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Detoxification of Sarin, an Acetylcholinesterase Inhibitor, by Recombinant Organophosphorus Acid Anhydrolase

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Pesticide waste and chemical stockpiles are posing a potential threat to both the environment and human health. There is currently a great effort toward developing effective and economical methods for the detoxification of these toxic organophosphates. In terms of safety and economy, enzymatic biodegradation has been recommended as the most promising tool to detoxify these toxic materials. To develop an enzymatic degradation method to detoxify such toxic organophosphorus compounds, a gene encoding organophosphorus acid anhydrolase (OPAA) from genomic DNA of *Alteromonas haloplanktis* C was subcloned and expressed. The enzyme consists of a single polypeptide chain with a molecular weight of 48 kDa. It demonstrates strong hydrolyzing activity on sarin, an acetylcholinesterase inhibitor. Moreover, its high activity is sustained for a considerable length of time. It is projected that the recombinant OPAA can be applied as an enzymatic tool that can be used not only for the detoxification of pesticide wastes, but also for the demilitarization of chemical stockpiles.

Keywords: Organophosphorus acid anhydrolase (OPAA), Detoxification, Sarin (isopropyl methylphosphonofluoridate)

Introduction

Organophosphorus compourds are extensively used as agricultural and domestic pesticides. These include insecticides, fungicides, and herbicides (McDaniel *et al.*, 1988). Over 30 million kilograms of these compounds are used annually in the United States (FAO, 1983). The usefulness of these compounds is due to their major effect on the central and peripheral nervous system via acetylcholinesterase (AChE) inhibition. Indeed, organophosphorus compounds of certain structures are so toxic that they are commonly used as nerve gas agents (Robinson, 1980). High level exposure of these compounds causes both acute and serious health consequences, and eventually results in delayed chronic cholinergic and neurological toxicity (Ali *et al.*, 1983).

Even though the efficacies of many pesticides are very useful, the residual compounds pose a significant threat to field contamination, such as soil and water contamination, as well as to the accumulation of these pesticides in food products. In addition to such pesticide wastes, there is another category of toxic chemical compounds that pose even severer global concern to human beings. They are chemical weapons that were produced by many countries during World Wars I & II, as well as the Cold War. Currently a huge amount of chemical stockpiles exist throughout the world. These troublesome chemical compounds have been recognized as one of the hot international issues that need to be resolved. Thanks to the efforts of many western countries over a couple of decades, however, it was finally agreed that these chemical stockpiles should be destroyed under the supervision of the Organization for the Prohibition of Chemical Weapons (OPCW). OPCW came into existence in 1997 to implement the provisions of the Chemical Weapons Convention (CWC). Its eventual goal is to realize the vision of a world free of chemical weapons (Krutzsch *et al.*, 1994).

These two chemical categories, pesticide wastes and chemical warfare stockpiles, should be removed and detoxified as soon as possible, lest they contaminate soil, water and food products. To achieve this, much effort is currently being made to develop an effective and economical method to detoxify these toxic organophosphates. Among the many methods, enzymatic biodegradation stands out, because of its potential use in detoxification and demilitarization of these extremely toxic materials. This method is being recommended as a very promising tool by which some toxic organophosphates can be detoxified both economically and

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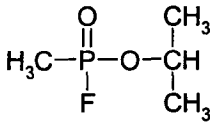

Fig. 1. Chemical structure of sarin (isopropylmethylphosphonofluoridate).

safely.

In connection with the new trends, we initially attempted in this paper to find a potential enzymatic candidate that can degrade chemical stockpiles very effectively. For this, we subcloned a OPAA gene from *Alteromonas haloplanktis* and examined the hydrolytic activity of its gene product on sarin (isopropyl methylphosphonofluoridate) (Fig. 1). Sarin is one of the major portions of the chemical stockpiles that are causing global concerns. It is the very nerve gas that the terrorist, Japanese cult Aum Shinrikyo, used at the subway stations in Tokyo, 1995.

We observed that the enzyme could degrade sarin very effectively in a way that might be used and applied in the development of an enzymatic decontaminate, not only for field detoxification, but also for the destruction of the troublesome chemical stockpiles.

Materials and Methods

Bacterial strains and plasmids *Alteromonas haloplanktis* C and BL21 bacterial strains were obtained from the American Type Culture Collection. They were cultured at 37°C in a Pseudomonas bathycetes medium (3 g yeast extract, 10 g of protease peptone, 24 g of NaCl, 0.7 g of KCl, 5.3 g of $MgCl_2$, 7 g of $MgSO_4 \cdot 7H_2O$) and in a LB medium (10 g of tryptone, 5 g of yeast extract 10 g of NaCl) respectively (Klose *et al.*, 1989). The OPAA gene that was obtained from the chromosomal gene of *Alteromonas haloplanktis* C was subcloned into an expression vector, pQE-30, that resulted in recombinant DNA, pQE-OPAA.

Plasmid pQE-30 contains 6 consecutive histidine codons at the downstream of the translation initiation codon. The recombinant plasmid pQE-OPAA, when expressed, will give rise to 6 consecutive histidine amino acids at the N-terminal region. The cells were harvested by centrifugation (7,500 × *g*) for 10 min and stored at –20°C.

Oligonucleotide probes and PCR The DNA sequence of the OPAA gene from *Alteromonas haloplanktis* C was cloned and sequenced in 1997 by Cheng *et al.* Its gene is composed of 1,700 bp. The translation initiation and termination codons are located at the 277th and 1596th base pair from the transcription initiation codon, respectably (Cheng *et al.*, 1997). To amplify the DNA fragments that contained the entire translational region of the OPAA gene, except for the translation initiation codon, two kinds of oligomer DNA were synthesized in order to get proper restriction sites for subcloning (Fig. 2). The oligonucleotide DNAs were ordered from Ransom Hill Bioscience (Ramona, CA, USA).

As a template DNA for the polymerase chain reaction (PCR), total chromosomal DNA of the *Alteromonas haloplanktis* C strain was used (Cheng *et al.*, 1997). After amplification by PCR, the PCR products were fractionated on a 1% agarose gel (Fig. 3). The size of the DNA fragments that were determined by PCR was coincident with the theoretical calculate, 1,388 bp.

primer No. 1 :
5' CGGGCATGCGGATCCGAAAAATTAGCCGTTTTATAC 3'
BamHI

primer No. 2 :
5' CTACCCGGGGTCGACCGCGTATTGACTAACTCGTCT 3'
SalI

Fig. 2. Primer DNAs for the synthesis of OPAA gene by PCR.

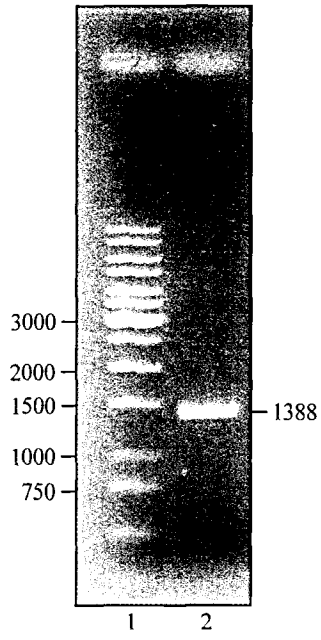

Fig. 3. Agarose gel electrophoresis of the PCR product containing the OPAA gene (from 270th bp to 1631th bp) and its flanking regions. Lane 1, marker DNAs; Lane 2, PCR products (1388 bp).

Subcloning and expression To subclone the OPAA gene into an expression vector pQE-30, the plasmid DNA and PCR products were double-digested with BamH I and Sal I, and ligased with T4 DNA ligase. The resulting recombinant plasmid DNAs, pQE-OPAA (Fig. 4), were then transformed into the *E. coli* strain BL21 and the clones that were resistant to *ampicillin* were selected.

To get expression, transformed bacteria BL21 were cultured in a LB medium that contained *ampicillin* (100 μg/ml). When OD_{600} of the bacterial culture reached 0.4~0.5, the culture media was supplemented with 1 mM IPTG to stimulate induction of protein expression. Incubation was kept for 18~24 h until it reached stationary phase. As was described in the previous section, plasmid pQE-OPAA harbors 6 consecutive histidine codons at the downstream region of the translation initiation codon. Therefore, OPAA, when expressed, is tagged with 6 histidines at the N-terminal region (Fig. 4). The 6 histidine tag has a high chemical

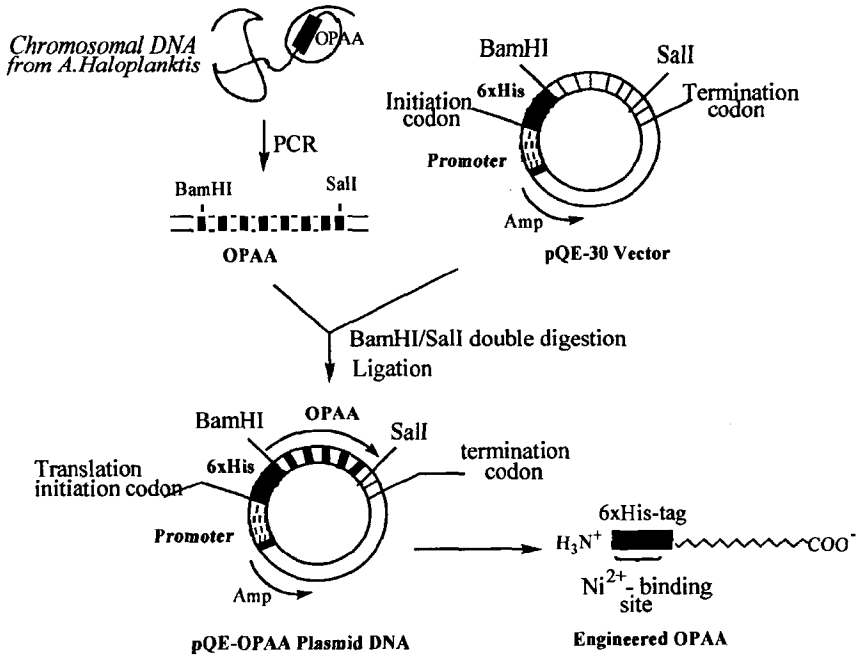

Fig. 4. Subcloning of plasmid pQE-OPAA and its gene product.

affinity to metal ions such as Ni^{2+}. The expression of engineered OPAA was initiated with the translation initiation codon of pQE-30, but stopped by the translation termination codon of the original OPAA gene.

Purification of OPAA The bacterial culture was harvested, washed twice with an ammonium carbonate buffer (50 mM $(NH_4)_2CO_3$ (pH 7.5), 0.1 mM $MnCl_2$ and 10 mM β-mercaptoethanol), and resuspended in a lysis buffer that contained lysozyme (Ni-NTA spin column kit, QIAGEN). The lysate was further lysed using French Pressure and sonicator, and centrifuged to get an aqueous supernatant (crude extract). The crude extract was then applied to an affinity column (Ni-NTA HisSorb Strips, QIAGEN). The affinity column was packed with solid matrix to which nickel ions (Ni^{2+}) were tightly bound. The nickel ions (Ni^{2+}) have high affinity to 6 histidine-tagged proteins. The column was washed with a washing buffer and eluted. The eluant from the affinity column was directly used for both SDS-polyacrylamide gel electrophoresis and an OPAA activity test.

SDS-polyacrylamide gel electrophoresis Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed by the method of Laemmli (Laemmli, 1970) using 7.5% polyacrylamide gel. The proteins in the gel were visualized by staining with Coomassie blue. Two kinds of samples were prepared, one with the whole cell lysate prepared from transformed BL21 and the other from the affinity column eluant.

Enzyme assay OPA anhydrase activity was routinely assayed by monitoring the fluoride release from sarin (isopropyl methylphosphonofluoridate) with a F^--specific electrode, as has often been described in the literature (Hoskin *et al.*, 1982, 1984; Landis, 1986). The reaction was monitored for 5 min. at 25°C in a 10 ml volume containing 50 mM $(NH_4)_2CO_3$ (pH 8.7), 0.1 mM $MnCl_2$, and 3 mM sarin. OPAA eluted from the affinity column was used without further purification.

For an enzyme assay, three kinds of enzyme extract were prepared; a whole cell extract from untransformed BL21, a crude extract prepared from transformed BL21 bacteria that were lysed and centrifuged, and an eluate from an affinity column. The OPAA that was eluted from the affinity column was used for an activity test without further purification. One unit of OPAA activity is defined as catalyzing the release of 1.0 μmole of F^- per min. Specific activity is expressed as units per milligram of protein. Protein concentration was determined colorimetrically using a Coomassie blue assay (Pierce Co, Rockford, IL) with bovine serum albumin (BSA).

Results

Confirmation of expressed OPAA protein by molecular weight To examine the molecular weight of the expressed OPAA, the proteins that were obtained from the affinity column were analyzed by electrophoresis on 7.5% polyacrylamide gel. A whole cell lysate from an untransformed BL21 strain was also fractionated as a control. The native OPAA consisted of 440 amino acid residues that are equivalent to a molecular weight of 48. 5kDa. As shown in Fig. 5, when fractionated on polyacrylamide gel, the expected OPAA band appeared at the position around 48 kDa. This result shows that the protein band that appeared around 48 kDa on the SDS-PAGE is the OPAA that is expressed in the BL21 strain that was transformed with the pQE-OPAA plasmid.

Hydrolytic activity of OPAA on sarin The Hydrolytic

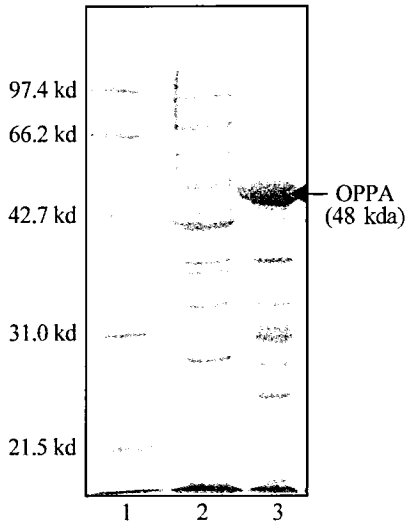

Fig. 5. Analysis of proteins by SDS-polyacrylamide gel electrophoresis (7.5%). Lane 1, Marker proteins; Lane 2, Whole cell lysate (untransformed); Lane 3, Affinity column eluate.

activity of OPAA on sarin was determined by measuring the amount of fluoride ion that was released from sarin with a F^--specific electrode at various times (Fig. 6). There are two reasons why sarin was chosen as a model for the biodegradation of toxic organophosphates in this study. Primarily, it is one of the major portions of the chemical stockpiles, but another important feature is that its chemical structure is very similar to soman (1,2,2-trimethylpropyl methylphosphono-fluoridate), another major constituent of chemical stockpiles. If OPAA from *Alteromonas haloplanktis* C has a hydrolyzing activity on sarin, it is strongly assumed that it may also have activity on soman.

In each assay, 3 mM sarin was used as a substrate in a 10 ml reaction mixture. When all of the sarin in the reaction mixture was degraded by OPAA, the total amount of F^- reached 30 μmol with monitoring by the F^--specific electrode.

A test of the hydrolytic activity was performed with 3 kinds of cell extracts; whole cell extract prepared from untransformed BL21, crude extract prepared from transformed BL21, and affinity column eluate. The whole cell extract that was prepared from untransformed BL21 showed almost no detectable hydrolytic activity. However, the crude extract showed a considerable amount of hydrolytic activity with the complete degradation of 3 mM sarin in 300 s. When assayed with the enzyme that was eluted from the affinity column, nearly half of the sarin in the reaction mixture was degraded after only 10 s, and gave complete degradation after 100 s. Also, after 100 s the amount of F^- arrived at 30 μmol and showed no more change in the total amount of F^-. This means that all of the 3 mM sarin added in the reaction mixture was degraded completely in 100 s. These results clearly indicate that the OPAA has a potential hydrolytic activity on sarin. The enzyme is also supposed to have a good hydrolytic activity on soman, because sarin has a similar chemical

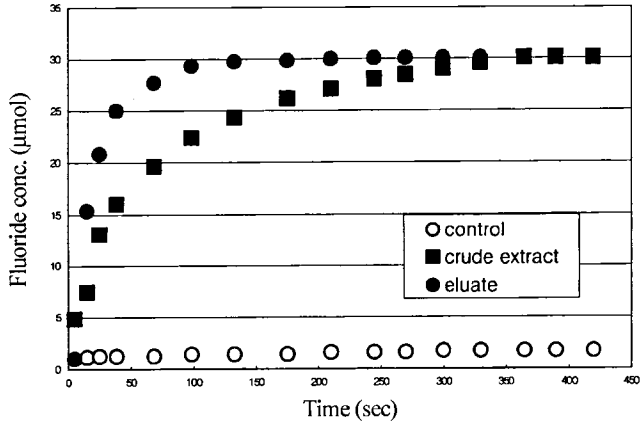

Fig. 6. Hydrolyzing activity of OPAA on sarin. Control, Whole cell extract prepared from BL21 bacteria; Crude Extract, Whole cell extract of transformed BL21; Eluate, Eluate from affinity column applied with crude extract.

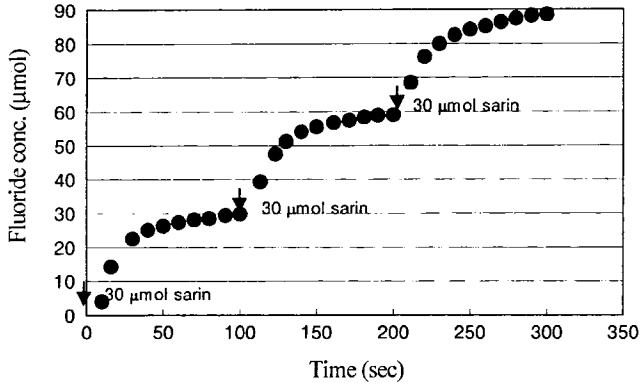

Fig. 7. Degradation of sarin added in a consecutive manner.

structure to that of soman.

Enzyme activity over an extended period To examine the activity of OPAA over an extended period, each 30 μmole sarin was consecutively added 3 times into the 10 ml reaction solution every 100 s (Fig. 7). The amount of OPAA in the reaction mixture was kept constant with the initial amount of OPAA, 50 mg per ml reaction buffer. After the start of the reaction, the initial additive of sarin was completely degraded in 100 s, which showed the same result (Fig. 6). In another 100 s, the second additive of sarin was also degraded completely. The same result happened with the third additive of sarin. After each round of reaction, the amount of fluoride ion released into the solution reached 30 μmole. This result indicates that all of the substrate added in each round of assay was completely degraded without causing any interference of the former addition of sarin in the degradation capacity of the latter portion. This result further indicates there was no detectable loss of OPAA activity in the reaction solution. It kept its potential activity for a considerable period of time.

Discussion

Globally, hundreds of thousands of tons of chemical stockpiles exist and need to be destroyed. Therefore, there has been a great deal of effort to develop both effective and economical methods to detoxify chemical stockpiles.

Traditionally chemical treatment, incineration, and landfills detoxified these compounds. Chemical methods are problematic in that large volumes of acids and alkali are used and subsequently must be disposed. Incineration, by all accounts, seems to be the most effective method for the destruction of these compounds. But, this method has met serious public opposition because of the toxic emissions into the air. Landfills are also not the recommended method in that it causes soil and groundwater contamination.

In an effort to degrade hazardous wastes such as chemical stockpiles, enzymatic biodegradation has recently been recommended by the US Army as the most promising decontamination system from an economic and safety point of view (Lin, 1997).

So far, several kinds of organisms such as, squid (Hoskin *et al.*, 1982), protozoan (Landis *et al.*, 1987), clams (Anderson *et al.*, 1988), mammals (Little *et al.*, 1986), and soil bacteria (Attaway *et al.*, 1987) were reported to harbor enzymes, called organophosphorus acid anhydrolases (OPAA). These catalyze the hydrolysis of a variety of toxic acetylcholinesterase-inhibiting organophosphates.

Of these organisms, naturally occurring bacterial isolates that harbor bacterial OPAA have received considerable attention. They have proved to be the most effective and economical tools for the detoxification of the toxic organophosphates (Sethunathan *et al.*, 1973; Munnecke *et al.*, 1974).

The first bacterial OPAA was detected from both *Flavobacterium* sp. (ATCC 27551) and *Pseudomonas diminuta* MG (Mulbry *et al.*, 1986). Although the OPAA was originally detected from two different organisms, their genes were later determined to be identical (Sedar *et al.*, 1982; Harper *et al.*, 1988; McDaniel *et al.*, 1989; Mulbry *et al.*, 1989). These enzymes were shown to degrade an extremely broad spectrum of organophosphorus compounds, such as parathion, along with other commonly used organophosphorus insecticides, dursban, paraoxon, coumaphos, diazinon, fensulfothion, and cyanophos (Brown, 1980; Chiang *et al.*, 1985).

Another OPAA from *Alteromonas* sp. strain JD 6.5 has also been purified to homogeneity (Brown, 1980; Chiang *et al.*, 1985). It too has high activity toward a wide range of organophosphorus compounds. Another OPAA from *Alteromonas undina* was also purified and characterized (Cheng *et al.*, 1993). It possesses hydrolyzing activity on the pesticides-including insecticides, fungicides, and herbicides.

Recently, another type of OPAA from *Alteromonas haloplanktis* was detected (Liu *et al.*, 1995). Although this enzyme was originally known as human prolidase (a dipeptidase that hydrolyzes X-Pro, dipeptides), later it was determined that this enzyme also harbors hydrolytic activity on a variety of pesticides (Cheng *et al.*, 1997).

Most of the enzymes that were described previously have been tested mainly on pesticides, rather than on chemical warfare, such as sarin and soman. However, in this paper, we tried to detect the hydrolytic activity of OPAA from *Alteromonas haloplanktis* C on the chemical warfare agent, sarin. As determined from these results, it has potential hydrolytic activity on sarin. Moreover it sustains its high activities for a considerable length of time. These results, which consider that sarin is similar in its chemical structure to soman that consists of another major portion of the chemical stockpiles, strongly suggest that OPAA from *Alteromonas haloplanktis* C may be used as an enzymatic tool that can be applied to the destruction of chemical stockpiles.

However, there are several limitations in using OPAA to detoxify both environmental pesticide wastes and chemical stockpiles. Purified OPAA is very expensive. Moreover, once used in a reaction solution, it cannot be reused. A solution may be to immobilize both native and recombinant OPAA onto solid matrix, such as nylon (membrane, powder, and tubing), porous glass, and silica beads. Such immobilized enzyme can then be applied to enzyme reactors for the detoxification of organophosphates (Munnecke, 1979; Caldwoll *et al.*, 1991). However, in most cases, these kinds of detoxification processes are expensive, because large amounts of purified OPAA are required.

Such economic problems can be eliminated if whole cells, rather than the enzyme itself, are immobilized onto the support (such as in an immobilized-cell bioreactor). However, the use of immobilized cells in a bioreactor has several disadvantages. Potentially, the most serious problem is the mass-transport limitation of substrates and products across the cell membrane, because the outer membrane can act as a permeability barrier that may inhibit substrates from interacting with the enzymes contained within the cell.

The diffusional resistance can be eliminated if OPAA is displayed on the surface of the cell membrane (Fanclaco *et al.*, 1992). This will allow substrates to interact with the enzyme at the outer-membrane and remove the diffusional membrane-barrier. Therefore, recombinant cells with surface-expressed OPAA will give a very promising tool to maximize its enzymatic effects on the large-scale and long-term detoxification of pesticide wastes and demilitarization of chemical stockpiles.

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