Screening Bacteria in the Presence of Organophosphorus Compounds

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Abstract

Organophosphorus acid anhydrolase (OPH) is an enzyme capable of degrading a wide range of Organophosphorus compounds (Dumas et al. 1989). The limited range of optimal activity prevents the use of OPH for commercial application. Directed Evolution shows great promise in the generation of a modified enzymes with improved functionality. For commercial application of OPH, improved activity at higher temperatures and lower pH would be required. The purpose of this experiment is to develop the parameters necessary for the selection of improved forms of \textit{opd}. \textit{Escherichia coli} and \textit{Lactococcus lactis} were selected for potential recipient cells of randomly-mutated forms of \textit{opd}. \textit{E. coli} was grown at 49°C and \textit{L. lactis} was grown at 37°C. Both \textit{E. coli} and \textit{L. lactis} were grown in the presence of different OP compounds at concentrations ranging from 0 mM to 16 mM and the minimum inhibitory concentration of each compound was determined which has the potential to serve as the basis for selection of an improved OPH based on growth of the recipient cells under selective conditions.

Introduction

Organophosphate (OP) compounds are highly toxic due to their ability to inhibit activity of acetylcholinesterase (AChE) which mediates the hydrolysis of acetylcholine, a neurotransmitter that plays an important role in transmitting nerve impulses in the brain, skeletal and muscular systems. Acetylcholine is hydrolyzed by AChE to avoid overstimulation of the nervous systems (Karpouzas & Singh 2006).

There has been an increase in concern about national security due to potential threats that OP compounds have for use as nerve agents for chemical warfare (Cho et al. 2004). In addition, the widespread use of OP compounds in agricultural, domestic, and veterinary use since 1973 has led to contamination of soil and water systems in all parts of the world (Karpouzas & Singh 2006). Improved methods for detection and decontamination of OP compounds are needed (Cho et al. 2004).

A group of enzymes classified as Organophophorus Acid Anhydrolases (OPAA) have the fortuitous ability to catalyze the hydrolysis of many highly toxic, acetylcholinesterase-inhibiting compounds, including chemical warfare G-type nerve agents and pesticides (Cheng et al. 1996).
OP compounds serve as a source of phosphorus and carbon for these microorganisms that poses an enzyme capable of hydrolyzing them (Karpouzas & Singh 2006).

The first OP-hydrolyzing enzyme gene to be cloned, sequenced, and expressed was organophosphorus acid hydrolase (OPH; EC 3.1.8.1). OPH was isolated from *Pseudomonas diminuta* MG and *Flavobacterium* sp. Strain ATCC 27551 (Cheng et al. 1996). OPH has received a lot of the attention due to its capability to degrade a wide range of OP compounds including chemical warfare agents and pesticides such as parathion, methyl parathion and fensulfothion, among many others (Dumas et al. 1989; Karpouzas & Singh 2006). OPH is a well-characterized metalloenzyme which typically contains one to two ions of zinc or cobalt (Cheng et al. 1996). OPH exhibits an optimal activity at 25°C and pH 7 (Dumas et al. 1989). Unfortunately, commercial application would reportedly require low pH and high temperature conditions that are well outside the activity range of the wild type enzyme (Kuchner & Arnold 1997).

Directed Evolution shows great promise in optimizing enzymatic activity for specific applications by generating vast sequence diversity followed by selection and screening of the mutated genes (Eijsink et al. 2005). Techniques such as error-prone PCR, chemical mutagenesis or UV irradiation have been used to randomly change gene sequences (Kuchner & Arnold 1997) thereby generating large numbers of related proteins with different amino acid sequences.

Taguchi et al. performed directed evolution for the cold adaptation of subtilisin BPN’, a mesophilic and industrially useful alkaline serine protease, by chemical mutagenesis of a wild-type subtilisin gene. The mutated genes were transformed into *Escherichia coli* JM109 and plated and incubated at 37°C overnight and then further incubated for 2 days at 10°C. Screening of the mutant subtilisins showing activity under these low temperature conditions were
determined by the initial rate of clear-zone formation around the transformed colonies. The mutant enzymes were then isolated and purified and an assay was carried out for both the wild-type and mutant form enzyme at various temperatures by monitoring enzymatic hydrolysis of AAPF. One candidate mutant was found showing 100% increase in subtilisin activity at 10°C (1998).

Cho et al. performed directed evolution on OPH to improve the hydrolysis of a poorly hydrolysable substrate, chlorpyrifos. DNA shuffling was performed following rapid pre-screening for improved OPH variants involving a plate assay based on the formation of clear haloes due to chlorpyrifos hydrolysis. Variants were then isolated and purified. Variants were then used to hydrolyze chorpyrifos which was measured by changes in absorbance at 276 nm. The best variant exhibited a 715-fold increase in specific activity as compared to the wild-type OPH (2004).

A critical part of a directed evolution application is the selection process. Ideally, selection could be tied to bacterial survival such that cells expressing an improved version of the enzyme would out-compete those cells possessing the wild-type version. Accordingly, an effective selection method must be developed before improvements in OPH activity at elevated temperatures and lower pH levels can be attempted.

*E. coli* and *L. lactis* are two bacteria which might be used to select for improved activity of OPH. *E. coli* has an optimum growth temperature of 37°C but has been shown to grow at 49°C (Fotadar et al. 2005) and may be a potential host for the selection of elevated temperature OPH variants. *L. lactis* is a lactic acid bacterium with an optimal growth temperature of 37°C and the ability to thrive at low pH; this strain may serve as a potential host for the selection of low pH OPH variants.
Both PMSF and DFP are serine protease inhibitors. At elevated temperatures *E. coli* expresses PepQ, a protease that degrades denatured proteins (Park et al. 2004). Due to the accumulation of misfolded proteins at elevated temperatures, cells lacking PepQ are unable to survive above 42°C. The inhibition of PepQ by DFP and/or PMSF may serve as the basis for a selection mechanism that is able to prevent growth of those cells that lack OP hydrolyzing activity at elevated temperatures.

The purpose of this experiment is to develop the parameters necessary for the selection of *opd* gene variants, through a directed evolution protocol, that exhibit improved activity at elevated temperatures or lower pH levels.

**Methods**

**Bacterial strains and plasmid.** *E. coli* JM109 was grown in LB media at 37°C for routine maintenance. *L. lactis* was maintained in MRS media at 37°C. For comparative studies, OPH was expressed with pKL*opd* in *E. coli*. Ampicillin was included in the medium at 150 µg/ml to produce selective pressure for the maintenance of this plasmid.

**Organophosphorus compounds.** Paraoxon, Coumaphos, Demeton-S, Parathion, and Methyl-Parathion were obtained from Chem Service, Inc (West Chester, PA). Diisopropylfluorophosphate (DFP) and Phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich, Inc (St. Louis, MO). Paraoxon was dissolved in deionized water to 13 mM. Coumaphos was dissolved in toluene to 11 mM. Demeton-S was dissolved in methanol to 15 mM. DFP was dissolved in isopropanol to 2.7 M. Parathion was dissolved in toluene to 57 mM. Methyl-Parathion was dissolved in toluene to 94 mM. Phenylmethylsulfonyl fluoride...
(PMSF) was dissolved in isopropanol to 100 mM. After preparing OP compound solutions, the solution were stored at -20°C.

**Temperature-related assays.** *E. coli* JM109 was incubated at 49°C in the presence of the each OP at concentrations of 0 mM to 16 mM. The minimum inhibitory concentration under these growth conditions was determined for each assay. Upon identification of the minimum inhibitory concentration, the assay was repeated with *E. coli* JM109-pKLopd at the minimum inhibitory concentration to determine the effect of OPH expression on growth. Each assay was prepared and incubated for 16 hours. The optical density of each sample was measured at 600 nm.

**Acidity-related assays.** *L. lactis* was grown at 37°C in the presence of each OP at concentrations of 0 mM to 16 mM. Each assay was prepared and incubated for 16 hours. The optical density of each sample was measured at 600 nm.

**Results**

![Graph showing absorbance at 600 nm for L. lactis grown in the presence of OP compounds with various concentrations of methyl parathion, DFP, paraoxon, parathion, coumaphos, demeton S, and PMSF.](image)
Fig. 1 – The growth of *L. lactis* in the presence of the different OP compounds shows an over-all decreasing trend with increased concentration of each OP compound. The minimum inhibitory concentration was determined for each OP compound based on the absence of growth of *L. lactis*. The toxic effects of the solvents: methanol, isopropanol, and toluene occurred at volumes greater than the volume of the minimum inhibitory concentration.

![E. coli JM109 Grown in the Presence of OP Compounds at 49°C](image)

Fig. 2 - *E. coli* JM109 grown in the presence the OP compounds shows an overall decreasing trend with increasing concentrations. The minimum inhibitory concentration was determined based on the absence of growth of *E. coli*. The toxic effects of the solvents: methanol, isopropanol, and toluene occurred at volumes greater than the volume of the minimum inhibitory concentration.
Fig. 3 – In the presence of PMSF at 37°C growth was observed even at 4 mM which is the minimum inhibitory concentration at 49°C (Fig. 2).

Table 1 – The minimum inhibitory concentrations at which no growth was observed for both *E. coli* JM109 and *L. lactis* are listed. Both *E. coli* JM109 and *E. coli* JM109-pKLopd have the same minimum inhibitory concentrations.

<table>
<thead>
<tr>
<th>OP Compounds</th>
<th><em>E. coli</em> JM109</th>
<th><em>E. coli</em> JM109-pKLopd</th>
<th><em>L. lactis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF</td>
<td>4 mM</td>
<td>4 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>DFP</td>
<td>16 mM</td>
<td>16 mM</td>
<td>16 mM</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>14 mM</td>
<td>14 mM</td>
<td>14 mM</td>
</tr>
<tr>
<td>Coumaphos</td>
<td>n.a.</td>
<td>n.a.</td>
<td>12 mM</td>
</tr>
<tr>
<td>Demeton S</td>
<td>n.a.</td>
<td>n.a.</td>
<td>10 mM</td>
</tr>
<tr>
<td>Parathion</td>
<td>8 mM</td>
<td>8 mM</td>
<td>12 mM</td>
</tr>
<tr>
<td>Methyl Parathion</td>
<td>10 mM</td>
<td>10 mM</td>
<td>14 mM</td>
</tr>
</tbody>
</table>

**Discussion**

In the presence of each OP compound, the growth of both *E. coli* JM109 and *L. lactis* decreased with increase in OP concentration. The concentrations of Coumaphos and Demeton S could not be used for *E. coli* JM109 to determine the minimum inhibitory concentration due to the amount of toluene and methanol in the solution which alone caused death to *E. coli* JM109 cells.
The growth of *E. coli* at 37°C at 4 mM of PMSF and the absence of growth of at 49°C at 4 mM of PMSF suggests that at 49°C, PepQ is being inhibited. Further assays can be carried out at 37°C with *E. coli* in the presence of each of the other OP compounds to determine if the same outcome is observed. If the same outcomes are observed as with PMSF, it would further clarify that PepQ is being inhibited in all cases at 49°C, thus preventing growth of cells at the minimum inhibitory concentrations.

The minimum inhibitory concentrations were determined based on the lowest concentration of OP compound observed to inhibit the growth of the bacterial cells. Table 1 depicts the minimum inhibitory concentrations which may serve as the parameters necessary for selection of potential mutated forms of the *opd* gene. *E. coli* JM109 and *L. lactis* will serve as recipient cells for the mutated forms of the *opd* gene. Selection will be based on the presence of growth at the minimum inhibitory concentrations of each OP compound which would indicate the presence of an improved OPH enzyme.

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**References**


