, mevinphos dichlorvos and crotoxyphos. This assay is based on the substrate (i.e., protons)-dependent fluorescence quenching of FITC-labeled phosphotriesterase enzyme and monitored using a KinExA fluorescence analyzer. The assay is rapid and relative simple, and allows semi-automated sample handling. In addition, the assay can tolerate 10% methanol without changes in the measured activity. The immobilized PTE shows a relatively high thermal stability that is suitable for potential application as an environmental monitoring tool. Its long term stability at room temperature also makes this enzyme an ideal tool for long term monitoring of chemical or biological detoxification process.

This assay detects the production of protons, $H^+$, and the analyte is measured in a buffered the solution. Like any method (e.g. potentiometric, OPH) employing the production or consumption of $H^+$ ions, the main disadvantage for this assay is the effect of the buffer capacity of the assay medium on the calibration curves for organophosphate substrates. This assay may still be useful for a number of environmental applications. Most
of the environmental water matrices measured for this project showed alkalinity levels in the mM concentration range. If the calibration curve is prepared in a similar matrix as the environmental sample, this internal variability can be controlled. One potential application that was explored in this project involves monitoring the bioremediation of coumaphos. This potential application involves monitoring the bioremediation of coumaphos. In this case, the coumaphos concentrations were relatively high and the buffer capacity of the medium were well defined. A relatively rapid analysis of coumaphos for this application could provide an alternative to the HPLC method that is currently used.

In order to develop an assay with a higher sensitivity for continuous and in situ applications, there is a need to reduce pretreatment steps required for sample preparation and controlling or eliminating the buffer capacity effects. Future work in this area might involve coupling the enzyme to a solid support which could assist in the extraction of the compounds of interest. Nevertheless, due to the assay’s versatility in detecting a wide range of organophosphate pesticides, this enzyme assay mechanism may be particularly useful in the design of biosensors for bioremediation process control applications.
APPENDIX I

Micro BCA Protein Assay

Reagent

Reagent A, contains sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH.

Reagent B, contains 4% bicinchoninic acid (BCA) in water.

Reagent C, contains 4% cupric sulfate, pentahydrate in water.

Prepare Micro Working Reagent

1. Mix 2 parts of Micro Reagent C + 48 parts of Micro Reagent B.

2. Then, add 50 parts of Micro Reagent A and mix well.

Test Tube Assay Procedure

1. Prepare a set of protein standards of known concentration by diluting the stock BSA (bovine serum albumin) solution provided in the same diluent as your unknown samples.

2. Pipet 1.0 ml of each standard or unknown protein sample into the appropriately labelled test tube. For blanks, use 1.0 ml of diluent.

3. Add 1.0 ml working Reagent to each tube. Mix well.
4. Incubate all tubes at 60 °C for 60 minutes.

5. After incubation, cool all tubes to room temperature.

6. Measure the absorbance at 562 nm of each tube vs. water reference.

7. Prepare a standard curve by plotting the net (blank corrected) absorbance at 562 nm vs. protein concentration. Using this standard curve, determine the protein concentration for each unknown protein sample.

* In my experiment, used 1.5 ml.
APPENDIX II

The Absorbance Scan Spectra of FITC and FITC-labeled PTE
APPENDIX III

Lineweaver-Burk Peciprocal Plot: \(1/v \text{ versus } 1/[S]\)

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

A. Lineweaver-Burk plot for the PTE with varying concentrations of paraoxon in 50 mM buffer, pH9.0.

\[
y = 0.3721x + 0.6215
\]

\(R^2 = 0.9863\)

B. Lineweaver-Burk plot for the FITC-labeled PTE (F/P=0.7) with varying concentrations paraoxon in 50 mM buffer, pH9.0.

\[
y = 0.294x + 0.5559
\]

\(R^2 = 0.9841\)
APPENDIX IV

*p*-Nitrophenol Extinction Coefficient at Different pH

1. Calculate *p*-Nitrophenol Extinction Coefficient at Different pH:

\[ \text{HA} \rightleftharpoons H^+ + A^- \]

\[ K_a = \frac{[H^+][A^-]}{[HA]} \]

\[ \frac{[HA]}{[A^-]} = 10^{pK_a - pH} \]

\[ [HA] = [A^-]10^{pK_a - pH} \]

Beer’s Law:

\[ A = \varepsilon_{\text{app}}bc \]

\[ = \varepsilon_{\text{app}}b\left( [A^-] + [HA] \right) \]

\[ = \varepsilon_{\text{app}}b\left( [A^-] + [A^-]10^{pK_a - pH} \right) \]

\[ = \varepsilon_{\text{app}}b[A^-](1 + 10^{pK_a - pH}) \]

when pH=9.0, [HA] ≈ 0:

\[ A = \varepsilon b[A^-] \]

\[ \varepsilon_{\text{app}} = \varepsilon \left( 1 + 10^{pK_a - pH} \right)^{\dagger} \] (Equation 1)

HA: *p*-nitrophenol.

\( \varepsilon_{\text{app}} \): apparent extinction coefficient.

\( \varepsilon \): extinction coefficient, 17000 M\(^{-1}\)·cm\(^{-1}\) [Omburo, 1993].

\( pK_a \): dissociation constant, 7.16.

b: light path length, 1 cm.

77
2. Extinction Coefficient from Experiment Measurement and Equation 1:

<table>
<thead>
<tr>
<th>pH</th>
<th>( \varepsilon_{\text{experiment}} )</th>
<th>( \varepsilon_{\text{app}} = \varepsilon \left( 1 + 10^{pK_a - pH} \right)^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>1.66E+03</td>
<td>1.10 E+03</td>
</tr>
<tr>
<td>6.5</td>
<td>4.17E+03</td>
<td>3.05 E+03</td>
</tr>
<tr>
<td>7.0</td>
<td>9.30E+03</td>
<td>6.95 E+03</td>
</tr>
<tr>
<td>7.5</td>
<td>1.48E+04</td>
<td>1.17 E+04</td>
</tr>
<tr>
<td>8.0</td>
<td>1.75E+04</td>
<td>1.49 E+04</td>
</tr>
<tr>
<td>9.0</td>
<td>2.01E+04</td>
<td>1.68 E+04</td>
</tr>
</tbody>
</table>

\( \rho \)-nitrophenol extinction coefficient at different pH. ■ calculated the \( \varepsilon_{\text{app}} \). ▲ experiment determined \( \varepsilon_{\text{experiment}} \).
APPENDIX V

FITC-labeled PTE Beads Thermostability

The fluorescence tracing of KinExA assay for paraoxon (100 μM) in 0.1 mM HEPES buffer containing 100 mM NaCl buffer, pH 8.0 after heating treatment of the immobilized enzyme. The starting fluorescence intensities were adjusted.
APPENDIX VI

KinExA assay for pesticides in 0.1 mM HEPES-MES, 100 mM NaCl buffer, pH 8.0. The error bars are the standard deviations of the three measurements.
KinExA assay for pesticides in 1.0 mM HEPES-MES, 100 mM NaCl buffer, pH 8.0. The error bars are the standard deviations of the three measurements.


VITA

Graduate College
University of Nevada, Las Vegas

Yan Wang

Local Address:
1555 E. Rochelle Ave. #234
Las Vegas, NV89119

Home Address:
2025 Rose Coral Road
Las Vegas, NV89106

Degree:
Bachelor of Science, Biochemical Engineering, 1983
East China University of Science and Technology

Master of Science, Chemistry, 1998
University of Nevada, Las Vegas

Thesis Title: Development of A Phosphotriesterase Enzyme Assay for Determination of Organophosphate Pesticides

Thesis Examination Committee:
Chairperson, Dr. Kim R. Rogers, Ph. D.
Committee Member, Dr. Brian Johnson, Ph. D.
Committee Member, Dr. Spencer Steinberg, Ph. D.
Graduate Faculty Representative, Dr. David James, Ph. D.