Investigation of the Catalytic Mechanism and Biosensing Potential of Phosphotriesterases

by

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ABSTRACT

INVESTIGATION OF THE CATALYTIC MECHANISM AND BIOSENSING POTENTIAL OF PHOSPHOTRIESTERASES

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University of Guelph, 2011

This thesis describes the characterization of SsoPox, a lactonase with promiscuous phosphotriesterase activity from the hyperthermophilic archaeon, *Sulfolobus solfataricus*, and the potential of the phosphotriesterase from *Brevundimonas diminuta* (PTE_{Bd}) to function as an organophosphate sensor. Arg-223 and Tyr-99 of SsoPox are not essential for lactonase activity, however substitution of a phenylalanine in place of Tyr-97 abolished lactonase activity while reducing paraoxonase activity by 20-fold. Substrate specificity of SsoPox can be modulated through the partial blockage of the hydrophobic binding tunnel adjacent to the active site. The specificity constant for \( N\-(3\text{-oxo-decanoyl})\-L\text{-homoserine lactone} \) decreased 37-fold when a phenylalanine was introduced in place of Leu-226. PTE_{Bd} was expressed and purified from *Pseudomonas putida* and, like SsoPox, can be immobilized to Disruptor™ paper. The immobilized enzyme can be used to detect five organophosphates at concentrations as low as 50 \( \mu \text{M} \). Incubation of PTE_{Bd}-immobilized sensors at different temperatures proved that the enzyme is stable for at least 40 days at 23.5°C without any detectable change in activity.
Acknowledgements

I would first like to extend an enormous thanks to my advisor, Dr. Stephen Seah, for his patience, kind support and down-to-earth nature. I could not have asked for a better supervisor and it is thanks to Stephen’s guidance that I was able to hone my scientific skill set and succeed in my time as a graduate student.

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Filomena Ng laid the groundwork for my project and taught me much of what I used over the course of these two years and I am grateful for all that she has given me. Undergraduate students Sarah Massey and Dave Collings were instrumental in the timely completion of several aspects of my project and their work was very much appreciated.

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Finally, I’m thankful to all of my family and friends (especially those from the 4th floor Science Complex) for keeping me positive through failed experiments and unexpected results.
Author’s Declaration of Work Done

I hereby declare that I performed all of the work described in this thesis unless otherwise stated.
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AHL</td>
<td>N-acyl homoserine lactone</td>
</tr>
<tr>
<td>C4-HSL</td>
<td>N-butyryl-L-homoserine lactone</td>
</tr>
<tr>
<td>C10-HTL</td>
<td>Decanoyl homoserine thiolactone</td>
</tr>
<tr>
<td>CBD</td>
<td>Cellulose-binding domain</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>LP</td>
<td>Leader peptide</td>
</tr>
<tr>
<td>OP</td>
<td>Organophosphate</td>
</tr>
</tbody>
</table>
| 3-oxo-C6-HSL | N-(β-ketocaproyl)-L-homoserine lactone  
(also N-(3-oxohexanoyl)-L-homoserine lactone) |
| 3-oxo-C8-HSL | N-(3-oxo-octanoyl)-L-homoserine lactone |
| 3-oxo-C10-HSL| N-(3-oxo-decanoyl)-L-homoserine lactone |
| 3-oxo-C12-HSL| N-(3-oxo-dodecanoyl)-L-homoserine lactone |
| PLL          | Phosphotriesterase-like lactonase |
| PLL\textsubscript{Dr} | Phosphotriesterase-like lactonase from \textit{Deinococcus radiodurans} |
| PLL\textsubscript{Gk} | Phosphotriesterase-like lactonase from \textit{Geobacillus kaustophilus} |
| PTE\textsubscript{Ar} | Phosphotriesterase from \textit{Agrobacterium radiobacter} |
| PTE\textsubscript{Bd} | Phosphotriesterase from \textit{Brevundimonas diminuta} |
| RMSD         | Root mean squared deviation |
| SDS-PAGE     | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
CHAPTER 1: LITERATURE REVIEW

This thesis describes the study of two enzymes that are capable of degrading organophosphates (OPs); SsoPox from the thermophilic archaeon, *Sulfolobus solfataricus*, and the phosphotriesterase from the mesophilic soil bacterium, *Brevundimonas diminuta* (PTE<sub>Bd</sub>). SsoPox and PTE<sub>Bd</sub> are homologous enzymes and share approximately 32% amino acid sequence identity; however each exhibits a preference for different substrates. SsoPox is foremost a lactonase capable of hydrolyzing *N*-acyl homoserine lactones (AHLs) but possesses promiscuous phosphotriesterase activity that resembles that of PTE<sub>Bd</sub>. PTE<sub>Bd</sub> is capable of degrading organophosphate pesticides and chemical warfare agents with a specificity constant towards its best substrate, paraoxon, approaching the rate of diffusion (Dumas *et al*., 1989; Caldwell *et al*., 1991). Due to the high thermostability of SsoPox it has become the focus of many studies aiming to create novel biotechnological applications. With the help of recent structural information the catalytic mechanism and the basis for AHL acyl-chain length specificity of SsoPox will be explored in this thesis. The high efficiency and broad substrate specificity of PTE<sub>Bd</sub> is being investigated for its usefulness in the bioremediation of OP-contaminated environments and the protection of organisms poisoned by these toxic compounds. The immobilization of PTE<sub>Bd</sub> to paper for the purpose of creating an OP-detecting biosensor will be described.

1.1 Organophosphates

OPs belong to a class of highly toxic, synthetic compounds that are commonly used as pesticides, petroleum additives, plasticizers and, historically, chemical warfare agents (Raushel, 2002; Singh, 2009). OPs share a similar chemical structure (see Figure...
1.1) consisting of a phosphorus center bonded to an oxygen or sulphur atom (in oxon and thiol forms, respectively) and ester linkages to phenoxy or alkyl groups (Merone et al., 2005). The toxicity of these compounds (Table 1.1) arises from their ability to be efficiently absorbed through the skin, lungs and gastrointestinal tract where they can irreversibly inhibit acetylcholinesterase (Chambers and Levi, 1992; Costa, 2006). When active, acetylcholinesterase removes acetylcholine from synaptic nerve junctions by cleaving it into acetate and choline, thus regenerating the potential for signal transmission between neurons. OPs inhibit acetylcholinesterase activity by phosphorylating a critical serine residue that is required for catalysis. The build-up of acetylcholine in the human nervous system results in an acute cholinergic crisis characterized by convulsions, bronchoconstriction, salivation, bradycardia, behavioural incapacitation, muscle weakness, coma and ultimately death due to respiratory failure (Bunce, 1994; Lejeune et al., 1998).

More than one hundred OP pesticides are distributed worldwide (accounting for approximately 38% of total pesticide usage) and it is estimated that approximately 30,000 tonnes of OP pesticide are applied annually to crops in the United States alone (Singh, 2009). Despite the high toxicity of some OPs they are considered safer than precursors such as dichloro-diphenyl-trichloroethane (DDT), dieldrin and other organochlorines which can bioaccumulate in the environment and affect non-target organisms. OPs possess relatively short half-lives (Table 1.2) and are prone to photolysis as well as microbial assimilation which rapidly degrades them into products that are many orders of magnitude less toxic (Figure 1.2) (Ragnarsdottir, 2000). Despite these short theoretical half-lives the degradation of these compounds varies immensely as a function of sunlight
Figure 1.1 Structures of various organophosphate compounds. Organophosphates are composed of a phosphorous centre bound to an oxygen or sulphur atom, two alkoxy groups and one other substituent.
Table 1.1  LD$_{50}$ toxicity values for selected organophosphate compounds.  Data obtained from the Hazardous Substance Data Bank (http://toxnet.nlm.nih.gov/index.html).

<table>
<thead>
<tr>
<th>Compound</th>
<th>LD$_{50}$/Rat oral (mg/kg)</th>
<th>LD$_{50}$/Mouse oral (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organophosphate pesticides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>paraoxon</td>
<td>1.8</td>
<td>0.76</td>
</tr>
<tr>
<td>parathion</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>terbufos</td>
<td>2</td>
<td>5.4</td>
</tr>
<tr>
<td>azinphos-methyl</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>methyl-parathion</td>
<td>14</td>
<td>NA</td>
</tr>
<tr>
<td>phosmet</td>
<td>113</td>
<td>160</td>
</tr>
<tr>
<td>chlorpyrifos (Dursban)</td>
<td>151</td>
<td>152</td>
</tr>
<tr>
<td>coumaphos $^1$</td>
<td>$&gt; 240$</td>
<td>NA</td>
</tr>
<tr>
<td>malathion</td>
<td>5,843</td>
<td>4,059</td>
</tr>
<tr>
<td><strong>Organophosphate nerve agents</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VX $^2$</td>
<td>0.012</td>
<td>NA</td>
</tr>
<tr>
<td>sarin</td>
<td>0.55</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Organochlorines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dieldrin</td>
<td>38.3</td>
<td>38</td>
</tr>
<tr>
<td>DDT</td>
<td>100</td>
<td>150 - 300</td>
</tr>
</tbody>
</table>

$^1$ Data obtained from the U.S. Environmental Protection Agency (2006)

$^2$ O-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate

NA: No data available
Table 1.2  Comparison of the environmental half-lives of selected organophosphate compounds. Data reproduced from Singh (2006). Australian and United Kingdom soil samples were treated with OP pesticide solutions and incubated for 3 – 4 hours. The various OPs were solvent extracted and analyzed by HPLC. Half-life was estimated by linear regression of the logarithmic plot of concentration versus time of incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Half-life in soil (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chlorpyrifos</td>
<td>16 – 120</td>
</tr>
<tr>
<td>coumaphos</td>
<td>24 – 1,400</td>
</tr>
<tr>
<td>diazinon</td>
<td>11 - 21</td>
</tr>
<tr>
<td>dimethoate</td>
<td>2 - 41</td>
</tr>
<tr>
<td>glyphosphate</td>
<td>30 - 174</td>
</tr>
<tr>
<td>parathion</td>
<td>30 – 180</td>
</tr>
</tbody>
</table>
Figure 1.2 General reaction scheme for organophosphate hydrolysis by PTEBd and the degradation of paraoxon into less toxic products. (A) Organophosphates are degraded enzymatically by hydrolysis of the phosphoester bonds. (B) The degradation of paraoxon yields non-toxic diethyl phosphate and 500-fold less toxic p-nitrophenol. LD₅₀ values (mouse, oral) were obtained from the Hazardous Substance Data Bank (U.S. National Library of Medicine, 2011).
exposure, temperature, soil pH and microbial composition. As a result many terrestrial and aquatic ecosystems throughout the world suffer from OP contamination and their accumulation can severely impact populations of non-target organisms (Singh, 2009). For example, the annual death of hundreds of fish in freshwater streams on Prince Edward Island, Canada, is attributed to the run-off contamination of azinphos-methyl from crop fields (Hassall, 1990). A study in 1995 by Simcox et al. found that soil from agricultural properties in eastern Washington State were contaminated with OPs in the millimolar concentration range.

The presence of OPs in the environment has led to the development of sensitive detection techniques. Current methods involve washing large environmental samples with a solvent, such as perchloric acid or sulphuric acid-dissolved sodium bicarbonate, which is subsequently concentrated and analyzed by liquid or gas chromatography coupled with mass spectrometry to identify the contaminants present (U.S. Department of Health, 2001). This system is time-consuming, expensive and requires advanced equipment and trained professionals. Therefore a simple and affordable alternative for rapid OP detection would provide an enormous advantage to conservation and environmental bioremediation efforts.

In addition to their environmental hazards, OPs pose a danger in the industrial setting where they are synthesized and the ingestion of these compounds in suicide attempts is a global concern (Singh, 2009). Bird et al. (2008) have estimated that every year approximately 3,000,000 poisonings and 300,000 deaths can be attributed to OP ingestion. The safe disposal of these compounds is essential from an environmental and human health perspective. Common methods of disposal include incineration and
alkaline hydrolysis (Felsot et al., 2003) but these techniques are expensive and yield products that are damaging to the environment. As a result, biodegradation of OPs using microbial enzymes has become the focus of many studies.

1.1.1 Phosphotriesterase Activity of PTE_{Bd} and SsoPox

Enzymes capable of hydrolyzing the phosphoester bonds of OPs are found in several different organisms including *B. diminuta* (formerly *Pseudomonas diminuta*), various archaea, protozoa, clams, squids, hogs, rabbits, sheep, hamsters, locusts and humans (Main, 1960; Mehrotra et al., 1969; Geldmacher-von et al., 1973; Lenz et al., 1973; Whitehouse and Ecobichon, 1975; Zech and Wigand, 1975; Hoskin et al., 1984; Landis et al., 1987; Dumas et al., 1989; Merone et al., 2005). In general the best characterized and most active OP-degrading enzymes are found in bacteria and are referred to as phosphotriesterases, organophosphorus hydrolases, OP-degrading enzymes, paraoxonases or parathionases (Raushel, 2002; Merone et al., 2005). No natural substrate has been identified for these enzymes and they are believed to have emerged within the last 70 years in response to widespread OP pesticide use (Yang et al., 2003). Not all bacteria in possession of organophosphate-degrading enzymes are capable of utilizing the resulting products. The diversity within the microbial soil community is such that co-metabolic symbiosis likely ensures the continued assimilation of these products into the biosphere.

Among the first OP-degrading bacteria isolated from soil samples in the 1970s were *Flavobacterium* sp. ATCC 27551 and *B. diminuta* (Sethunathan and Yoshida, 1973; Munnecke and Hsieh, 1976). Despite being encoded by genes located on non-homologous vectors, the phosphotriesterases of *Flavobacterium* sp. and *B. diminuta* are
identical and will hereafter be referred to as PTE_{Bd} (Harper et al., 1988; McDaniel et al., 1988). PTE_{Bd} is the best characterized OP-hydrolyzing enzyme and is capable of degrading a broad spectrum of pesticides and nerve agents (Dumas et al., 1989). The best substrate for PTE_{Bd} is paraoxon towards which it exhibits a specificity constant of 4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} (Dumas et al., 1989; Caldwell et al., 1991).

Native PTE_{Bd} is membrane-associated and targeted to the periplasmic face of the inner membrane by the twin arginine translocation (Tat) pathway (Gorla et al., 2009). A 39 kDa enzyme is predicted from the encoding gene however purification yields a truncated 35 kDa protein (Mulbry and Karns, 1989). This truncation results from the post-translational cleavage of a 29 residue N-terminal signal sequence during Tat-assisted membrane association (Gorla et al., 2009). A crystal structure has been determined for PTE_{Bd} (PDB ID: 1HZY) (Benning et al., 1994).

The localization of genes encoding PTE_{Bd} on non-homologous vectors led evolutionary microbiologists to suggest that horizontal gene transfer played an important role in the distribution of OP-degrading enzymes (Siddavattam et al., 2003). Little else was known about the evolutionary origin of phosphotriesterases until the discovery of a chromosomal gene encoding SsoPox, an enzyme that is structurally and functionally similar to PTE_{Bd}, in the hyperthermophilic archaeon, \textit{S. solfataricus} (Merone et al., 2005). \textit{S. solfataricus} possesses an optimal growth temperature of 80°C (Merone et al., 2005) and therefore must utilize highly thermostable enzymes for survival. Merone et al. (2005) have previously determined the half-life of SsoPox to be 4 hours at 95°C and 30 minutes at 100°C. The thermostability of SsoPox makes it an attractive candidate in biotechnology research since it implies resistance against other harsh conditions such as...
the presence of solvents, detergents, and proteases (Jaenicke and Bohm, 1998). At 70°C SsoPox exhibits a specificity constant of $4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for its best OP substrate, paraoxon, which is 4 orders of magnitude lower than that of PTE$_{Bd}$ (Caldwell et al., 1991; Merone et al., 2005).

1.2 SsoPox Lactonase Activity

The reason SsoPox exhibits such low paraoxonase activity is because its primary enzymatic function is not as a phosphotriesterase but rather as a lactonase. Lactonase activity occurs in the same active site as phosphotriesterase activity but exploits the unique structural features of SsoPox to bind lactone substrates (Elias et al., 2008). These features will be described in more detail in Section 1.5. Lactonases are enzymes that hydrolyze lactone compounds by cleaving the lactone ring (Figure 1.3). SsoPox possesses a broad substrate specificity for AHLs and exhibits the highest specificity constant, $3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, towards $N$-(3-oxo-decanoyl)-homoserine lactone (3-oxo-C10-HSL). AHLs are clinically relevant because they act as quorum sensing molecules in some bacteria and can regulate the activation of virulence factors. Therefore lactonases may attenuate this process and prohibit these cellular processes from occurring. Quorum sensing will be discussed in more detail in Section 1.2.1.

The evolutionary relevance of AHL hydrolysis by SsoPox remains to be seen given that $S. solfataricus$ dwells in boiling water springs where lactones cannot exist stably. Quorum sensing-like systems have been observed in archaea. For example the release of extracellular proteases by $Natronococcus occultus$ has been linked to the production of autoinducing acylated homoserine lactones (Paggi et al., 2003).
Figure 1.3 General reaction scheme for AHL hydrolysis by SsoPox. Enzymatic AHL hydrolysis results in the opening of the lactone ring.
Conversely, studies looking to substantiate true quorum sensing systems in archaea have failed to do so. An investigation into the formation of biofilms by the acidophile, *Ferroplasma acidarmanus*, failed to identify any signalling molecules, including AHLs, but instead found that biofilm formation is likely initiated in response to anaerobic growth conditions (Baker-Austin *et al.*, 2010). To date no quorum-related pathway involving AHLs has been confirmed in archaea.

### 1.2.1 *N*-acyl Homoserine Lactones and Quorum Quenching

Many bacteria sense their population density through a sophisticated cell-to-cell communication system that elicits the expression of specific genes when the population density reaches a critical threshold. This so-called quorum sensing pathway can exert transcriptional control over a variety of biological functions, including virulence and biofilm formation, and thus plays an important role in bacterial pathogenesis (Dong *et al.*, 2007). Quorum sensing relies on extracellular signalling molecules such as AHLs, fatty acid derivatives and oligopeptides to carry out this intercellular cross-talk (Dong *et al.*, 2007). AHL-mediated quorum sensing is one of the best understood mechanisms of intercellular communication and over 70 bacterial species have been identified that produce AHL-type autoinducers (Dong *et al.*, 2007). For instance, the quorum sensing mechanism of *Vibrio fischeri*, a bacterium that has proven invaluable to our understanding of quorum sensing systems, utilizes *N*-(3-oxohexanoyl)-homoserine lactone (3-oxo-C6-HSL) and *N*-(3-oxooctanoyl)-homoserine lactone (3-oxo-C8-HSL) along with the LuxI/LuxR family of transcription factors to elicit the expression of genes involved in bioluminescence. More clinically relevant examples of AHLs as quorum signalling molecules are *N*-(3-oxododecanoyl)-homoserine lactone (3-oxo-C12-HSL) and
$N$-butyryl-homoserine lactone (C4-HSL) which activate the lasR/I and rhlR/I transcription systems, respectively, in \textit{Pseudomonas aeruginosa}. \textit{P. aeruginosa} is an opportunistic pathogen capable of colonizing solid surfaces and causing dangerous nosocomial infections (Hidron \textit{et al}., 2008; Kerr and Snelling, 2009). Due to increasing antimicrobial resistance and its ability to form protective biofilms, \textit{P. aeruginosa} is a serious concern within the healthcare sector (Hidron \textit{et al}., 2008; Tart and Wozniak, 2008). Together, 3-oxo-C12-HSL and C4-HSL control the pathogenicity of \textit{P. aeruginosa} by prompting the expression of genes encoding virulence factors which subsequently harm host cells and inactivate host proteins during infection. It is interesting to note that the lasR/I and rhlR/I system is capable of compensating for the loss of either of the two AHL signals (Latifi \textit{et al}., 1996; Dekimpe and Deziel, 2009). This illustrates the importance of disabling multiple signalling molecules to fully inhibit quorum sensing.

Disruption of this intercellular communication with the aim of reducing quorum-related expression of virulence factors is referred to as quorum quenching and can be achieved through the hydrolysis of signalling molecules by specific hydrolases. For example, AHL acylases can be employed to hydrolyze AHL autoinducers into the appropriate fatty acid and homoserine lactone (Leadbetter and Greenberg, 2000; Huang \textit{et al}., 2003; Lin \textit{et al}., 2003; Park \textit{et al}., 2005). A recent study by Ng \textit{et al}. (Ng \textit{et al}., 2011) demonstrated that membrane-immobilized SsoPox significantly reduced the production of quorum-related virulence factors, elastase, protease and pyocyanin, when present in a culture of \textit{P. aeruginosa}. Quorum quenching is an attractive alternative to biocides as a method of biocontrol since the development of bacterial resistance is less
likely to occur as less selective pressure is exerted on the bacterial population (Mullard, 2009).

1.3 General 3D Structures of PTE\textsubscript{Bd} and SsoPox

SsoPox and PTE\textsubscript{Bd} are structurally similar and are both classified in the amidohydrolase superfamily of enzymes. Enzymes in this family possess the same (β/α)\textsubscript{8} structural motif containing a mono- or binuclear metal center and are capable of catalyzing a variety of different reactions. Examples of amidohydrolases include phosphotriesterase, urease, adenosine deaminase, renal dipeptidase, dihydroorotase and various hydantoinases (Seibert and Rauschel, 2005).

PTE\textsubscript{Bd} and SsoPox are homodimeric with each protomer resembling a distorted TIM-barrel (β/α)\textsubscript{8} structure with a binuclear metal center (Figure 1.4) (Benning \textit{et al.}, 1994). The α-carbon atoms of PTE\textsubscript{Bd} and SsoPox overlap reasonably well with a root mean squared deviation (RMSD) of 1.05 Å (Elias \textit{et al.}, 2008). The active site of each protomer is not associated with the dimer interface therefore each protomer is thought to possess the capacity for OP hydrolysis. Both PTE\textsubscript{Bd} and SsoPox possess unique active site topology (Figure 1.5) which will be discussed in more detail in Section 1.6.

The presence of metal cofactors in the active site is crucial for enzyme activity. Omburo \textit{et al.} (1992) reported that the two Zn\textsuperscript{2+} present in native PTE\textsubscript{Bd} protomers can be substituted with Co\textsuperscript{2+}, Ni\textsuperscript{2+}, Cd\textsuperscript{2+} and Mn\textsuperscript{2+} to yield active enzyme, however Co\textsuperscript{2+} appears best for activity and has been shown to improve thermostability (Rochu \textit{et al.}, 2002; Yang \textit{et al.}, 2003; Rochu \textit{et al.}, 2004). The metal ions are designated α and β, indicating the more buried and solvent-exposed cations, respectively. The crystal structure of SsoPox reveals each protomer to contain Fe\textsuperscript{3+} and Co\textsuperscript{2+} in the α and β
Figure 1.4 Protomeric and dimeric structure of PTE$_{Bd}$ and SsoPox. (A) The protomer of PTE$_{Bd}$, shown on left as a forest green cartoon, is a distorted (β/α)$_8$-barrel with a binuclear metal centre (shown as red spheres). The surface representation of dimeric PTE$_{Bd}$ is shown on the right from the same perspective as the protomer. (B) The protomer of SsoPox is shown in bright orange on left as a cartoon with its binuclear metal centre represented by red spheres. SsoPox dimer is shown on the right. Image generated using PyMOL (Delano, 2002).
Figure 1.5  Important differences in the active site topology of PTE<sub>Bd</sub> and SsoPox.

Several conserved residues contribute to the active site topology of PTE<sub>Bd</sub> and SsoPox but key differences contribute to significant functional disparity. (A) PTE<sub>Bd</sub> co-crystallized (PDB ID: 3CAK) in the presence of diethyl phosphate, a product of paraoxon hydrolysis. Specific residues are highlighted to illustrate important differences with SsoPox. (B) SsoPox co-crystallized with C10-HTL, an inhibitory lactone mimic. This panel illustrates residues corresponding with those shown in Panel A and their contribution to active site topology. This thesis explores the possible importance of Arg-223 and Tyr-97 in stabilization of the negative reaction intermediate formed during AHL turnover in SsoPox. Image generated using PyMOL (Delano, 2002).
positions, respectively, however for catalysis the $\alpha$-cation (iron) likely possesses an oxidation state of +2 instead of +3 (Elias et al., 2008). Like PTE$_{Bd}$, metal incorporation studies have been performed with SsoPox and Cd$^{2+}$ was found to be the optimal cation for both phosphotriesterase and AHL lactonase activity (Ng, 2009). Six residues are in direct coordination with the cations in the active site of both enzymes (Figure 1.6). In PTE$_{Bd}$ H55, H57 and D301 coordinate the $\alpha$-cation, H201 and H230 are complexed with the $\beta$-cation and carboxylated K169 bridges both metals. The crystal structure of PTE$_{Bd}$ also reveals the presence of a bridging water molecule which is important in catalysis (described in Section 1.4). The ligands in coordination with the divalent metal center of SsoPox are identical to those of PTE$_{Bd}$: H22, H24 and D256 complex with the $\alpha$-cation, H170 and H199 interact with the $\beta$-cation and bridging both metals are carboxylated K137 and a catalytic water molecule.

The most prominent structural differences between SsoPox and PTE$_{Bd}$ are the absence of a 15 residue segment in SsoPox that corresponds to loop 7 of PTE$_{Bd}$, elongation of loop 8 in SsoPox and the truncation of SsoPox at the N- and C-termini (Figure 1.7). The lengthened loop 8 of SsoPox is close to the active site and forms a tunnel which is believed to be important for AHL binding (Elias et al., 2008). This tunnel is lined primarily by hydrophobic residues: L228, L226, W278, T265, A275, L274 and F229. The acyl-chain of the lactone mimic, decanoyl homoserine thiolactone (C10-HTL), co-crystallized with SsoPox (PDB ID: 2VC7) fits perfectly into the tunnel (Figure 1.8).

SsoPox possesses several unique structural features which may contribute to its high thermostability. For instance, it contains numerous amino acids that are involved in
Figure 1.6  Comparison of the ligand geometry of the PTE$_{Bd}$ and SsoPox binuclear core. Residues important in cation coordination are shown in PTE$_{Bd}$ (A) and SsoPox (B). Although it is not represented in the crystal structure, the metal-bridging Lys-169 of PTE$_{Bd}$ is believed to be carboxylated similar to Lys-137 of SsoPox. Image generated using PyMOL (Delano, 2002).
Figure 1.7 Superposition of $\text{PTE}_{\text{Bd}}$ and SsPox highlighting structural differences. $\text{PTE}_{\text{Bd}}$ is shown in forest green and SsPox in bright orange. SsPox exhibits truncated N- and C-termini, loss of a loop that corresponds to loop 7 in $\text{PTE}_{\text{Bd}}$, and an elongated loop 8 which forms a hydrophobic binding tunnel adjacent to the active site. Image generated using PyMOL (Delano, 2002).
Figure 1.8  Structure of the hydrophobic binding tunnel of SsoPox. Loop 8 of SsoPox forms a large hydrophobic tunnel adjacent to the metal-containing active site (cations shown as red spheres) that accommodates the acyl-chain of C10-HTL (shown as grey sticks) perfectly. Image generated using PyMOL (Delano, 2002).
the formation of salt bridges including N- and C-terminal residues (Elias et al., 2008). The ionic interactions involved in salt bridging are favourable at high temperatures and help stabilize protein structure (Das and Gerstein, 2000; Szilagyi and Zavodszky, 2000). Half of the 76 charged surface residues of SsoPox participate in salt bridging and contribute significantly to enzyme thermostability. The importance of salt bridging in SsoPox is further illustrated by Del Vecchio et al. (2009) who identified salt bridge networks in which as many as seven residues participate in charge-charge interactions. Another determinant of thermostability in SsoPox is the increased surface area of contact at the dimer interface (Del Vecchio et al., 2009).

1.4 Proposed Catalytic Mechanism for Organophosphate Hydrolysis by SsoPox and PTE_{Bd}

A model for OP hydrolysis has been proposed (Wong and Gao, 2007; Elias et al., 2008) and is illustrated in Figure 1.9. Upon substrate binding, OP hydrolysis proceeds via an S_{N2}-like mechanism in which the bi-metallic center activates the bridging water molecule to its catalytic hydroxide form. The activated hydroxide carries out nucleophilic attack on the phosphorus center of bound OP substrate to form a penta-coordinated transition state that bridges the two metals while Co^{2+} stabilizes the developing negative charge on the phosphoryl oxygen. The bond between the phosphorus center and leaving group is broken, leaving the intermediate coordinated to both metals. Finally the phosphorus moiety is expelled and its displacement is coupled with the regeneration of a catalytic water molecule.
Figure 1.9 Proposed catalytic mechanism of organophosphate hydrolysis by SsoPox and PTE\textsubscript{Bd}. In the paraoxonase reaction a catalytic water molecule is activated to hydroxide by the bimetallic centre. The activated nucleophile attacks the phosphoryl centre of bound OP via an S\textsubscript{N}2-like mechanism in which the negatively charged intermediate is stabilized by both divalent metal ions. The bond between the phosphate and leaving group is broken leaving the intermediate coordinated to both metals. Expulsion of the phosphorus moiety allows for enzymatic regeneration via uptake of a catalytic water molecule from the bulk solvent.
1.5 Proposed Catalytic Mechanism for AHL Hydrolysis by SsoPox

SsoPox has been co-crystallized with a lactone mimic, C10-HTL (Elias et al., 2008), for which the enzyme has no activity. This structure led Elias et al. (2008) to propose a catalytic mechanism for lactonase activity in SsoPox. Recently, Chow et al. (2010) have proposed minor additions to the reaction scheme which are included below. The catalytic mechanism of AHL hydrolysis is illustrated in Figure 1.10.

Substrate binding is facilitated by the interaction of the acyl-chain with the hydrophobic binding tunnel adjacent to the active site. The head group is positioned above the binuclear center and it is believed that the planar face is properly oriented through interactions between Y97 and the carbonyl oxygen of the lactone ring. The bimetallic center activates a bridging molecule to a catalytic hydroxide ion which performs nucleophilic attack on the polarized carbonyl bond, forming a tetrahedral intermediate which is stabilized by the β-cation. The electron pair from the carbonyl oxygen is transferred back to the carbonyl carbon, cleaving the lactone ring. Finally, substrate is released and regeneration of the catalytic water molecule takes place.

Due to the conservation of active site tyrosines among related lactonases with promiscuous phosphotriesterase activity and lactonases from the metallo-β-lactamase family, Elias et al. (2008) postulated that Y97 is critical for lactonase activity. Merone et al. (2010) generated a Y97W variant that exhibited comparable lactonase activity to wild type however no further investigation into the importance of this residue has been reported.
Figure 1.10  Proposed catalytic mechanism of AHL hydrolysis by SsoPox. For the lactonase reaction a water molecule is activated to a hydroxide which carries out a nucleophilic attack on the polarized carbonyl bond of bound lactone substrate. The electron pair of the carbonyl oxygen are reclaimed by the carbon, opening the lactone ring. The negatively charged intermediate is stabilized by the divalent metal ions and a proton donor. The identity of the proton donor is still under investigation.
1.6 Investigation of the Catalytic Differences Between \( \text{PTE}_{\text{Bd}} \) and \( \text{SsoPox} \)

\( \text{PTE}_{\text{Bd}} \) is capable of degrading a broad range of OPs, including parathion, coumaphos, diazinon, cyanophos and chlorpyrifos (known commercially as Dursban) albeit with lower efficiency than paraoxon (Dumas et al., 1989). This wide substrate specificity has made \( \text{PTE}_{\text{Bd}} \) the focus of many site-directed mutagenesis and directed evolution studies aimed at modifying its specificity to include other OP substrates, such as chlorpyrifos (Cho et al., 2004) and nerve agents such as sarin, soman and VX (Reeves et al., 2008). \( \text{SsoPox} \) and \( \text{PTE}_{\text{Bd}} \) share approximately 32% sequence identity and possess significant structural similarities therefore \( \text{PTE}_{\text{Bd}} \) may be a useful paradigm for studies on \( \text{SsoPox} \).

The proposed catalytic mechanisms described in Section 1.4 and Section 1.5 both involve the activation of a water molecule to a nucleophilic hydroxide but the proton shuttling mechanism that accompanies this process is not well understood. Aubert et al. (2004) initially proposed that D301, which coordinates the \( \alpha \)-cation and catalytic water molecule in \( \text{PTE}_{\text{Bd}} \), forms a hydrogen bond network with H254 and D233 to shuttle protons from the active site into the bulk solvent. An H254R variant constructed in a site-directed mutagenesis study by DiSioudi et al. (1999) resulted in a 3-fold reduction in \( k_{\text{cat}}/K_M \) towards paraoxon but increased specificity towards demeton S. They proposed that H254 is important in defining active site structure and that H254R imparts flexibility to the active site enabling the enzyme to accommodate large substrates such as demeton S. Conversely, a directed evolution study by Roodvelt and Tawfik (2005) led to an H254R \( \text{PTE}_{\text{Bd}} \) variant and possessing a 2-fold higher specificity for paraoxon. If this amino acid played an important role in catalysis we would expect to observe more
consistent and dramatic changes in the catalytic parameters of these variants. Furthermore, histidine is not strictly conserved among related phosphotriesterases. For example, the phosphotriesterase of *Agrobacterium radiobacter* (PTE$_{Ar}$) possesses an arginine (R254) in the position corresponding to H254 in PTE$_{Bd}$. Despite this difference, PTE$_{Ar}$ exhibits a specificity constant towards parathion that is 7-fold higher than that of PTE$_{Bd}$. In addition, substitution of arginine with a histidine in PTE$_{Ar}$ does not alter this value (Horne *et al.*, 2006). When R223 of SsoPox, corresponding to H254 in PTE$_{Bd}$, was substituted with a histidine, Ng (2009) and Elias *et al.* (2008) separately reported a reduction in paraoxon specificity of at least two orders of magnitude. If histidine were important for proton shuttling we would expect to see a reduction in paraoxonase activity in the H254R PTE$_{Bd}$ variant and an increase in paraoxonase activity in the R254H and R223H variants of PTE$_{Ar}$ and SsoPox, respectively. Since these substitutions did not change the activity of PTE$_{Bd}$ or PTE$_{Ar}$ and resulted in a dramatic reduction in the activity of SsoPox, we surmise that H254 is not essential for proton shuttling during paraoxon hydrolysis.

So far we have only described the phosphotriesterase activity of PTE$_{Bd}$; however it has been shown to possess lactonase activity. Roodveldt *et al.* (2005) demonstrated that PTE$_{Bd}$ is capable of hydrolyzing 5-thioethyl-$\gamma$-butyrolactone with a $k_{cat}/K_M$ of 1.1 x $10^3$ M$^{-1}$ s$^{-1}$. The lactonase mechanism of PTE$_{Bd}$ remains unknown. An H254R variant increased the specificity constant of PTE$_{Bd}$ for 5-thioethyl-$\gamma$-butyrolactone by 33-fold (Roodveldt and Tawfik, 2005) while an R223H SsoPox variant reduced efficiency towards the same substrate by 200-fold (Elias *et al.*, 2008). The R223H variant of SsoPox also exhibited a 20-fold reduction in the $k_{cat}/K_M$ value towards 3-oxo-C8-HSL.
and 3-oxo-C10-HSL (Ng, 2009). These results suggest that arginine in this position may be important for lactonase activity.

1.7 Phosphotriesterase-like Lactonases

The amidohydrolase superfamily is known to contain enzymes which possess promiscuous secondary activities. A good example is dihydroorotase, which catalyzes the conversion of carbamoyl aspartate to dihydroorotate but also has weak paraoxonase activity (Roodveldt and Tawfik, 2005). Similarly, the ability of PTE<sub>Bd</sub> to hydrolyze lactones was just discussed (Roodveldt and Tawfik, 2005) and SsoPox functions foremost as a lactonase but exhibits promiscuous phosphotriesterase activity (Afriat et al., 2006). The structural similarities between SsoPox and several other AHL lactonases with promiscuous phosphotriesterase activity have led Afriat et al. (2006) to propose the creation of a subgroup of enzymes denoted phosphotriesterase-like lactonases (PLLs).

PLLs have been identified in several mesophilic organisms (Mycobacterium avium subsp. paratuberculosis, Mycobacterium tuberculosis, Rhodococcus erythropolis, Deinococcus radiodurans) as well as thermophiles (Geobacillus stearothermophilus, Geobacillus kaustophilus, and Sulfolobus islandicus) and even an acidophile (Sulfolobus acidocaldarius) (Afriat et al., 2006; Porzio et al., 2007; Chow et al., 2009; Hawwa et al., 2009; Xiang et al., 2009; Chow et al., 2010; Gotthard et al., 2011). These proteins share approximately 30% identity (Figure 1.11) and superimpose with an RMSD of less than 2.0 Å. Like SsoPox, each of these enzymes requires divalent metal cations to function. It is proposed that investigation of these enzymes may provide clues as to the evolutionary origin of the phosphotriesterase activity. The high activity and broad substrate specificity of PTE<sub>Bd</sub> is believed to have evolved within the last 70 years from a pre-existing
Figure 1.11 Sequence alignment of SsoPox, \( \text{PTE}_\text{Bd} \) and various phosphotriesterase-like lactonases. Amino acids highlighted in blue are conserved active site residues involved in the coordination of the binuclear metal centre. Residues corresponding with R223, Y97 and Y99 in SsoPox are highlighted in red. Included are the sequences of AhlA from \( R. \text{erythropolis} \) (PLL_Re), Dr0930 of \( D. \text{radiodurans} \) (PLL_Dr), GKL of \( G. \text{kaustophilus} \) (PLL_Gk) and \( \text{PTE}_\text{Ar} \) of \( A. \text{radiobacter} \) (PLL_Ar).
hydrolase (Dumas et al., 1989; Scanlan and Reid, 1995; Buchbinder et al., 1998; Raushel and Holden, 2000). PLLs like SsoPox provide a tangible link to PTE_{Bd} and it has been suggested that evolution from a lactonase to a potent paraoxonase could occur in as short as several years (Merone et al., 2008). Current understanding of the evolutionary origin of SsoPox and related PLLs remains incomplete.

Creation of this classification has allowed researchers to investigate individual PLLs while contributing to the general understanding of closely related enzymes. For example, a study performed in 2009 examined the efficacy of loop swapping and loop-specific residue substitution between PTE_{Bd} and the PLL of D. radiodurans (PLL_{Dr}) which exhibits very low paraoxonase activity with a $k_{cat}/K_M$ of 0.8 M$^{-1}$ s$^{-1}$ (Xiang et al., 2009). The binuclear metal centers of PLL_{Dr} and PTE_{Bd} are essentially identical but major differences are found within the composition and conformation of their eight respective $\beta\alpha$ loops. Production of a chimeric protein in which the $\beta\alpha$ loops close to the active site of PLL_{Dr} were replaced with the corresponding loops of PTE_{Bd} failed to bestow heightened paraoxonase activity and also resulted in the expression of largely insoluble protein (Xiang et al., 2009). This indicates that the inherent structural information embedded within the loops of PTE_{Bd} is insufficient to bestow activity to related enzymes. In a second experiment, loop residues responsible for forming the substrate-binding pocket of PTE_{Bd} were substituted into corresponding positions in PLL_{Dr} and resulted in a 16-fold increase in paraoxonase activity. This demonstrates the importance of hypothesis-driven experimentation in the investigation of PLLs.

A recent directed evolution study characterized variants of the PLL from G. kaustophilus (PLL_{Gk}). Chow et al. (2010) sought to analyze the importance of three
residues, Y99, E101 and R230, corresponding to Y97, Y99 and R223 in SsoPox, for lactonase activity. An E101N/R230I double mutant exhibited a 72-fold increase in $k_{\text{cat}}/K_M$ towards 3-oxo-C12-HSL and possessed lactonase activity towards C4-HSL - a substrate that cannot be hydrolyzed by the wild type enzyme. Chow et al. (2010) also observed changes in the colour of PLL$_{Gk}$ when variants were concentrated to 50 mg/mL. For example wild type PLL$_{Gk}$ is purple at high concentrations but the aforementioned E101N/R230I variant appears brown. They attributed these observations to the modulation of a charge-transfer complex established between Y99 and the β-cation. Furthermore they suggested that the residue in position 101 may dictate the positioning of the stabilizing tyrosine, thus indirectly influencing active site geometry. This tyrosine is highly conserved among PLLs and the group is currently formulating a structural explanation for these observations.

1.8 Protein Immobilization

One of the aims of this thesis is the immobilization of PTE$_{Bd}$, therefore protein immobilization will be briefly reviewed here. Protein immobilization techniques have been extensively studied and are important for the development of new biotechnologies. Examples of potential applications include sensors capable of detecting the presence of a particular analyte and the construction of continuous flow bioreactors that either break down contaminants or carry out reactions to produce specific products (Cabana et al., 2009; Tee and Kaletunc, 2009; Zhao et al., 2009). There are three principal methods of protein immobilization - covalent linkage, entrapment and non-covalent interactions – and each has its strengths and weaknesses. Covalent modification, for example, involves the introduction of reactive groups to a compound that are capable of covalently bonding.
the appropriate substituents of a targeted protein. There is no stronger method of protein
immobilization than covalent linkage and theoretically it can be applied to any protein,
however, this technique involves harsh chemical treatments which can disrupt enzyme
activity and the orientation of immobilized protein may render a significant proportion
inactive. Entrapment methods involve the polymerization of a porous material around
active enzyme. While entrapment does not require enzyme modification and can be used
to immobilize virtually any enzyme, the conditions involved in polymerization may be
denaturing and matrix composition may impede or even prohibit analyte diffusion.

This thesis involves the expression of protein for the purpose of immobilization
using two separate ionic, or non-covalent, immobilization techniques. Non-covalent
immobilization does not involve harsh chemical treatments or polymerization conditions
and can be accomplished by generating recombinant proteins containing affinity tags.
For example, a cellulose-binding domain (CBD) can be fused to the N- or C-terminus of
the target protein for affinity-based adsorption to cellulosic materials (Liu et al., 2009;
Kyla-Nikkila et al., 2010; Velikodvorskaya et al., 2010). Not only does the presence of a
CBD allow for selective immobilization onto a variety of cellulose substrates but it
creates the potential for a single-step purification method in which CBD-tagged protein is
purified from a heterogeneous protein mixture via elution through a cellulose matrix
(Ong et al., 1993). The primary disadvantage of non-covalent immobilization is enzyme
loss due to leaching. Ionic or hydrophobic protein-substrate interactions are affected by
storage factors such as temperature, pH and ionic strength (Taylor, 1991) therefore
dissociation is difficult to avoid. Another disadvantage of this technique, specifically
with respect to a fused affinity module, is inactivation of the protein or insolubility due to
improper folding in the presence of an exogenous domain. Richins et al. (2000) observed that a PTE$^{Bd}$ fusion protein containing the CBD of cellulose-binding protein A, from Clostridium cellulovorans, at the C-terminus exhibited no enzyme activity whereas an N-terminally fused variant was unaffected. Immobilization using CBD-enzyme fusions is appealing for a number of reasons. First, cellulose-based materials are relatively inexpensive and exist in a variety of different forms including fibres, powders and papers. Second, cellulose substrate has a low affinity for nonspecific protein binding (Levy and Shoseyov, 2002). Finally, CBD-protein constructs can be expressed in common hosts such as E. coli (Tomme et al., 1996).

A recent technology called nano alumina, manufactured by the Argonide Corporation in Sanford, Florida (Tepper and Kaledin, 2007), holds significant potential in the development of protein-immobilized biotechnologies. Nano alumina is a positively charged matrix of aluminum oxide nanoparticles electroadhesively bound to microglass fiber (Tepper and Kaledin, 2007). Nano alumina has traditionally been employed to filter water and is capable of the high retention of submicron particulates including proteins, viral particles and even arsenic (Tepper and Kaledin, 2007; Karim et al., 2009; Tepper, 2009; Gibbons et al., 2010). The Ahlstrom Corporation has recently obtained the rights to manufacture a nano alumina-cellulose amalgam under the brand name Disruptor™. Disruptor™ is a positively charged paper capable of strongly binding proteins through electrostatic interactions with negatively charged surface groups. Ng et al. (2011) first used Disruptor™ for the immobilization of SsoPox in quorum-quenching assays and reported a protein immobilization efficiency of 95%.
1.8.1 Organophosphate Biosensors

One of the many practical applications of immobilized proteins is the creation of a membrane-based biosensor that can detect the presence of a particular compound. For instance, immobilization of PTE_Bd or acetylcholinesterase on paper can create a litmus-like OP-detecting system. The inconvenience of current OP detection methods has been mentioned previously and biosensors may enable us to develop faster, more affordable, single-step systems.

OP biosensors have been successfully created using PTE_Bd (Lei et al., 2005) or acetylcholinesterase (Hossain et al., 2009) as the active component. PTE_Bd-immobilized biosensors allow for the direct detection of OPs whereas acetylcholinesterase-immobilized sensors work by inhibition. Acetylcholinesterase is commercially available and the sensors that have been constructed exhibit remarkably low detection thresholds, however this method is not without its disadvantages. There are numerous acetylcholinesterase inhibitors in nature, for example fungal aflatoxins or carbamate-based insecticides, which can elicit false positives on acetylcholinesterase-dependent sensors. These compounds can also irreversibly bind the enzyme limiting a sensor to one treatment. Liu and Lin (2006) created an OP sensor by immobilizing acetylcholinesterase to a carbon nanotube. In the absence of inhibitors, acetylcholinesterase hydrolyzes acetylthiocholine to produce acetate and thiocholine. Thiocholine is an electroactive species and generates an amperometric response. In the presence of an inhibitor, no thiolactone would be produced and no signal would be detected. The lowest detection threshold of this sensor for paraoxon is 0.4 pM.
The lowest detection threshold found for a PTE_{Bd}-immobilized biosensor was reported by Lei et al. (2005). This sensor involved the adsorption of engineered *Pseudomonas putida* cells expressing PTE_{Bd} on their surface to a membrane wrapped around a carbon paste electrode. Similar to the acetylcholine-based sensor just described, this method relies on the generation of an amperometric signal. OPs are degraded by PTE_{Bd} into *p*-nitrophenol which is subsequently hydrolyzed into the electroactive compounds, benzoquinone and hydroquinone, by native *P. putida* enzymes. The presence of these species triggers an amperometric signal that can detect paraoxon at concentrations as low as 1 nM (Lei et al., 2005). Although these electronic signal-based sensors are highly sensitive, they require trained professionals to operate and are not easily deployable in the field. Hossain et al. (2009) have developed a highly sensitive colorimetric sensor by coupling acetylcholinesterase and indophenyl acetate in a paper-based bilateral flow system. This system relies on the inhibition of acetylcholinesterase to prevent hydrolysis of indophenyl acetate into indophenoxide, a blue product. The absence of blue colour indicates the presence of inhibitor and paraoxon concentrations as low as 1 nM can be detected (Hossain et al., 2009). Although more practical for field use, this sensor remains non-specific due to its dependence on acetylcholinesterase.

An inexpensive device with a long shelf-life that is easily deployed in the field by nonprofessionals would be beneficial to public health and environmental protection efforts. PTE_{Bd} possesses broad substrate specificity and is the most active OP-degrading enzyme known. Despite limitations to its thermal stability, this protein has a reported half-life of 3 months when covalently immobilized to polyurethane foam (Lejeune and
Russell, 1996) and Richins et al. (2000) observed an activity loss of only 15% in immobilized CBD-PTE_{Bd} after 30 days.

1.9 Project Overview

The aim of this thesis is two fold:

1) to better understand the substrate specificity and catalytic mechanism of SsoPox

2) to explore the potential of PTE_{Bd} in the development of a biosensor for the detection of OPs

With respect to SsoPox, this thesis focuses on four residues. Residue R223 of SsoPox was previously implicated in the shuttling of protons away from the active site during paraoxonase catalysis. This hypothesis has been ruled out by contradictory evidence from several research groups studying corresponding residues in PTE_{Bd} and various PLLs however the importance of this residue in AHL lactonase function has not been explored in depth. The crystal structure of SsoPox complexed with C10-HTL suggests that R223 may be capable of stabilizing the negative reaction intermediate formed during AHL lactonase activity. This role is currently attributed to Y97 which is also believed to assist in AHL binding by positioning the lactone ring in a catalytically productive orientation prior to turnover. The neighbouring residue, Y99, has also been suggested to play an indirect role in substrate binding through shared hydrogen bond interactions with Y97. SsoPox possesses a hydrophobic binding tunnel adjacent to the active site that is capable of accommodating the acyl-chain of AHL substrates. SsoPox
has been found to exhibit a preference for AHLs with longer acyl-chains (Ng, 2009). Residue L226, located near the tunnel mouth, contributes to tunnel structure and is oriented in such a way that it may be modified to alter substrate specificity.

To achieve the objectives outlined above, the substrate specificity of SsoPox variants R223K, R223Q, Y97F, Y99F, Y97F/Y99F and L226F were determined for both the paraoxonase and AHL lactonase reactions. We hypothesized that substitution of R223 with a similar amino acid, lysine, would yield no change in either activity. Conversely, substitution with a shorter, neutral residue, glutamine, was expected to reduce both paraoxonase and AHL lactonase activity. We also hypothesized that removal of the hydroxyl group of Y97 via phenylalanine substitution would significantly reduce lactonase activity but that Y99F would exhibit a less drastic effect. Finally we sought to simultaneously increase and decrease the substrate specificity of SsoPox for short- and long-chain AHLs, respectively, by introducing a bulky L226F substitution into the proposed hydrophobic binding tunnel adjacent to the active site.

PTE\textsubscript{Bd} possesses high catalytic activities for a broad range of OPs and could be harnessed for biotechnological applications. The project was funded by the Sentinel Bioactive Paper Network, a Canadian consortium that seeks to create high-value cellulose-based products, therefore PTE\textsubscript{Bd} was immobilized to paper substrate in order to generate a biosensor for the detection of OPs.
CHAPTER 2: MATERIALS AND METHODS

All components of solutions, buffers and media mentioned in this section are listed in Appendix I.

2.1 Chemicals

All OPs, N-acyl homoserine lactones and m-cresol purple were purchased from Sigma-Aldrich (Oakville, ON, Canada) or Cayman Chemical (Ann Arbor, MI, USA). Restriction enzymes and T4 DNA ligase were obtained from Invitrogen (Burlington, ON, Canada) or New England BioLabs (Pickering, ON, Canada) with the exception of FastDigest restriction enzymes which were acquired from Fermentas Life Sciences (Burlington, ON, Canada). All other chemicals were analytical grade and obtained from Sigma-Aldrich and Fisher Scientific (Nepean, ON, Canada).

2.2 Bacterial Strains and Plasmids

*E. coli* BL21 (λDE3) and *P. putida* KT2442 were grown in Luria-Bertani (LB) broth. pT7-7 (Studier and Moffatt, 1986) was used as a cloning vector and pBTLT7 and pVLT-31 (De Lorenzo *et al.*, 1993) were used for expression in *E. coli* BL21 (λDE3) and *P. putida* KT2442, respectively. pBTLT7 is a derivative of the broad host range vector, pBTL4 (Lynch and Gill, 2006) containing the T7 promoter and RBS (ribosomal binding sequence) from pT7-7 (Baker *et al.*, 2009). Strains containing pT7-7 were cultured in media containing 100 µg/mL ampicillin and those containing pBTLT7 or pVLT-31 were grown in 15 µg/ml tetracycline.
2.3 DNA Manipulation

2.3.1 Plasmid DNA Purification

The Plasmid DNA Kit (Bio Basic, Markham, ON, Canada) was used to purify DNA for the high copy plasmids, pT7-7 and pBTLT7. Bacteria were cultured in a 15 mL test tube overnight at 37°C in 6 mL of LB media and 100 µg/mL of ampicillin, shaking at 210 rpm. Cells were harvested by centrifugation at 17,000 x g and DNA was prepared according to the manufacturer’s instructions.

pVLT-31 is a low copy plasmid and was purified using the alkaline lysis method described by Sambrook and colleagues (1989). Bacterial cultures were grown in 15 mL test tubes overnight at 37°C in two 6 mL aliquots of LB supplemented with 15 µg/mL tetracycline, shaking at 210 rpm. Cells were collected by centrifugation at 17,000 x g for 3 minutes. The supernatant was discarded and the pellet was resuspended in 200 µL of Solution I. Cell lysis was achieved through the addition of 200 µL of Solution II followed by several rapid inversions to mix. To neutralize the mixture, 200 µL of ice-cold Solution III were added and the samples were placed on ice for 10 minutes. Centrifugation at 21,000 x g for 15 minutes separated denatured genomic DNA and proteins from soluble plasmid DNA. To extract plasmid DNA from the remaining cellular debris 250 µL of chloroform:iso-amyl alcohol (24:1, v/v) and 250 µL of Tris-saturated phenol were added to the sample. After vortexing for 10 seconds the sample was centrifuged at 17,000 x g for 10 minutes. The plasmid DNA, now present in the aqueous fraction, was transferred to a new tube and treated with 500 µL of isopropanol. After several rapid inversions and incubation at 20-25°C for 2 minutes precipitated DNA was harvested by centrifugation at 17,000 x g for 10 minutes. Isopropanol was carefully
removed and 50 µL of 70% ethanol was used to wash the pellet. Following centrifugation at 17,000 x g for 5 minutes the ethanol was removed and the sample was placed in a biological safety cabinet for approximately 20 minutes to air-dry. Finally, the DNA pellet was resuspended in 20-30 µL of sterile double-distilled H2O and stored at -20°C.

2.3.2 DNA Purification from Agarose Gel

Following agarose gel electrophoresis, the gel fragment of interest was excised and DNA was extracted using a commercial Gel Extraction Kit (Bio Basic, Markham, ON, Canada) according to the manufacturer’s instructions.

2.3.3 DNA Ligation

Ligation reactions were carried out to insert various SsoPox mutants and PTEBd into pT7-7, pBTL7 and pVLT-31. Reaction mixtures contained final concentrations of 1X ligase buffer, 400 U ligase (New England BioLabs, Pickering, ON, Canada) and the vector and target insert at a molar ratio of 3:1. The ligation mixture was incubated at 15°C overnight prior to transformation by electroporation into E. coli BL21 (λDE3) or P. putida KT2442.

2.3.4 Transformation by Electroporation

Transformations were performed according to the method described by Sambrook et al. (1989). Fifty microlitres of electrocompetent cells and 2 µg of plasmid DNA were pipetted into an electroporation cuvette with a gap size of 1 mm and tapped gently to mix. For transformation into E. coli BL21 (λDE3) the following electroporation parameters were used: 1.25 kV, 200 Ω, 25 µF. For transformation into P. putida KT2442 cells the following parameters were used: 1.25 kV, 400 Ω, 25 µF. Next, the mixture was
resuspended in 500 µL of SOC and incubated at 37°C, shaking at 210 rpm for 1 hour. Following incubation the mixture was plated on a LB agar plate supplemented with the appropriate antibiotic and incubated for 37°C overnight.

2.3.5 Screening for Positive Transformants

Restriction analysis of isolated plasmids was carried out to identify colonies which possess vector containing the appropriate insert. Single colonies were sampled and their plasmid DNA harvested following the methods described in Section 2.3.1. Resulting DNA samples were treated with the appropriate restriction enzymes to excise the gene of interest from the plasmid. Following digestion each sample was loaded onto an agarose gel for analysis. DNA from positive clones was sequenced at the Guelph Molecular Supercenter (University of Guelph) for confirmation.

2.3.6 Agarose Gel Electrophoresis

1% (w/v) agarose gels were prepared in TAE buffer. Samples were mixed with DNA loading buffer and loaded into the wells. Electrophoresis was carried out at 100 V for 1 hour before being stained in ethidium bromide (0.5 µg/mL) for 30 minutes and visualized under UV light. GeneRuler 1 kb DNA Ladder (Fermentas, Burlington, ON, Canada) was electrophoresed as a molecular weight standard.

2.4 Site-specific Mutagenesis

All site-directed mutagenesis was performed using pT7-7 containing the codon-optimized gene from *S. solfataricus* previously constructed in the Seah lab (Figure 2.1) (Ng, 2009). Flanking the gene encoding SsoPox are *XbaI* and *HindIII* restriction sites for relocation into pVLT-31.
**Figure 2.1** Plasmid maps for recombinant constructs used for SsoPox cloning and overexpression. The codon-optimized genes encoding SsoPox variants (black arrow) were generated in pT7-7 (A) which contains a β-lactamase (bla) gene and confers ampicillin resistance (Studier and Moffatt, 1986). An XbaI and HindIII digestion excises the gene and ribosome binding sequence (rbs) for transfer to the *P. putida* expression vector, pVLT-31 (B), as described in Section 2.4. pVLT-31 confers resistance to tetracycline (*Tet^R*) (De Lorenzo et al., 1993). Graphical representations are not drawn to scale.
The constructs encoding the R223K and R223Q SsoPox variants were created previously by Filomena Ng (unpublished) using site-directed mutagenesis via the Stratagene QuickChange protocol. Primers are shown in Table 2.1. PCR cycling parameters were 95°C for 30 seconds; 15 cycles of 95°C for 30 seconds, 48°C for 1 minute, and 68°C for 1 minute 15 seconds, and finally 68°C for 5 minutes.

The genes encoding the Y97F, Y99F, Y97F/Y99F and L226F variants were synthesized by site-directed mutagenesis via the modified QuickChange method involving partially overlapping primers described by Liu and Naismith (2008). Primer sequences are displayed in Table 2.1. Platinum Pfx polymerase (Invitrogen, Burlington, ON, Canada) was used for PCR and cycling parameters were 95°C for 5 minutes; 20 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 5 minutes and a final 5 minute extension step at 72°C.

2.5 Preparation of Recombinant PTE_Bd Constructs

A codon-optimized gene encoding PTE_Bd with the native leader peptide at the N-terminus and a C-terminal CBD was synthesized by BioBasic (Markham, ON, Canada) and inserted into the vector pUC57 (Figure 2.2). The encoded CBD was derived from the β-1,4-xylanase (Cex) of C. fimii but contains a serine-arginine at its N-terminus instead of a serine-proline and also possesses a shorter linker sequence (see Appendix II). The desired PTE_Bd constructs were obtained through the use of the appropriate restriction sites flanking the leader peptide (ClaI) and CBD (HpaI). Desired constructs were inserted into pBTLT7 using NdeI and HindIII restriction sites prior to transfer into pVLT-31 through the utilization of XbaI and HindIII sites (Figure 2.3).
Table 2.1 Primers for site-specific mutagenesis of SsoPox. SsoPox variants were generated using either the Stratagene QuickChange protocol or the partially overlapping primer method described by Liu and Naismith (2008). Mutated bases giving rise to altered codons are underlined.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>R223K&lt;sub&gt;forward&lt;/sub&gt;</td>
<td>5’- CTTCATCGGTCTGGATAAGTACGGCCTGGACCTG-3’</td>
</tr>
<tr>
<td>R223K&lt;sub&gt;reverse&lt;/sub&gt;</td>
<td>5’- CAGGTCCAGGCCGTACTTTATCCAGACCCGATGAAG-3’</td>
</tr>
<tr>
<td>R223Q&lt;sub&gt;forward&lt;/sub&gt;</td>
<td>5’- CATCGGTCTGGATCAGTACGGCCTGGACCTG-3’</td>
</tr>
<tr>
<td>R223Q&lt;sub&gt;reverse&lt;/sub&gt;</td>
<td>5’- CAGGTCCAGGCCGTACTTGATCCAGACCCGATG-3’</td>
</tr>
<tr>
<td>Y97F&lt;sub&gt;forward&lt;/sub&gt;</td>
<td>5’- CCGGTATCTTTCATCTATATCGACCTGCCCCGGTTC-3’</td>
</tr>
<tr>
<td>Y97F&lt;sub&gt;reverse&lt;/sub&gt;</td>
<td>5’- GATATAGATGAGATACCGGTACCCGCACAACCAG-3’</td>
</tr>
<tr>
<td>Y99F&lt;sub&gt;forward&lt;/sub&gt;</td>
<td>5’- CATCAGTTTTATCGACCTGCGTTCTACTTCC-3’</td>
</tr>
<tr>
<td>Y99F&lt;sub&gt;reverse&lt;/sub&gt;</td>
<td>5’- GGTGTAAGAGATGTAGATACCGGTACCCGC-3’</td>
</tr>
<tr>
<td>Y97F/Y99F&lt;sub&gt;forward&lt;/sub&gt;</td>
<td>5’- CTTCATCTTTATCGACCTGCGTTCTACTTCC-3’</td>
</tr>
<tr>
<td>Y97F/Y99F&lt;sub&gt;reverse&lt;/sub&gt;</td>
<td>5’- GGTGTAAGAGATTAAGATACCGGTACCCGC-3’</td>
</tr>
<tr>
<td>L226F&lt;sub&gt;forward&lt;/sub&gt;</td>
<td>5’- GTTACGGCTTTGACCTGTCTGACCCGATCCAGTG-3’</td>
</tr>
<tr>
<td>L226F&lt;sub&gt;reverse&lt;/sub&gt;</td>
<td>5’- CAGGTCCAAAAGCCGTACCCGATCCAGACCAGATG-3’</td>
</tr>
</tbody>
</table>
**Figure 2.2** DNA sequence for the codon-optimized gene encoding PTE\textsubscript{Bd} fused with the gene encoding the CBD from *C. fimi*. The gene encoding PTE\textsubscript{Bd} was engineered to allow for expression with and without leader peptide and a CBD from *C. fimi*. Bolded in pink are the *Clai* restriction sites flanking the leader sequence which is highlighted by a dotted underline. Bold blue bases represent *HpaI* restriction sites for the removal of the underlined CBD sequence. Silent mutations to remove rare codons are shown bolded in green. The *Ndel* and *HindIII* restriction sites are outlined in red and black at the beginning and end of the gene, respectively.
Figure 2.3 Plasmid maps for recombinant constructs used for PTE_{Bd} and PTE_{Bd-CBD} cloning and overexpression. The codon-optimized genes encoding PTE_{Bd} (black arrow, panel A) and PTE_{Bd-CBD} (black box with attached green arrow, panel B) were cloned into pBTLT7 via Ndel and HindIII restriction sites. pBTLT7 is a modified form of pBTL-4 containing a T7 promoter and confers resistance to tetracycline (Tet\textsuperscript{R}) (Baker \textit{et al.}, 2009). Graphical representations are not drawn to scale.
2.6 Protein Overexpression

2.6.1 Mini-Scale Expression of PTE\textsubscript{Bd}

Small-scale expression studies were carried out with recombinant \textit{E. coli} containing PTE\textsubscript{Bd}, PTE\textsubscript{Bd}-CBD, His-PTE\textsubscript{Bd} and His-PTE\textsubscript{Bd}-CBD using 3 mL batch cultures in LB using 15 mL test tubes. At logarithmic phase (optical density of 0.6) IPTG and CoCl\textsubscript{2} were added to final concentrations of 1 mM and 0.2 mM, respectively. For each construct an uninduced control was performed. Following overnight incubation at 37\textdegree C, shaking at 210 rpm, cells were collected and resuspended in 50 \textmu L of B-PER II (Thermo Scientific, Rockford, IL, USA) before being vortexed for 1 minute. Cell debris was pelleted via centrifugation at 20,000 x g and both the supernatant containing soluble protein and pellet containing insoluble protein were analyzed via SDS-PAGE.

2.6.2 Large-Scale Expression of SsoPox and PTE\textsubscript{Bd}

Large-scale SsoPox and PTE\textsubscript{Bd} expression was achieved following a modified version of the protocol reported by Ng \textit{et al.} (2011). Cultures of recombinant \textit{P. putida} containing the appropriate plasmid were prepared in 2 L flasks containing 1 L of LB broth supplemented with the appropriate antibiotics. At logarithmic phase (optical density between 0.4 and 0.6) IPTG and CoCl\textsubscript{2} were added to the cultures at final concentrations of 0.5 mM and 0.2 mM, respectively. Following overnight incubation at 37\textdegree C with shaking at 210 rpm, cells were collected by centrifugation at 3000 x g. The cells were washed once with 250 mL of 20 mM HEPES (pH 8.0) and harvested by recentrifugation in order to remove contaminating salts and residual LB broth prior to storage at -20\textdegree C.
2.6.3 Cell Lysis and Pretreatment of Lysate

Harvested cells were resuspended in 20 mM HEPES, 0.2 mM CoCl$_2$ (pH 8.0) (3 mL lysis buffer per gram of cells) and treated with lysozyme (final concentration of 0.2 mg/mL) for 20 minutes at 20–25°C room temperature before being frozen at -20°C overnight. After thawing the cells were disrupted by sonication in six 1-minute cycles on ice before being centrifuged at 58,500 x $g$ for 20 minutes to pellet cellular debris. At this stage clarified lysate containing PTE$_{bd}$ was prepared for the purification process, however, the thermostability of SsoPox was exploited through a series of heat treatment steps prior to column loading. Specifically, clear lysate was incubated at 60°C for 15 minutes and centrifuged at 75,600 x $g$ for 20 minutes to precipitate thermally sensitive host proteins. This procedure was repeated at 70°C prior to column chromatography.

2.7 SsoPox Purification

Clarified lysate was loaded onto a Source 15Q anion exchange column (2 x 15 cm; GE Healthcare, Baie d’Urfe, QC, Canada) that had been equilibrated with 20 mM sodium HEPES (pH 8.0). The column was washed with three column volumes of equilibration buffer followed by a linear gradient of buffer containing NaCl from 0 to 1 M over ten column volumes. Fractions exhibiting paraoxonase activity were eluted with buffer containing 0.2 M NaCl and were pooled prior to concentration. Concentration was achieved through repeated washing with storage buffer (20 mM HEPES, 0.2 mM CoCl$_2$, pH 8.0) over an Amicon filtration unit fitted with a YM10 filter (Millipore, Nepean, ON, Canada). Finally protein was stored in the aforementioned buffer at -80°C.
2.8  PTE\textsubscript{Bd} Purification

2.8.1 PTE\textsubscript{Bd} Purification Using Ni-NTA Chromatography

His-tag purification was performed using Qiagen Ni-NTA Agarose resin (Toronto, ON, Canada). Clarified lysate was incubated on a rotator for 1 hour at 4\(^\circ\)C in 2 mL of Ni-NTA resin that had been equilibrated with 15 mL of 50 mM NaH\textsubscript{2}PO\textsubscript{4}, 300 mM NaCl, 10 mM imidazole lysis buffer (pH 8.0). The resin and lysate were added to a column and washed with 50 mM NaH\textsubscript{2}PO\textsubscript{4}, 300 mM NaCl, 20 mM imidazole (pH 8.0) until 100 \(\mu\text{L}\) of flow through no longer reacted with 25 \(\mu\text{L}\) of 1X Bradford dye. Ten millilitres of 50 mM NaH\textsubscript{2}PO\textsubscript{4}, 300 mM NaCl buffer containing concentrations of imidazole ranging from 20 mM to 250 mM were added to the column and collected in 1 mL fractions. Fractions testing positive for protein content using the aforementioned Bradford test were pooled, concentrated using a Millipore YM10 filter and stored in 20 mM HEPES, 0.2 mM CoCl\textsubscript{2} (pH 8.0) at -80\(^\circ\)C.

2.8.2 PTE\textsubscript{Bd} Purification Using Anion Exchange and Size Exclusion Chromatography

Purification of PTE\textsubscript{Bd} was accomplished using two liquid chromatography treatments. Clear lysate was first loaded onto a Source 15Q anion exchange column and washed as described in Section 2.7. Fractions positive for paraoxonase activity were eluted in the flow through bulk which was pooled and concentrated using 20 mM HEPES, 0.2mM CoCl\textsubscript{2} (pH 8.0) before being loaded into a HiLoad 26/60 Superdex 200 size-exclusion column (GE Healthcare, Baie d’Urfe, QC, Canada) that had been equilibrated with 20 mM HEPES containing 0.15 M NaCl (pH 8.0). Fractions exhibiting paraoxonase activity eluted at 195 mL buffer. These fractions were pooled, concentrated
using a Millipore YM10 filter and stored in 20 mM HEPES, 0.2 mM CoCl$_2$ (pH 8.0) at -80°C.

2.9 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE gels were composed of a 10% (w/v) polyacrylamide separating gel and 5% (w/v) polyacrylamide stacking gel. Samples were treated with SDS loading buffer and boiled for 2 minutes at 100°C before being loaded into the wells of the gel. Samples were electrophoresed at 10 mA with a maximum voltage of 150 V for 1.5 hours in 1X SDS buffer. Visualization of the bands was achieved by staining the gel in Commassie stain solution for 30 minutes and destaining overnight in destaining solution. PageRuler Unstained Protein Ladder (Fermentas, Burlington, ON, Canada) was used as a molecular mass marker.

2.10 Protein Concentration Determination

Protein concentration was determined using the Bradford assay (Bradford, 1976). A standard curve was prepared using bovine serum albumin and protein storage buffer (20 mM HEPES, pH 8.0, 0.2 mM CoCl$_2$). Twenty microlitre samples between 0.1 mg/mL and 1 mg/mL were combined with 980 μL of 1X Bradford reagent and the absorbance was measured at 595 nm. Protein samples of unknown concentration were mixed in the same way and their concentrations determined from the standard curve.

2.11 Enzyme Assays

2.11.1 Determination of SsoPox Catalytic Parameters with Paraoxon

Paraoxonase activity was measured spectrophotometrically by monitoring the production of $p$-nitrophenol at 405 nm ($\varepsilon = 16.5$ cm$^{-1}$ mM$^{-1}$) (Ng, 2009) using a Varian Cary 100 Bio UV-Visible spectrophotometer equipped with a thermojacketed cuvette.
holder at 25°C. Reactions were performed in a total volume of 1 mL containing 20 mM Tris-HCl (pH 8.0), 0.02 mM CoCl₂, 10% acetonitrile and 20 μg/mL enzyme. All assays were at least duplicated and paraoxon concentrations used were between 0.1 x \( K_M \) and 3 x \( K_M \). Linear regression was used to calculate the reaction velocity at each substrate concentration and data was fitted by non-linear regression to the Michaelis-Menten equation using LEONORA software (Cornish-Bowden, 1995). A unit of enzyme activity is defined as the amount of enzyme required to catalyze the conversion of 1 μmol of substrate per minute.

2.11.2 Determination of SsoPox Catalytic Parameters with Various AHLs

Lactonase activity was measured using a pH indicator assay similar to that described by Afriat et al. (2006). The pH indicator, \( m \)-cresol purple decreases in absorbance at 560 nm (ε = 2.41 cm⁻¹ mM⁻¹) as the pH decreases due to the formation of carboxylic acid products (Ng, 2009). Determination of kinetic parameters was achieved using a BMG FLUOstar Optima microplate reader at 25°C in 96-well plates with a pathlength of 0.6 cm. Reaction mixtures contained 2 mM Tris-HCl (pH 8.0), 160 mM NaCl, 0.3 mM \( m \)-cresol purple, 0.02 mM CoCl₂, 2% dimethyl sulfoxide, 20 μg/mL enzyme and \( N \)-acyl homoserine lactone substrates at concentrations between 0.4 x \( K_M \) and 3 x \( K_M \). Linear regression was utilized to determine the reaction velocity at each substrate concentration. Data was fitted by non-linear regression to the Michaelis-Menten equation using LEONORA software (Cornish-Bowden, 1995).

2.12 Examining PTE\(_{Bd}\) Activity Towards Various Organophosphates

Ten different compounds were tested with PTE\(_{Bd}\) in solution. Table 2.2 lists the OPs tested and their respective method of detection and Figure 2.4 illustrates the
Table 2.2  Tested organophosphates and their respective methods of detection. The suitability of 10 OPs as test substrates for the PTE\textsubscript{Bd} biosensor prototype was determined by monitoring reactions for the production of visualizable products. The production of $p$-nitrophenol generates a visible yellow colour; 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB), also known as Ellman’s reagent, reacts with the free sulfhydryl groups of hydrolysis products to produce a yellow product, 5-thio-2-nitrobenzoic acid; and azinphos-methyl and coumaphos degradation yield compounds which fluoresce when exposed to UV light.

<table>
<thead>
<tr>
<th>Method of detection</th>
<th>Organophosphate</th>
<th>Chemical detected</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colourful product</td>
<td>paraoxon</td>
<td>$p$-nitrophenol product</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>methyl-paraoxon</td>
<td>$p$-nitrophenol product</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>parathion</td>
<td>$p$-nitrophenol product</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>methyl-parathion</td>
<td>$p$-nitrophenol product</td>
<td>yellow</td>
</tr>
<tr>
<td>Indirectly visualizable product</td>
<td>chlorpyrifos</td>
<td>5-thio-2-nitrobenzoate</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>phosmet</td>
<td>5-thio-2-nitrobenzoate</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>terbufos</td>
<td>5-thio-2-nitrobenzoate</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>malathion</td>
<td>5-thio-2-nitrobenzoate</td>
<td>yellow</td>
</tr>
<tr>
<td>Fluorescent product</td>
<td>azinphos-methyl</td>
<td>3-methyl-1,2,3-benzotriazin-4-one ($\lambda_{ex} = 314\text{nm}, \lambda_{em} = 394\text{nm}$)</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>coumaphos</td>
<td>chlorferron ($\lambda_{ex} = 355\text{nm}, \lambda_{em} = 460\text{nm}$)</td>
<td>blue</td>
</tr>
</tbody>
</table>
Figure 2.4 Chemical basis for the detection of organophosphates using visible products, DTNB and fluorescence. (A) $p$-nitrophenol, a yellow substance, is produced from the hydrolysis of paraoxon. (B) Phosmet degradation yields a free sulfhydryl group which reacts with 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) to generate intensely yellow 5-thio-2-nitrobenzoic acid. (C) The degradation of azinphos-methyl produces a product that fluoresces yellow under UV light. Visible products are boxed in yellow.
A

\[
\begin{align*}
\text{paraoxon} & \xrightarrow{\text{PTE}_{\text{Me}}} \text{diethyl phosphate} + \text{p-nitrophenol} \\
& \xrightarrow{\text{H}_2\text{O}} \text{dimethyl sulfanyl phosphonite} + 5,5'-\text{dithiobis(2-nitrobenzoic acid)}
\end{align*}
\]

B

\[
\begin{align*}
\text{phosmet} & \xrightarrow{\text{PTE}_{\text{Me}}} \text{dimethyl sulfanyl phosphonite} + \text{2-methylisoindole-1,3-dione} \\
& \xrightarrow{\text{H}_2\text{O}} \text{dimethyl sulfanyl phosphonite} + \text{5-thio-2-nitrobenzoic acid} + \text{5-[dimethoxy(sulfanylidyne)-\(\lambda_2\)-phosphanylidene]-2-nitrobenzoic acid}
\end{align*}
\]

C

\[
\begin{align*}
\text{azinphos-methyl} & \xrightarrow{\text{PTE}_{\text{Me}}} \text{dimethyl sulfanyl phosphonite} + \text{3-methyl-1,2,3-benzotriazin-4-one} \\
& \xrightarrow{\text{H}_2\text{O}} \text{314 nm (\(\lambda_{\text{excitation}}\))} + \text{394 nm (\(\lambda_{\text{emission}}\))}
\end{align*}
\]
chemical nature of each detection system. Degradation of paraoxon, methyl-paraoxon, parathion and methyl-parathion generates bright yellow $p$-nitrophenol which was observed by the naked eye. The products of phosmet, chlorpyrifos (Dursban), malathion and terbufos cannot be measured directly but their hydrolysis can be measured using 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB). Degradation of these substrates yields a free sulfhydryl group which reacts with DTNB to form 2-nitro-5-thiobenzoate, a yellow compound which absorbs at 412 nm ($\varepsilon = 14.15 \text{ cm}^{-1} \text{ mM}^{-1}$). Colour development using DTNB was measured spectrophotometrically when qualitative assessment proved difficult. The product of coumaphos and azinphos-methyl degradation is fluorescent and was detected using a 3UV-38 3UV handheld UV lamp (Fisher Scientific, Nepean, ON, Canada) emitting at a 302 nm wavelength. All reactions were permitted to proceed for a maximum of 24 hours prior to qualitative assessment.

2.13 PTE$_{Bd}$ Biosensor

2.13.1 Immobilization

Seven micrograms of PTE$_{Bd}$ were spotted onto circular discs (6 mm in diameter) of Disruptor™ Grade 4601 (cellulose, un laminated) nano alumina membrane (Ahlstrom, Mount Holly Springs, PA, USA) and incubated at 4°C for 45 minutes to allow for binding. As a control, enzyme storage buffer (20 mM HEPES, pH 8.0, 0.2 mM CoCl$_2$) was spotted. Following incubation, three 5-minute washes were carried out on a shaker using 50 mL of storage buffer to remove any unbound protein. Detection of protein shedding was carried out qualitatively by testing the final wash buffer for paraoxonase activity. All solutions were sterilized by filtration through 0.45 μm nylon filters (Millipore, Nepean, ON, Canada) and washes were performed under sterile conditions.
2.13.2 Immobilization Efficiency Determination

To determine the amount of protein immobilized on the membrane, 20 μg of PTE\textsubscript{Bd} were spotted onto circular discs (diameter of 6 mm) of Disruptor\textsuperscript{TM} functionalized membrane. As a control, enzyme storage buffer (20 mM HEPES, pH 8.0, 0.2 mM CoCl\textsubscript{2}) was spotted. The enzyme-spotted and control membranes were incubated at 4°C for 1 hour to allow for binding. Two enzyme-immobilized discs were washed in a 1.5 mL microfuge tube with 500 μL of enzyme storage buffer for 15 minutes on a rotator. The protein concentration of the wash solution was determined by Bradford assay (Bradford, 1976). For the standard curve, 50 μL of bovine serum albumin in concentrations ranging from 4 μg/mL to 40 μg/mL were mixed with 150 μL of 1X Bradford reagent. Absorbance was measured at 590 nm using a BMG FLUOstar Optima microplate reader in a 96-well plate. To determine the quantity of protein present in the wash, 50 μL of the wash sample were mixed with 150 μL of 1X Bradford reagent. After measuring absorbance the protein concentration was interpolated from the standard curve. The amount of protein immobilized would be the difference between the total amount of protein spotted and the amount of protein in the wash.

2.13.3 Determination of Immobilized PTE\textsubscript{Bd} Detection Thresholds

Different concentrations of each OP were prepared in order to determine the lowest concentration detectable on Disruptor\textsuperscript{TM}-immobilized PTE\textsubscript{Bd}. Excess storage or wash buffer was pipetted from the surface and edges of each biosensor prior to treatment. Twenty-five microlitres of each OP solution was spotted onto separate samples and incubated at 20-25°C for up to 1 hour before being observed for colour and fluorescence development.
2.13.4 Long-term Stability Assay with Immobilized \( \text{PTE}_{\text{Bd}} \)

A 40 day trial was conducted at three different storage temperatures, -15.5°C, 4°C and 23.5°C to determine the long-term viability of immobilized \( \text{PTE}_{\text{Bd}} \). Samples and controls were prepared as described in Section 2.13.1 prior to storage in 500 \( \mu \)L of 20 mM HEPES (pH 8.0), 0.2 mM CoCl\(_2\) at the appropriate temperature. Every 2 to 4 days samples were removed from storage buffer and treated in duplicate with 25 \( \mu \)L of 1 mM paraoxon, parathion or coumaphos as described in Section 2.13.3. Quantification of colour intensity was achieved by scanning samples into a computer and analyzing the image using ImageJ software (Rasband, 1991).
CHAPTER 3: DETERMINATION OF THE IMPORTANCE OF RESIDUES IN SSOPOX THAT ARE INVOLVED IN AHL LACTONASE AND PARAOXONASE ACTIVITIES

A comparison of the active site residues of SsoPox and related phosphotriestersases reveals the substitution of R223 in SsoPox with a histidine in PTE_{Bd}. This histidine has been suggested to play an important role in OP hydrolysis by relaying a proton from D301 (D256 in SsoPox) during catalysis (Aubert et al., 2004). Although this hypothesis could explain the reduced paraoxonase activity of SsoPox relative to PTE_{Bd}, an R223H SsoPox variant possessed 360-fold reduced activity towards paraoxon (Ng, 2009) and no supporting evidence exists to substantiate this claim. The same R223H variant was found to exhibit specificity constants towards lactone substrates that were significantly lowered. For instance the $k_{cat}/K_M$ was 230-fold reduced for 5-thiobutyl-$\gamma$-butyrolactone (Elias et al., 2008), 20-fold reduced for 3-oxo-C8-HSL, and 200-fold reduced for 3-oxo-C10-HSL (Ng, 2009). This suggests that this residue could be important for AHL hydrolysis. We hypothesized that the residue in position 223 could play a role in stabilization of the negative reaction intermediate formed during AHL turnover (Figure 1.10). To test this theory R223 was replaced with lysine and glutamine. Lysine is chemically similar to arginine and possesses a positive charge whereas glutamine has no charge and therefore should be unable to stabilize any negative intermediary. R223K and R223Q variants were generated by site-specific mutagenesis and their kinetic parameters determined.

The importance of Y97 and Y99 in SsoPox and various PLLs has been discussed in the literature. For example, in addition to its suggested role as a stabilizing residue
during AHL hydrolysis, the phenolic hydroxyl of Y97 is thought to hydrogen bond with the carbonyl oxygen of lactone substrate and position the lactone ring in a catalytically productive orientation (Vanhook et al., 1996; Elias et al., 2008) (Figure 3.1). Y99 may also be important for this orientation since it hydrogen bonds and contributes to the proper positioning of the phenol ring of Y97 (Chow et al., 2010). Despite these hypotheses little has been done to investigate the relevance of these residues. Here we examine the importance of the hydrogen-bonding capacity of Y97 and Y99 on paraoxonase and AHL lactonase activity by generating Y97F, Y99F and Y97F/Y99F variants and measuring their kinetic parameters.

A hydrophobic binding tunnel adjacent to the active site of SsoPox is thought to assist AHL binding through the accommodation of acyl-chains. This theory is supported by the high efficiency of SsoPox towards substrates with longer hydrophobic tails (Ng, 2009) and the inhibitor-bound structure of SsoPox shows the acyl-chain of C10-HTL in the tunnel. The structure of SsoPox reveals a leucine in position 226 which is important for tunnel formation. Protein modeling suggests that substitution of L226 with a large hydrophobic residue may introduce a steric impediment to the tunnel thus affecting the specificity of the enzyme towards AHLs. To examine the impact of this modification on the substrate specificity of SsoPox, an L226F variant was constructed by site-specific mutagenesis and its kinetic parameters were determined for a variety of AHL substrates.

SsoPox is a thermostable enzyme and possesses lactonase and promiscuous phosphotriesterase activities which make it an attractive candidate for the reduction of bacterial virulence caused by quorum sensing and the detoxification of pesticide pollutants. Although kinetic activity dramatically improves at higher temperatures with
Figure 3.1  Schematic showing the proposed role of Tyr-97 and Tyr-99 in AHL binding in SsoPox. Hydrophobic interactions between the acyl-chain of the substrate, shown here as 3-oxo-C8-HSL, and the binding tunnel formed by loop 8 of SsoPox likely contribute to initial binding and general orientation of AHL substrate. Tyr-97 and Tyr-99 have been implicated in the proper positioning of the lactone ring through shared hydrogen bond interactions (shown as blue dotted lines) and hydrogen bonding between the lactone carbonyl and the phenolic hydroxyl of Tyr-97. Distances indicated were measured in PyMOL (Delano, 2002) using the C10-HTL-bound SsoPox structure.
this thermostable enzyme (Elias et al., 2008), high temperature causes a shift in the pK\textsubscript{a} of the pH indicator, \textit{m}-cresol purple, and AHLs are thermally unstable therefore all activity determinations were performed at 25ºC for this thesis.

3.1 Results

3.1.1 Generation of SsoPox Variants

Site-directed mutagenesis was attempted on pVLT-31 containing the codon-optimized gene encoding SsoPox however no corresponding 11 kb band was observed following PCR and gel electrophoresis indicating that amplification had not been achieved. The 2.5 kb, high copy number plasmid, pT7-7, was instead used for mutagenesis and transformed into \textit{E. coli} BL21 (\textlambda DE3) prior to excision and religation of the mutated SsoPox genes into pVLT-31 for expression in \textit{P. putida} KT2442. Once in pVLT-31 all mutations were verified by sequencing.

3.1.2 Purification of SsoPox Variants

SsoPox variants were purified by heat treatment and anion exchange chromatography and yielded an average of approximately 8 mg of protein per litre of culture (3 mg per gram of cells, wet weight). This result is similar to that reported by Ng et al. (2011) for wild type SsoPox and variant R223H. The molecular mass of the purified protein as estimated by SDS-PAGE was 35 kDa (Figure 3.2), which is in agreement with the molecular mass predicted from the amino acid sequence.

3.1.3 Substrate Specificity of the R223K and R223Q SsoPox Variants

Progress curves were collected for five minutes for paraoxonase and AHL lactonase reactions and data was fitted to the Michaelis-Menten equation as demonstrated in Figure 3.3 and Figure 3.4. The kinetic parameters for AHL lactonase activity in the
Figure 3.2  Coomassie Blue-stained SDS-polyacrylamide gel of all SsoPox variants purified from *P. Putida*. Gel loaded with standard molecular weight marker (lane 1) and pure wild type SsoPox (lane 2) as well as variants R223K (lane 3), R223Q (lane 4), Y97F (lane 5), Y99F (lane 6), Y97F/Y99F (lane 7) and L226F (lane 8). Molecular masses from the protein standard are indicated to the left of the gel.
Figure 3.3 Y97F/Y99F specific activity versus paraoxon concentration curve. The assay mixture contained 20 mM Tris-HCl (pH 8.0), 0.02 mM CoCl₂, 10% acetonitrile and various concentrations of paraoxon. Data points shown represent the average of values measured in triplicate and were fitted by non-linear regression to the Michaelis-Menten equation using the program Leonora (Cornish-Bowden, 1995).
Figure 3.4  Y99F specific activity versus 3-oxo-C8-HSL concentration curve. The assay mixture contained 2 mM Tris-HCl (pH 8.0), 160 mM NaCl, 0.02 mM CoCl₂, 2% dimethyl sulfoxide, 0.3 mM m-cresol purple and varying concentrations of 3-oxo-C8-HSL. Data points shown represent the average of values measured in triplicate and were fitted by non-linear regression to the Michaelis-Menten equation using the program Leonora (Cornish-Bowden, 1995).
R223K and R223Q variants was less than 20-fold relative to wild type (Table 3.1). Both amino acid substitutions led to a 3- and 10-fold increase in $K_M$ and 4- and 6-fold increase in $k_{cat}$, respectively. The resulting specificity constants for both variants remained similar to that of wild type.

The parameters for paraoxonase activity were also determined for both mutants (Table 3.1). Due to slight increases in $K_M$ and 2- and 20-fold reductions in $k_{cat}$, the catalytic efficiencies ($k_{cat}/K_M$) of R223K and R223Q decreased by 10- and 40-fold, respectively.

### 3.1.4 Substrate Specificity of the Y97F, Y99F and Y97F/Y99F SsoPox Variants

The Y97 residue is proposed to be important for proper positioning of the lactone ring prior to AHL hydrolysis (Elias et al., 2008). Kinetic parameters determined for Y97F, Y99F and Y97F/Y99F variants are summarized in Table 3.2. Variants Y97F and Y97F/Y99F had no detectable AHL lactonase activity towards 3-oxo-C8-HSL or 3-oxo-C10-HSL, the two best substrates known for SsoPox. No other AHL substrates were tested with these two variants. Y97F and Y97F/Y99F exhibited measurable paraoxonase activity with a 20-fold and 2-fold reduction in specificity constants, respectively, relative to wild type. Supplementation of Y97F and Y97F/Y99F paraoxonase reaction assays with up to 100 times the original concentration of CoCl$_2$ did not affect the reaction rate (Table 3.3). The Y99F substitution did not significantly alter $k_{cat}/K_M$ towards either substrate.

### 3.1.5 Substrate Specificity of the L226F SsoPox Variant

SsoPox is capable of hydrolyzing a variety of AHLs with different acyl-chain lengths and substitutions but appears to exhibit higher specificity constants towards
Table 3.1  Comparison of the kinetic parameters of the wild-type SsoPox and R223H, R223K and R223Q variant enzymes. Activity measurements were performed in triplicate at 25°C. Paraoxonase reactions contained 20 mM Tris-HCl (pH 8.0), 0.02 mM CoCl₂, and 10% acetonitrile. AHL lactonase assays contained 2 mM Tris-HCl (pH 8.0), 160 mM NaCl, 0.02 mM CoCl₂, 2% dimethyl sulfoxide and 0.3 mM m-cresol purple.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Paraoxonase activity</th>
<th>3-oxo-C8-HSL lactonase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_M$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>SsoPox$^1$</td>
<td>1.3 ± 0.2</td>
<td>0.50 ± 0.01</td>
</tr>
<tr>
<td>R223H$^1$</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>R223K</td>
<td>2.8 ± 0.3</td>
<td>0.026 ± 0.0008</td>
</tr>
<tr>
<td>R223Q</td>
<td>5.1 ± 0.6</td>
<td>0.26 ± 0.01</td>
</tr>
</tbody>
</table>

$^1$ Data reported by Ng (2009)

* $K_M$ for substrate is too high to determine individual kinetic constants therefore $k_{cat}/K_M$ was determined from the slope of the specific activity versus substrate concentration graph.
Table 3.2  Comparison of the kinetic parameters of the wild-type SsoPox and Y97F, Y99F and Y97F/Y99F variant enzymes. Activity measurements were collected in triplicate and standard deviations are provided.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Paraoxonase activity</th>
<th>3-oxo-C8-HSL lactonase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_M$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>SsoPox$^1$</td>
<td>1.3 ± 0.2</td>
<td>0.50 ± 0.01</td>
</tr>
<tr>
<td>Y97F</td>
<td>0.75 ± 0.08</td>
<td>0.017 ± 0.0004</td>
</tr>
<tr>
<td>Y99F</td>
<td>2.6 ± 0.2</td>
<td>1.2 ± 0.02</td>
</tr>
<tr>
<td>Y97F/Y99F</td>
<td>0.54 ± 0.03</td>
<td>0.12 ± 0.002</td>
</tr>
</tbody>
</table>

$^1$ Data reported by Ng (2009)
ND: $k_{cat}$ is lower than the detection limit of 0.005 s$^{-1}$
Table 3.3  Specific activity of SsoPox variant Y97F towards paraoxon in reactions containing 10- and 100-fold excess CoCl₂. No change in specific activity was observed when the CoCl₂ concentration of a 1 mL, 10 mM paraoxonase reaction containing Y97F was increased from 0.02 mM to 0.2 mM and 2 mM.

<table>
<thead>
<tr>
<th>Concentration of CoCl₂ (mM)</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>0.0768</td>
</tr>
<tr>
<td>0.2</td>
<td>0.0784</td>
</tr>
<tr>
<td>2.0</td>
<td>0.0779</td>
</tr>
</tbody>
</table>
longer AHLs. The only significant change in specificity of the L226F variant was observed with 3-oxo-C10-HSL for which L226F exhibited a specificity constant 37-fold lower than that of the wild type (Table 3.4). The specificity constant towards C4-HSL, 3-oxo-C6-HSL and 3-oxo-C12-HSL improved 3-fold however specificity decreased 3-fold for 3-oxo-C8-HSL. The kinetic parameters of L226F towards paraoxon were similar to the wild type SsoPox (Table 3.5).

3.2 Discussion

The arginine in position 223 of SsoPox is invariably conserved among PLLs. The 360-fold decrease in catalytic efficiency towards paraoxon by R223H reported by Ng (2009) and 1000-fold decrease observed by Elias et al. (2008) refutes the previously held theory that histidine acts as a proton-shuttling residue and is essential for paraoxonase function. Substitution of the corresponding histidine in PTE_Bd with arginine does not alter paraoxonase activity (Hill et al., 2003; Yang et al., 2003; Roodveldt and Tawfik, 2005) and further calls into question this hypothesis. The R223H variant had a significant impact on AHL lactonase activity, reducing efficiency towards 3-oxo-C8-HSL by 20-fold. However the side chain of histidine is significantly different in terms of size and charge from arginine, thus more conservative substitutions of R223 are required to shed light on the role of this residue in AHL lactonase activity. The crystal structure of C10-HTL-bound SsoPox indicates a hydrogen bond between R223 and the carbonyl oxygen of the lactone ring (Figure 3.5). Correspondingly, the structure reported for PLL_Gk in complex with C4-HSL displays the same interaction between the lactone ring and R230 (Chow et al., 2010). This suggests that R223 may perform a stabilizing role on
Table 3.4  Comparison of the AHL lactonase activity of the wild-type SsoPox and L226F variant enzyme. All activity measurements were performed in triplicate and standard deviation values are included.

<table>
<thead>
<tr>
<th>AHL Substrate</th>
<th>SsoPox</th>
<th>L226F</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4-HSL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_M$ (mM)</td>
<td>22 ± 3</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>7 ± 0.4</td>
<td>11 ± 0.5</td>
</tr>
<tr>
<td>$k_{cat}/K_M$ (x 10$^3$ M$^{-1}$ s$^{-1}$)</td>
<td>0.32 ± 0.04</td>
<td>0.98 ± 0.1</td>
</tr>
<tr>
<td>3-oxo-C6-HSL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_M$ (mM)</td>
<td>5.6 ± 0.9</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>0.52 ± 0.05</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>$k_{cat}/K_M$ (x 10$^3$ M$^{-1}$ s$^{-1}$)</td>
<td>0.092 ± 0.005</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>3-oxo-C8-HSL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_M$ (mM)</td>
<td>0.16 ± 0.03</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>1.0 ± 0.4</td>
<td>10 ± 0.4</td>
</tr>
<tr>
<td>$k_{cat}/K_M$ (x 10$^3$ M$^{-1}$ s$^{-1}$)</td>
<td>6.6 ± 1</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>3-oxo-C10-HSL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_M$ (mM)</td>
<td>0.050 ± 0.0001</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>1.5 ± 0.2</td>
<td>8.4 ± 0.5</td>
</tr>
<tr>
<td>$k_{cat}/K_M$ (x 10$^3$ M$^{-1}$ s$^{-1}$)</td>
<td>290 ± 50</td>
<td>7.8 ± 1</td>
</tr>
<tr>
<td>3-oxo-C12-HSL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_M$ (mM)</td>
<td>0.17 ± 0.002</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>0.95 ± 0.03</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>$k_{cat}/K_M$ (x 10$^3$ M$^{-1}$ s$^{-1}$)</td>
<td>5.5 ± 0.1</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

$^1$ Data for all substrates except C4-HSL reported by Ng (2009)
Table 3.5  Comparison of the paraoxonase activity of the wild-type SsoPox and L226F variant enzyme. Activity measurements were performed in triplicate and standard deviation values are indicated.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (x 10$^3$ M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SsoPox $^1$</td>
<td>1.3 ± 0.2</td>
<td>0.50 ± 0.01</td>
<td>0.40 ± 0.06</td>
</tr>
<tr>
<td>L226F</td>
<td>5.8 ± 0.5</td>
<td>4.5 ± 0.1</td>
<td>0.78 ± 0.08</td>
</tr>
</tbody>
</table>

$^1$ Data reported by Ng (2009)
Figure 3.5  SsoPox active site topology showing residues interacting with bound inhibitor C10-HTL. Distances between Arg-223 and Tyr-97 and the carbonyl oxygen of C10-HTL are indicated. Image generated using PyMOL (Delano, 2002).
the negative reaction intermediate formed during AHL hydrolysis; a role currently attributed to Y97 in the literature.

To test this hypothesis R223K and R223Q variants were generated and characterized. A positively charged residue, lysine is capable of stabilizing the negative intermediate while glutamine, with a polar but neutral side chain, would lack this capacity. However the specificity constant of both variants for 3-oxo-C8-HSL did not differ from that of wild type. Recently, Chow et al. (2010) reported that an R230 substitution, corresponding to R223 in SsoPox, to aspartic acid in PLL-Gk reduced the $k_{\text{cat}}$ for 3-oxo-C8-HSL by 10-fold. If the arginine residue stabilizes the negatively charged intermediate during lactone hydrolysis one would expect a more significant decrease in $k_{\text{cat}}$ in the aspartic acid-substituted enzyme due to charge repulsion. Together, this result and our data do not support the hypothesis that R223 performs a stabilizing role during AHL lactonase activity. The $k_{\text{cat}}/K_M$ for paraoxon hydrolysis on the other hand was reduced by 10- and 40-fold in R223K and R223Q, respectively. Since no structure of SsoPox or other related PLL containing bound organophosphate is available, it is difficult to determine the role of this residue position in OP hydrolysis.

Like R223, Y97 and Y99 are well-conserved among PLLs. Y97 and its equivalent in other PLLS have been proposed to serve the following roles: (i) facilitating binding and the proper positioning of substrate via interactions with the carbonyl oxygen of the lactone ring (Vanhook et al., 1996). (ii) the stabilization of a negative reaction intermediate formed during hydrolysis (Elias et al., 2008), a role we attributed previously to R223. (iii) the coordination and retention of the β-metal cation (Xiang et al., 2009; Chow et al., 2010). Structurally dissimilar AHL lactonases unrelated to the PLL
subgroup, such as AiiA, a metallo-β-lactamase superfamily member from *Bacillus thuringiensis* (Liu *et al.*, 2005), also possess a tyrosine residue at the same location that serves a similar function. Chow *et al.* (2010) also recently proposed that Y99 may be indirectly involved in positioning the scissile bond of the lactone ring by hydrogen-bonding and positioning the phenolic oxygen of Y97. The crystal structure of SsoPox illustrates the potential for hydrogen-bonding between the phenolic hydroxyls in Y97 and Y99, which would support this theory. In PTE$_{Bd}$ on the other hand, positions 97 and 99 are tryptophan and phenylalanine, respectively. They contribute to the formation of a small hydrophobic binding cleft which is proposed to accommodate the phenoxy or alkyl leaving group formed during OP hydrolysis (Merone *et al.*, 2010). A hydrophobic cleft is also present in SsoPox but it is contributed by W263, adjacent to Y99, at the active site opening (Merone *et al.*, 2010).

Three variants, Y97F, Y99F and Y97F/Y99F were characterized in this thesis. The phenylalanine side chain is structurally similar to tyrosine but does not possess a $p$-hydroxyl group and lacks the capacity to form hydrogen bonds. Both Y97F and Y97F/Y99F variants completely lost activity towards 3-oxo-C8-HSL and 3-oxo-C10-HSL, however, they still possessed paraoxonase activity, indicating that the enzyme’s structure is not severely disrupted and binding of metal cofactor required for paraoxonase activity is not compromised due to the Y97F substitution. In contrast, the steady-state kinetic parameters for the Y99F variant for AHL and paraoxon hydrolysis were unaffected. It therefore appears that Y97 and its capacity to form hydrogen bonds is essential for AHL lactonase activity but not paraoxonase activity. More specifically
these results support the previous hypotheses that (i) Y97 is important for AHL binding, and (ii) Y97 stabilizes the negative reaction intermediate during AHL hydrolysis.

Although Y97 and Y99 are not critical for paraoxonase activity they appear to have an influence on the kinetic parameters for this substrate. In particular, the kinetic parameters for Y97F towards paraoxon were 20-fold reduced but those of the double mutant, Y97F/Y99F, were similar to the wild type enzyme. This suggests that interactions between these two residues (hydrogen bonding in the case of the wild type enzyme and hydrophobic interactions in the case of the Y97F/Y99F variant) has a positive effect on paraoxonase activity. However this does not explain why the kinetic parameters for the Y99F single variant were largely unchanged from the wild type. Structural data of the variants may shed further light on the role of these residues in paraoxonase activity.

The final avenue of SsoPox characterization involved the modification of substrate specificity through changes in the large hydrophobic binding tunnel adjacent to the active site. This tunnel is formed by a flexible loop that is absent in PTE\textsubscript{Ide} and that is thought to be important for acyl-chain binding (Seibert and Raushel, 2005). L226 is not part of this loop but is located within the tunnel and its orientation relative to substrate in the C10-HTL-bound crystal structure suggests that it would make an excellent target for changing acyl-chain length specificity in SsoPox. SsoPox hydrolyzes longer chain AHLs such as 3-oxo-C8-HSL and 3-oxo-C10-HSL more efficiently than short chain AHLs like C4-HSL and 3-oxo-C6-HSL (Ng, 2009). Introducing phenylalanine at this position is expected to reduce the ability of SsoPox to bind long chain AHLs (Figure 3.6).
Figure 3.6  SsoPox structure illustrating possible changes in the hydrophobic binding tunnel upon L226F substitution. Structure of the binding tunnel in the wild type SsoPox (A) and the L226F variant (B) are shown. Substitution of L226 to phenylalanine led to a narrowing of the tunnel. Image generated using PyMOL (Delano, 2002).
To determine the impact of this change on the AHL specificity of SsoPox we kinetically characterized an L226F variant with AHLs possessing acyl-chains 4, 6, 8, 10 and 12 carbons in length. Introduction of L226F did not alter the AHL lactonase activity of SsoPox towards C4-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL or 3-oxo-C12-HSL but reduced the catalytic efficiency towards 3-oxo-C10-HSL by 37-fold.

The binding tunnel formed by loop 8 of SsoPox is large and the introduction of an L226F substitution does not block it completely. Elias et al. (2008) identified that loop 8 is more flexible than the core loops of SsoPox and is capable of accommodating substrate while remaining rigid enough for proper positioning. The RMSD of loop 8-forming α-carbons between free and substrate-bound SsoPox is 0.91 Å and the entire loop is involved in binding (Elias et al., 2008). It is likely that the flexibility of loop 8 is capable of compensating for any structural change resulting from an L226F substitution. While this explains the minor differences observed between specificity constants of wild type SsoPox and L226F towards C4-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL and 3-oxo-C12-HSL it is difficult to justify the 37-fold decrease in specificity towards 3-oxo-C10-HSL. The wild type SsoPox exhibits its highest efficiency towards 3-oxo-C10-HSL but 50-fold lower values towards both 3-oxo-C8-HSL and 3-oxo-C12-HSL. No hypothesis currently exists to explain this variation between substrates that differ in length by only two carbons. It is possible that the flexibility of loop 8 is perfectly suited for 3-oxo-C10-HSL binding in wild type but that an L226F substitution leads to a reduction in this flexibility. As the best substrate for SsoPox, the observed activity loss with 3-oxo-C10-HSL may be magnified compared to other, less efficiently hydrolyzed substrates. It is important to note that C10-HTL is an inhibitor that cannot be hydrolyzed by SsoPox and is thus bound
in a catalytically unproductive position in the co-crystallized structure. Therefore one must remain cautious when drawing structural explanations for substrate interactions from this model. Ultimately structural data for L226F and wild type complexed with 3-oxo-C10-HSL would permit a better explanation for the unusual pattern of activity loss observed with this amino acid substitution. Nonetheless these results support the hypothesis that modification of AHL substrate specificity is possible through changes to the residues lining the binding tunnel.
CHAPTER 4: GENERATION OF AN ORGANOPHOSPHATE BIOSENSOR USING PTE$_{Bd}$

In order to examine the potential of PTE$_{Bd}$ in biotechnological applications we must be able to produce appreciable quantities of the protein efficiently. Efforts have been made to purify PTE$_{Bd}$ using *Spodoptera frugiperda*, *Streptomyces lividans* and *E. coli* as expression hosts however the protein yield of these systems is between 4 and 30 mg/L of culture. Walker *et al.* (2002) have previously engineered recombinant *P. putida* KT2442 to express endogenous PTE$_{Bd}$ and fully degrade parathion into non-toxic products. However no attempts have been made to determine the amount of soluble PTE$_{Bd}$ produced in this system. Here we investigate the potential of the *P. putida* KT2442 expression system and fast performance liquid chromatography to harvest workable quantities of pure, soluble PTE$_{Bd}$.

Past work has been done to immobilize PTE$_{Bd}$ to solid supports, however this has traditionally been aimed at creating a method for detoxifying OP-contaminated samples from the environment (Caldwell *et al.*, 1991; Ghanem and Raushel, 2005) rather than engineering an OP-detecting product. Recent advancements in membrane-immobilization technologies have led to the production of nano alumina paper; an aluminum oxide and cellulose amalgam with an inherent positive charge. Capable of strongly binding large proteins and viral particles (Karim *et al.*, 2009), nano alumina membranes have been extensively researched as a filtration medium. Recent work in the Seah lab has demonstrated the potential of nano alumina to effectively bind SsoPox and inhibit the production of quorum-related virulence factors, elastase, protease and pyocyanin, in cultures of *P. aeruginosa* (Ng *et al.*, 2011). Here we attempt to immobilize
PTE\textsubscript{Bd} to nano alumina to create a novel biosensor capable of detecting various OP pesticides. We also examined the long-term stability and appropriate storage conditions for immobilized PTE\textsubscript{Bd} over a period of 40 days.

4.1 Expression and Purification of Codon-Optimized PTE\textsubscript{Bd}

The genes encoding PTE\textsubscript{Bd} and PTE\textsubscript{Bd-CBD} without a leader peptide were subcloned into the broad host range expression vectors pBTLT7 and pBTLT7His and transformed into \textit{E. coli} BL21 (\lambda\textsubscript{DE3}). Overexpression was achieved but the vast majority of protein was insoluble. A sample of cell extract from PTE\textsubscript{Bd-CBD} and His-PTE\textsubscript{Bd} cultures tested positive for paraoxonase activity and small amounts of protein corresponding to the expected size of PTE\textsubscript{Bd} were detectable on an SDS-polyacrylamide gel loaded with soluble lysate. Attempts were made to purify His-PTE\textsubscript{Bd} from clarified lysate using a Ni-NTA column. However, His-PTE\textsubscript{Bd} did not bind tightly to the column and all flow through and elution fractions tested positive for paraoxonase activity.

The gene encoding PTE\textsubscript{Bd} with the signal peptide codons removed was subcloned into pVLT-31 using \textit{XbaI} and \textit{HindIII} and transformed into \textit{P. putida} KT2442 where it yielded high amounts of soluble protein in LB media when grown at 37\textdegree C. Purification was achieved using anion exchange chromatography followed by a gel filtration step (Figure 4.1). The average yield of purified PTE\textsubscript{Bd} was 3.5 mg per litre of culture (1 mg of protein per gram of cells). The molecular mass of the purified protein was 34 kDa as estimated by SDS-PAGE, which agrees with the predicted molecular mass calculated from the amino acid sequence.
Figure 4.1  Coomassie Blue-stained SDS-acrylamide gel of PTE$_{Bd}$ purified from $P$. putida. Gel loaded with standard molecular weight marker (lane 1) and pure PTE$_{Bd}$ (lane 2). Molecular masses from the protein standard are indicated to the left of the gel.
4.2 Determination of Appropriate Organophosphates for Biosensor Testing

To determine which OP degradation products are readily visible, reactions containing PTE$_{Bd}$ were carried out with ten OPs in 1 mL cuvettes and observed for colour production. None of the reactions requiring DTNB (chlorpyrifos, phosmet, terbufos and malathion) produced a change that was visible to the naked eye. Azinphos-methyl did not produce any fluorescence after 24 hour incubation with PTE$_{Bd}$. Paraoxon, methyl-paraoxon, parathion, methyl-parathion and coumaphos degradation produced visible changes within 10 minutes therefore these OPs were used for future experimentation with membrane-immobilized PTE$_{Bd}$.

4.3 Immobilization of PTE$_{Bd}$ on Nano alumina Membrane

Known commercially as Disruptor™, nano alumina membrane has been shown to bind negatively charged biological particles including viruses and proteins (Sobsey and Jones, 1979). Seven micrograms of PTE$_{Bd}$ were manually spotted and incubated on Disruptor™ and washed three times with 50 mL of 20 mM HEPES (pH 8.0), 0.2 mM CoCl$_2$. Twenty five microlitres of 1 mM paraoxon were spotted on the resulting sample and an observable yellow colour developed within 5 minutes at 20-25ºC, indicating that immobilization was successful and that PTE$_{Bd}$ retained activity. Buffer from the final wash initially displayed significant paraoxonase activity however filtration through a 0.45 µm filter to remove Disruptor™ fibers eliminated this activity indicating that strong ionic adsorption had been achieved.

Immobilization efficiency was determined by quantifying the amount of protein present in the solution used to wash two Disruptor™ discs onto which a total of 40 µg
was applied. Of the 40 μg pipetted onto the membranes, 36 μg were immobilized, giving an immobilization efficiency of 90%.

4.4 Organophosphate Detection Thresholds with Immobilized PTE<sub>Bd</sub>

PTE<sub>Bd</sub> immobilized on Disruptor<sup>TM</sup> (7 μg of enzyme immobilized per disc) was tested for its ability to detect paraoxon, methyl-paraoxon, parathion, methyl-parathion and coumaphos (Figure 4.2). For OPs whose degradation yields p-nitrophenol, the lowest concentration at which colour change was visible was 0.1 mM. For coumaphos, fluorescence was visible at concentrations as low as 0.05 mM.

4.5 Stability Test with PTE<sub>Bd</sub> Biosensor

To determine the stability of PTE<sub>Bd</sub> on paper, Disruptor<sup>TM</sup>-immobilized enzyme was stored at three different temperatures, -15.5°C, 4°C and 23.5°C, for 40 days and colour intensities generated from paraoxon, parathion and coumaphos treatments were determined every 2 to 5 days. Development of fluorescence was qualitatively assessed while the production of p-nitrophenol was quantified using ImageJ software (Rasband, 1991). From the analysis of p-nitrophenol standards it was found that changes in yellow intensity only affect readings in the blue colour channel (Ng, 2009) (Figure 4.3). Following a 20 minute treatment, biosensors were scanned and the average blue channel value was determined from the generated RGB image file. Over the course of 40 days there was no evident change in the ability of PTE<sub>Bd</sub> to detect paraoxon, parathion (Figure 4.4) and coumaphos and no buffer-spotted controls tested positive for colour development. There was also no difference in stability detected between the various storage temperatures tested. Colour and fluorescence development, although permitted to
Figure 4.2 Evaluation of the paraoxon, parathion and coumaphos sensing ability of PTE$_{Bd}$ immobilized on nanoalumina membrane. Seven micrograms of PTE$_{Bd}$ were spotted onto Disruptor™ (Ahlstrom) membrane and washed three times with 50 mL of 20 mM HEPES (pH 8.0), 0.2 mM CoCl$_2$ prior to exposure to 25 μL of either 1 mM paraoxon (A), 1 mM parathion (B) or 1 mM coumaphos (C). After 5 minutes a clearly visible colour/fluorescence was observed for the enzyme-immobilized paper samples (i). No change was detected on the control papers which were spotted with enzyme storage buffer (ii).
Figure 4.3 Relationship between RGB intensities and $p$-nitrophenol concentration.

Colour intensity was quantified by measuring the average blue channel value from an RGB histogram generated from a digital image of a $p$-nitrophenol-spotted piece of Disruptor™ membrane. Shown here are the average values of each of the red, green and blue colour channels with increasing concentrations of $p$-nitrophenol. No changes were observed in red and green colour intensities up to a $p$-nitrophenol concentration of 10 mM. Measurements were performed in triplicate.
Figure 4.4 Stability of biosensor over 40 days when tested with 1 mM paraoxon and 1 mM parathion. Seven micrograms of PTE$_{Bd}$ were immobilized on Disruptor$^\text{TM}$ (solid symbols) and stored in buffer at 25.5°C (green), 4°C (black) and -15.5°C (blue). Negative controls (unfilled symbols) are Disruptor$^\text{TM}$ spotted with buffer (20 mM HEPES, pH 8.0, containing 0.2 mM CoCl$_2$) and stored under the same conditions. All sensors were tested with 1 mM paraoxon (A) or 1 mM parathion (B) for 20 minutes prior to scanning. Plotted data represent an average of duplicated samples.
occur over a period of 20 minutes, was clearly detectable after 5 minutes for the entirety of the study.

4.6 Discussion

PTE\textsubscript{Bd} is the best-characterized OP-degrading enzyme known and is capable of hydrolyzing paraoxon with catalytic efficiencies close to the diffusion limit. Due to its high activity and relatively broad substrate specificity (Dumas \textit{et al.}, 1989) it is well suited for applications in the detoxification of pesticide pollutants. Attempts to purify PTE\textsubscript{Bd} using bacterial systems have been hampered by difficulties with recombinant expression which is attributed to the presence of a 29-amino-acid-long N-terminal leader peptide used for periplasmic targeting (Gorla \textit{et al.}, 2009). Removal of this signal sequence has been demonstrated to improve expression in a bacterial host (Mulbry and Karns, 1989; Serdar \textit{et al.}, 1989). Indeed without a signal sequence we were able to observe appreciable levels of expression in \textit{E. coli} BL21 (λDE3). However, the protein was insoluble. N-terminally tagged His-PTE\textsubscript{Bd} did not bind to the Ni-column. This could be due to the His-tag being buried in the protein interior rendering it unable to interact with the column.

Expression of soluble PTE\textsubscript{Bd} appeared to improve significantly when introduced into \textit{P. putida} KT2442 as judged from SDS-PAGE gels. De Lorenzo \textit{et al.} (1993) have suggested that metalloenzymes express better in \textit{P. putida} because it possesses the cellular machinery necessary to efficiently incorporate metal cofactor into proteins and chaperone proper enzyme folding. This conclusion was reached after BphC, an enzyme requiring iron as a cofactor, was expressed in \textit{E. coli} and \textit{P. putida} and found to be ten times more active when purified from \textit{P. putida} (De Lorenzo \textit{et al.}, 1993). This could
explain the improved yield of soluble PTE\textsubscript{Bd} observed. Similarly, SsoPox, a homologue of PTE\textsubscript{Bd}, was expressed insolubly in \textit{E. coli} DH5\textalpha{} but not in \textit{P. putida} KT2442 (Ng \textit{et al.}, 2010).

Anion exchange and size-exclusion chromatography was sufficient for the purification of PTE\textsubscript{Bd} to near homogeneity. Purification from \textit{P. putida} KT2442 yielded 3.5 mg/L of culture, approximately 1 mg/g of cells (wet weight), which is comparable to other previously used systems that are summarized in Table 4.1. Our results are similar to those reported for \textit{E. coli} BL21 (Roodveldt and Tawfik, 2005) and \textit{E. coli} DH5\textalpha{} (Omburo \textit{et al.}, 1992) and more favourable than \textit{S. frugiperda} (fall armyworm) (Dumas \textit{et al.}, 1989). Roodveldt \textit{et al.} (2005) were able to obtain soluble PTE\textsubscript{Bd}, N-terminally fused to a maltose-binding domain, from recombinant \textit{E. coli} BL21 grown for at least 12 hours at 30\textdegree{}C and an additional 40 hours at 20\textdegree{}C post-induction. The introduction of a maltose-binding domain to the target protein has been shown to improve soluble yield in \textit{E. coli} (Kapust and Waugh, 1999) and can also be used as an affinity tag for purification. The reported 4 mg/L of culture is an optimistic estimate of soluble maltose-binding domain-fused PTE\textsubscript{Bd} since approximately 95% of expressed protein was insoluble (Roodveldt and Tawfik, 2005), which mirrors our observations using \textit{E. coli} expression systems. Since these results were reported for the fusion protein, not wild type PTE\textsubscript{Bd}, it is difficult to make a direct comparison with the purification of PTE\textsubscript{Bd} described in this thesis. It is also worth noting that the \textit{E. coli} BL21 expression method described by Roodveldt \textit{et al.} (2005) requires two different growth temperatures and an additional day for incubation compared to the method described here for \textit{P. putida} KT2442. Conversely, the purification of PTE\textsubscript{Bd} from \textit{E. coli} DH5\textalpha{} by Omburo \textit{et al.} (1992)
Table 4.1 Summary of protein yield from various PTE$_{Bd}$ expression systems. The expression and purification of PTE$_{Bd}$ has been attempted in bacterial and insect hosts. Expression in *P. putida* KT2442 is similar to that achieved in *E. coli* and *S. frugiperda* but the collection of secreted PTE$_{Bd}$ from *S. lividans* yields significant amounts of protein per culture volume.

<table>
<thead>
<tr>
<th>Expression host</th>
<th>Type</th>
<th>Protein yield</th>
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<tr>
<td></td>
<td></td>
<td>Per culture volume</td>
</tr>
<tr>
<td><em>P. putida</em> KT2442</td>
<td>Bacterial</td>
<td>3.5 mg/L</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (λDE3)</td>
<td>Bacterial</td>
<td>≤ 4 mg/L</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>Bacterial</td>
<td>NR</td>
</tr>
<tr>
<td><em>S. lividans</em> 66</td>
<td>Bacterial</td>
<td>10 – 30 mg/L</td>
</tr>
<tr>
<td><em>S. frugiperda</em></td>
<td>Insect</td>
<td>NA</td>
</tr>
</tbody>
</table>

NR: Not reported  
NA: Not applicable  
1 Data reported by Roodveldt *et al.* (2005)  
2 Data reported by Omburo *et al.* (1992)  
3 Data reported by Rowland *et al.* (1991)  
4 Data reported by Dumas *et al.* (1989)
achieved almost twice as much yield of protein than in *P. putida* KT2442 and exhibited 25-fold higher activity towards paraoxon. This was accomplished using a plasmid containing a *lac* promoter and a gene encoding a modified PTE$_{Bd}$ containing an N-terminal Met-Ile-Thr-Asn-Ser sequence derived from the N-terminus of the *lacZ* gene in place of the signal peptide (Mulbry and Karns, 1989). Cells were grown to stationary phase at 30°C (35 - 38 hours) prior to harvesting and purification involved four steps including protamine sulfate and ammonium sulfate precipitation followed by two gel-filtration columns. Again, because the PTE$_{Bd}$ purified from *E. coli* DH5α contained additional N-terminal amino acids it cannot be considered a true wild type PTE$_{Bd}$ expression system. In the future it would be interesting to clone our PTE$_{Bd}$ construct into a vector containing the *lac* promoter and attempt expression in *E. coli* DH5α following the protocol used by Omburo *et al.* (1992).

Expression of PTE$_{Bd}$ in *P. putida* KT2442 yielded 3-fold higher amounts of soluble protein per gram of cells compared to the baculovirus expression system (Dumas *et al.*, 1989). Adding to the disadvantage of this low yield system is the higher cost associated with insect cell cultures and a four day incubation cycle. In the 1980s and 1990s when this expression method was introduced, the focus was largely on the use of crude lysate to treat OP-contaminated solutions (Dumas *et al.*, 1989) or to examine *in situ* methods of detoxification (Phillips *et al.*, 1990). This may explain why no further optimization has been attempted. Despite the low yield and considerable growth time involved, PTE$_{Bd}$ purified from *S. frugiperda* exhibits the highest catalytic constant towards paraoxon of any recombinant PTE$_{Bd}$ known to date.
Another promising method of PTE\textsubscript{Bd} purification utilizes the bacterium, \textit{S. lividans}, because, being Gram positive, it can secrete soluble protein into the media thus simplifying downstream purification (Steiert \textit{et al.}, 1989; Rowland \textit{et al.}, 1991). Between 10 and 30 mg/L of PTE\textsubscript{Bd} were obtained by batch-feeding cultures of \textit{S. lividans} over several days before precipitating enzyme from the extracellular fluid with ammonium sulfate and redissolving it in buffer. This method yields markedly higher amounts of PTE\textsubscript{Bd}, of at least 3-fold improvement, with respect to the technique discussed in this thesis, however this method involves the maintenance of cultures over a period of many days and this requires additional resources and careful monitoring. Comparatively, expression in \textit{P. putida} KT2442 requires overnight incubation following induction and protein can be liberated by a short lysozyme incubation and sonication treatment. Despite these theoretical advantages PTE\textsubscript{Bd} purified from \textit{P. putida} KT2442 exhibited a specificity constant towards paraoxon that was 27-fold lower than that reported for insect cell-purified enzyme which possesses the highest documented $k\textsubscript{cat}/K\textsubscript{M}$ (Dumas \textit{et al.}, 1989). This reduction could be attributed to the presence of contaminating proteins that are visible on the SDS-polyacrylamide gel. Nevertheless, since our interests lay in the production of a PTE\textsubscript{Bd}-based biosensor, and the activity and purity attained using \textit{P. putida} KT2442 was sufficient to generate a colorimetric response on membrane, no further optimization was attempted.

Two different methods were attempted for immobilizing PTE\textsubscript{Bd} to paper. PTE\textsubscript{Bd}-CBD fusion protein would allow for purification and immobilization in a single step and selective immobilization would have been possible on a variety of cellulose-based substrates. However overexpressed PTE\textsubscript{Bd}-CBD was insoluble in the \textit{E. coli} host system.
The aggregation of insoluble protein could be explained by improper folding due to the presence of a 15 kDa C-terminal CBD from \textit{C. fimi}. Richins \textit{et al.} (2000) have previously reported that C-terminal fusion of a CBD from \textit{Cellulomonas} to PTE$_{Bd}$ yielded insoluble enzyme however N-terminal fusion generated soluble active protein. Due to time constraints and the questionable effect of the CBD on PTE$_{Bd}$ solubility we opted to continue expression and purification endeavors in \textit{P. putida} KT2442 using unfused PTE$_{Bd}$ rather than exploring alternative fusion constructs.

PTE$_{Bd}$ can be quickly and easily immobilized using ionic interactions with Disruptor™ nano alumina membrane. The interaction between PTE$_{Bd}$ and un laminated Disruptor™ is strong and the final wash solution has no detectable paraoxonase activity.

Ten OPs were tested for their potential for rapid visible colorimetric or fluorescent response on membrane-immobilized PTE$_{Bd}$. Table 4.2 lists the common applications of these OPs and their registration status with the U.S. Environmental Protection Agency. Reactions involving DTNB did not yield a visible increase in colour intensity. Ultimately work with DTNB was not pursued further because the compound itself exhibits a faint yellow colouration that would have made distinguishing the detection threshold difficult on Disruptor™. To demonstrate that the lack of fluorescence in the azinphos-methyl reaction resulted from insufficient hydrolysis, 250 µL of 10 M NaOH was added to a 24 hour old reaction to promote rapid degradation. After 5 minutes this positive control exhibited bright yellow fluroescence under UV light. It is important to note that paraoxon, methyl-paraoxon and parathion are banned in most countries due to their high toxicity (U.S. Environmental Protection Agency, 2011). In the interest of characterizing the first Disruptor™-immobilized PTE$_{Bd}$ prototype we chose to
Table 4.2  Tested organophosphates and their applications.  Paraoxon, methyl-paraoxon and parathion are currently banned by the U.S. Environmental Protection Agency due to their high toxicity to vertebrates and invertebrates.  Total phase-out of azinphos-methyl and methyl-parathion usage is scheduled to complete in 2012 and 2013, respectively. Data was provided by the U.S. Environmental Protection Agency (2011).

<table>
<thead>
<tr>
<th>Organophosphate</th>
<th>Type of pesticide</th>
</tr>
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<tbody>
<tr>
<td>paraoxon *</td>
<td>Crop</td>
</tr>
<tr>
<td>methyl-paraoxon *</td>
<td>Crop</td>
</tr>
<tr>
<td>parathion *</td>
<td>Crop</td>
</tr>
<tr>
<td>methyl-parathion</td>
<td>Crop</td>
</tr>
<tr>
<td>chlorpyrifos</td>
<td>Crop, Grass</td>
</tr>
<tr>
<td>phosmet</td>
<td>Crop, Household, Pets</td>
</tr>
<tr>
<td>terbufos</td>
<td>Crop</td>
</tr>
<tr>
<td>malathion</td>
<td>Crop, Household</td>
</tr>
<tr>
<td>azinphos-methyl</td>
<td>Crop</td>
</tr>
<tr>
<td>coumaphos</td>
<td>Livestock</td>
</tr>
</tbody>
</table>

* Products not registered with the U.S. Environmental Protection Agency
use these compounds in spite of their limited downstream suitability. Methyl-parathion and coumaphos remain relevant today however if this bioactive product is to ever be commercially feasible substantial investigation must be performed to identify other pesticides that produce detectable products when degraded by PTE_{Bd}. Current OP detection methods involve the analysis of soil or biomass digests that have been concentrated from large volumes by liquid or gas chromatography and mass spectrometry (U.S. Department of Health, 2001; Rissato et al., 2005). These techniques are highly sensitive and can determine whether food and environmental samples satisfy the safety limits for OPs. In Canada, soil and water screening techniques have a detection limit of 0.3 pM for OPs (Health Canada, 1986). The maximum acceptable concentration of parathion in drinking water, as designated by Health Canada, is 0.2 μM. The visual detection limit of paraoxon, methyl-paraoxon, parathion and methyl-parathion when spotted on Disruptor™-immobilized PTE_{Bd} is 100 μM which corresponds with the lowest visual detection threshold of p-nitrophenol (Table 4.3) (Ng, 2009). Due to the distinctive blue fluorescence that results from its degradation, coumaphos concentrations as low as 50 μM can be detected (Table 4.3). In light of this limitation some adjustments would be necessary in order to make the PTE_{Bd} biosensor more sensitive. For instance the sample could be concentrated prior to treatment. As previously mentioned, current methods of detection require the concentration of solvent extractant prior to analysis and these existing protocols could be modified for smaller volumes and use on a hand-held biosensor. A highly sensitive colorimetric sensor has been developed by Hossain et al. (2009) that employs a acetylcholinesterase-indophenyl acetate coupled system to detect paraoxon concentrations as low as 1 nM. This sensor relies on the inhibition of
Table 4.3 Lowest detection limits determined with membrane-immobilized PTE$_{Bd}$.

Seven micrograms of PTE$_{Bd}$ was spotted onto Disruptor™ (Ahlstrom) membrane and washed three times with 50 mL of 20 mM HEPES (pH 8.0), 0.2 mM CoCl$_2$ prior to exposure to 25 $\mu$L of varying concentrations of paraoxon, methyl-paraoxon, parathion, methyl-parathion and coumaphos. The detection threshold is defined as the lowest OP concentration that produced a visible colour response within 1 hour.

<table>
<thead>
<tr>
<th>Organophosphate</th>
<th>Lowest detectable concentration ((\mu)M)</th>
</tr>
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<tbody>
<tr>
<td>paraoxon</td>
<td>100</td>
</tr>
<tr>
<td>methyl-paraoxon</td>
<td>100</td>
</tr>
<tr>
<td>parathion</td>
<td>100</td>
</tr>
<tr>
<td>methyl-parathion</td>
<td>100</td>
</tr>
<tr>
<td>coumaphos</td>
<td>50</td>
</tr>
</tbody>
</table>
acetylcholinesterase to prevent the hydrolysis of indophenyl acetate into the blue product, indophenoxide. The weakness of this system is its susceptibility to false-positives as many compounds in nature are capable of inhibiting acetylcholinesterase, such as mycological aflatoxins or carbamate-based insecticides. Therefore further testing would be required to identify the compound responsible for a positive test result. This detection method employs capillary action through the membrane to move substrate over an enzyme-immobilized region of the sensor. This method could be translated into a PTE_{Bd}-based system wherein fresh substrate could come into contact with the enzyme to yield more $p$-nitrophenol thus resulting in an increase in the perceived yellow colour intensity.

When tested with 1 mM paraoxon, parathion and coumaphos, the sensors produced 100% of their original colour intensity after 40 days of storage under wet conditions. These findings agree with LeJune and Russell (1996), who demonstrated that covalently-immobilized PTE_{Bd} possesses a half-life of three months, and Richins et al. (2000) who observed only a 15% loss in activity in immobilized CBD-PTE_{Bd} after 30 days. To determine the true shelf-life of buffer-stored biosensor it would be necessary to complete an extended stability trial. It would also worthwhile to investigate the stability of PTE_{Bd} on Disruptor™ at elevated temperatures to emulate the outdoor environment where it may be used.

The stability curves shown in Figure 4.4 exhibit a significant amount of variability for both immobilized PTE_{Bd} and controls and several factors account for this. First, biosensors are placed between two transparencies prior to scanning. These transparencies contain imperfections in their surface which may alter the colour of the captured image. Secondly Disruptor™, as an unlaminated membrane, possesses a non-
uniform fibrous surface that may affect readings. Third, colour distribution across each biosensor was often irregular and depended on the presence of excess storage buffer and the exact region of pesticide addition to the membrane. Since the central area of each disc was selected for colour quantification this might affect readings. It is worth pointing out that the amount of variation shown on the stability curves is not significant enough to yield a visible difference in yellow colour intensity. For example, the colour observed on day 0 and day 40 for 4°C-stored biosensor tested with paraoxon was the same despite a difference in approximately 15 units.

There are advantages to using Disruptor™-immobilized PTE_Bd for the detection of OPs. PTE_Bd can be expressed and purified from bacteria and scaled for affordable, continuous, large-scale production in an industrial setting. In addition to being inexpensive, the simple addition and short incubation of protein on nano alumina membrane yields a sensor that can be used in the field without the need for expensive materials, special equipment and trained scientists. Before these advantages can be exploited, however, two primary obstacles must be overcome. First, PTE_Bd is active towards a broad range of OPs but we have only identified 5 that yield visible results within a practical time frame and only 2 of these are commonly used today in the developed world. Other methods for the detection of OP degradation products must be investigated and further optimization may be necessary. For example mutagenesis may be employed to increase the efficiency of PTE_Bd towards other substrates such as azinphos-methyl. The aforementioned second hurdle involves raising the sensitivity of Disruptor™-bound PTE_Bd so that it performs its function with environmentally and physiologically relevant concentrations of substrate.
CONCLUSIONS AND FUTURE WORK

We have successfully characterized the R223K, R223Q, Y97F, Y99F, Y97F/Y99F and L226F variants of SsoPox. R223 in SsoPox was hypothesized to stabilize a negative reaction intermediate formed during AHL hydrolysis. Substitution of R223 by lysine and glutamine does not alter AHL activity and refutes our proposed role for this residue. The Y97F and Y97F/Y99F variants completely eliminated AHL lactonase activity but retained 20- and 2-fold reduced paraoxonase activity, respectively. This demonstrates that Y97 is crucial for AHL hydrolysis and is consistent with the proposed theory that this tyrosine is responsible for either the initial binding of AHL substrate, or the stabilization of the negative reaction intermediate formed during AHL turnover, or both. Enzyme inhibition studies with Y97F in the presence of paraoxon and 3-oxo-C8-HSL would shed light on whether the lack of AHL activity in the Y97 variant is due to defective substrate binding or catalysis. Y99 does not appear essential for the paraoxonase or AHL lactonase activity of SsoPox as proposed for equivalent tyrosines in related PLLs.

SsoPox exhibits higher specificity constants towards long-chain AHLs. We hypothesized that the introduction of a bulky amino acid into the binding tunnel that accommodates the acyl-chain of AHLs would alter this preference to favour shorter substrates. The specificity constant of L226F did not change towards C4-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL or 3-oxo-C12-HSL but dropped 37-fold towards 3-oxo-C10-HSL. While we did not observe the predicted shift in substrate preference this finding does support the idea that tunnel modification can alter substrate specificity. It is difficult to conceptualize a structural explanation for the observed activity loss of L226F toward
3-oxo-C10-HSL however a crystal structure of L226F in complex with this or a longer substrate such as 3-oxo-C12-HSL could shed light on the nature of this interaction.

To our knowledge this thesis describes the first successful expression and purification of soluble PTE\textsubscript{Bd} from \textit{P. putida} KT2442. The average protein yield was 3.5 mg/L of culture and this method, involving two chromatographic steps, compares well to previous protocols using bacterial and baculovirus expression systems. To obtain a higher yield PTE\textsubscript{Bd} expression in \textit{P. putida} KT2442 will need to be better optimized. Alternatively, future work could involve the expression of recombinant PTE\textsubscript{Bd} containing no leader peptide in a \textit{lac} promoter-controlled \textit{E. coli} DH\textsubscript{5}\textalpha\ system similar to that described by Mulbry and Karns (1989) and Omburo (1992).

Furthermore we have developed a novel OP-detection system involving nano alumina membrane-bound PTE\textsubscript{Bd} that is capable of detecting the presence of paraoxon, methyl-paraoxon, parathion and methyl-parathion at concentrations as low as 100 \(\mu\)M and coumpahos as low as 50 \(\mu\)M. The immobilization of PTE\textsubscript{Bd} to Disruptor\textsuperscript{TM} was over 90\% efficient. A 40 day stability test of PTE\textsubscript{Bd} biosensor stored in three different temperatures revealed no detectable loss of efficacy. Further optimization is necessary to increase the sensitivity and substrate specificity of PTE\textsubscript{Bd} for biosensing. PTE\textsubscript{Bd} variants have been generated and exhibit higher specificity constants towards paraoxon, methyl-paraoxon, malathion, demeton S and nerve agents such as sarin, soman and VX (Yang \textit{et al.}, 2003; Cho \textit{et al.}, 2004; Reeves \textit{et al.}, 2008; Schofield and Dinovo, 2010; Tsai \textit{et al.}, 2010; Briseno-Roa \textit{et al.}, 2011). These findings hint at the potential application of membrane-immobilized PTE\textsubscript{Bd} as a chemical weapon-detecting sensor in the near future.
REFERENCES


APPENDIX I – Media and Solutions

**Luria-Bertani Broth**

1% (w/v) tryptone

0.5% (w/v) yeast extract

1% (w/v) NaCl

**Plasmid Preparation Solution I**

50 mM glucose

25 mM Tris-HCl (pH 8.0)

10 mM EDTA (pH 8.0)

200 µg/mL RNase A

**Plasmid Preparation Solution II**

1% (w/v) SDS

0.2 M NaOH

**Plasmid Preparation Solution III**

5 M sodium acetate (pH 5.2)  60 mL

Glacial acetic acid     11.5 mL

dH₂O              28.5 mL
**SOC**

2% (w/v) tryptone

0.5% (w/v) yeast extract

10 mM NaCl

2.5 mM KCl

10 mM MgCl₂

10 mM MgSO₄

20 mM glucose

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**DNA loading buffer (10 mL of 10X concentrate)**

Bromophenol blue 25 mg

10% SDS 20 µL

0.5 M EDTA (pH 8.0) 100 µL

Glycerol 3 mL

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**Lysis buffer for rapid supercoiled plasmid analysis (10 mL)**

Sucrose 1 g

KCl 0.447 g

0.5 M EDTA (pH 8.0) 100 µL

10% SDS 250 µL

2 N NaOH 500 µL

Bromophenol blue 5 mg
Lysis buffer for use with Ni-NTA agarose resin (1 L)

50 mM NaH$_2$PO$_4$ 6.90 g
300 mM NaCl 17.54 g
10 mM imidazole 0.68 g

Adjusted to pH 8.0 with NaOH

Wash buffer for use with Ni-NTA agarose resin (1 L)

50 mM NaH$_2$PO$_4$ 6.90 g
300 mM NaCl 17.54 g
20 mM imidazole 1.36 g

Adjusted to pH 8.0 with NaOH

Elution buffer for use with Ni-NTA agarose resin (1 L)

50 mM NaH$_2$PO$_4$ 6.90 g
300 mM NaCl 17.54 g
250 mM imidazole 17 g

Adjusted to pH 8.0 with NaOH
**SDS-PAGE Gels**

**10% separating gel (10 mL)**

- 30% (w/v) acrylamide: 3.3 mL
- 1.5 M Tris-HCl (pH 8.8): 2.5 mL
- 10% (w/v) SDS: 100 µL
- 10% (w/v) ammonium persulfate: 100 µL
- TEMED: 4 µL
- dH₂O: 4 mL

**5% stacking gel (3 mL)**

- 30% (w/v) acrylamide: 0.5 mL
- 1.0 M Tris-HCl (pH 6.8): 0.38 mL
- 10% (w/v) SDS: 30 µL
- 10% (w/v) ammonium persulfate: 30 µL
- TEMED: 3 µL
- dH₂O: 2.1 mL

**SDS loading buffer (10 mL of 2X concentrate)**

- 0.5 M Tris-HCl (pH 6.8): 2.5 mL
- 10% SDS: 4 mL
- Glycerol: 2 mL
- DTT: 0.23 g
- Bromophenol blue: 5 mg
**SDS running buffer (1 L of 5X concentrate)**

Tris  15 g  
Glycine  72 g  
SDS  5 g  

dH$_2$O was added to a final volume of 1 L

**TAE buffer (1 L of 50X concentrate)**

Tris  242 g  
Glacial acetic acid  57.1 mL  
0.5 M EDTA (pH 8.0)  100 mL

**Commassie stain solution**

Commassie Brilliant Blue R-250  0.4 mg  
Methanol  200 mL  
Acetic acid  40 mL  

dH$_2$O was added to a final volume of 600 mL

**Destain solution**

Methanol  100 mL  
Acetic acid  100 mL  

dH$_2$O was added to a final volume of 1 L
**5X Bradford reagent**

Commassie Brilliant Blue G-250  100 mg

85% phosphoric acid  100 mL

95% ethanol  50 mL

Commassie Brilliant Blue G-250 was dissolved in ethanol prior to the addition of phosphoric acid and water to a final volume of 200 mL. The solution was filtered through Whatman 1 filter paper.
APPENDIX II – Sequences

DNA sequence of the gene encoding PTE\textsubscript{Bd} containing leader peptide and CBD

ATGCAAACGCGTGCGTTGTGCTCAAGTCTGCGGCCGCCGCAGGAACTCTGCTCGGCGGCCTGGCTG
TTGGTGGCGCGAGGCGGTGGCTGATCGATCGGAGATGGTGGCTGGATCGATTGGCACAGGCGATCGGATC
AATACCGTGCGCGGTCCTACTACAATCTCTGAAGCGGGTTTCACACTGACTCACGAGCACATCTGCGG
CAGCTCGGCAGGATTCCTGGCGTGCTTGGCCAGAGTTCTTCGGTAGCCGCAAAGCTCTAGCGGAAAAGG
CTGTGAGAGGATTGCGCCGCGCCAGAGCGGCTGGCGTGCGAACGATTGTCGATGTGTCGACTTTCGATAT
CGGTCGCGACGTCAGTTTATTGGCCGAGGTTTCGCGGGCTGCCGACGTTCATATCGTGGCGGCGACCG
GTGGTTCGACCCGCACTTTCGATGCGATTGAGAGTGTAGAGGAAACTCAATATGGCATCGAAGACAC
CGGAATTAGGGCGGGCATTATCAAGGTCGCGACCACAGGCAAGGCGACCCCCTTCAGGAGTTAGTGT
TTAAAGCGCGGCCGCCGCCGCCGACGTGTGGCCACACCCCGT

GTCCGGTAACACCCTCACACGCGAGCAAGTCACGCAGCGATGGTAGACACAGCAGCCATTTTTGA
GTCCGAAGGCTTGCAGCCTTCACGGGTTCGTTGTATCTGGTCACAGCGATGATCTGACGATTTGTAGCT
ATCTCACCAGGCTCCTCGGCGCAGATACTTTCGTTCATGACATCCGGCGACAGTGAGTT

GGTCGTTAGATATGCGATGCACTACCGCGCTCTTGCGGCTGTCATCGTGCGAATGGTGCCCCGTGGAAC
GGTCTGGTCAGTCTGACACTGCTCCCTGCGGGCATTCGCGCAACACGGCTCT

CTTGATCAAGCCGCTCATCGAACCAGGTCATATGAAACAAATCCTCGTTTCGAATGACTGGCTGT
TCGGGTTTCTCGAGCTATGTACCAAAATCTGAGACGTAGATCGTGCAGGCGGACACCCGAGGATG
GCTCTCATATCCAGAGTGGACTTCTCCATACGAGAAAGGCGTCCACAGAGAAACCGCTGGC
AGGCACTATCGTGACTAACCAGGCGGGGTCTGTGATACCAGCCTTTGCGGGCTGTGTAACTCC
CAACCCCGACGCGGACGCCAACGCCAGCCAGCCACTACCGACCTGGCGCTGGGTTGCTAC

GTCTCGTGGGTTGGAATGATGGAACACGGGTATTTACGGCCAGGTAGCGGCTAAAAATACCGG
TAGCCCGCCTCGTTGATGGTGCCCTTACCGGACTTTACTGGCTGGAGCAGGTACCGTGGGCACAGGG
CGTGGAGACGACAGGTTACCGGCGGTTACCGGTGTAACCGGCTGGTGGTAGGACTGAAGCTT

AACATCCTGGCGGGTGCGCAGCCGCTCAGGTTGTTGCTTGGCCAGCCATCGCGACCAATCGCCGC
CCGACCAGCTTTTAGCTGAATGGTGCCCCGTGTACCGGTTGTTGAGTTAACTGAAGCTT