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Expression and Characterization of *Escherichia coli* Prolidase with Organophosphorus Compounds

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Abstract A relatively high homology between *Escherichia coli* prolidase and *Alteromonas* organophosphorous acid anhydrolase suggests that *E. coli* prolidase may have an activity to degrade toxic organophosphorous compounds. To confirm this suggestion, we cloned and expressed a prolidase gene (*pepQ*) of *E. coli* BL21. The recombinant *E. coli* prolidase that consisted of 443 amino acid residues exhibited activity and stereochemical selectivity against organophosphorous compounds, although its activity was two to three orders of magnitude less than that of the other organophosphorous acid hydrolase isolated from *Pseudomonas diminuta.*

Keywords: chemical warfare, detoxification, prolidase, organophosphorous compound

INTRODUCTION

Organophosphorous acid hydrolase (OPH; EC 3.1.8.1) and anhydrolase (OPAA; EC 3.1.8.2) enzymes have been under investigation since these enzymes catalyze the hydrolysis of many highly toxic organophosphorous compounds, including chemical warfare G-type nerve agents and pesticides [1-4]. OPH and OPAA have functional similarities in that both enzymes catalyze the hydrolysis of sarin (O-isopropyl methylphosphonofluoridate), and paraoxon. OPH has much higher catalytic activity on paraoxon than OPAA, whereas OPAA has significant soman (O-pinacolyl methylphosphonofluoridate) activity, which is not found in the native OPH.

The OPH genes (*opd*) were cloned from *Pseudomonas diminuta* MG and *Flavobacterium* sp. strain [5, 6], while the OPAA gene (*opaA*) was cloned from *Alteromonas* sp. strain JD6.5 [1]. The open reading frame (ORF) of the *opd* gene contains 975 bases, which encode a polypeptide of 325 amino acids with a molecular mass of 35 kDa, while the ORF of the *opaA* gene contains 1551 bases, which encode a polypeptide of 517 amino acid residues with a molecular mass of 59 kDa. Interestingly, amino acid sequence comparison of OPAA with the protein database showed no homology with OPH, but 50% and 30% homology with *Escherichia coli* prolidase and aminopeptidase P, respectively .

It is known that prolidase cleavages between amino acid and proline at the C terminus (NH_2–X-/Pro-COOH), whereas aminopeptidase P cleavages between any N-terminal amino acid and a proyl residue (NH_2–X-/Pro–Y-) [7]. The structure of *E. coli* aminopeptidase P reveals an active site contains a dinuclear manganese center and a high homology with *E. coli* prolidase [8]. Meanwhile, the recombinant OPAA exhibits prolidase activity with a high specific activity against Leu-Pro and Ala-Pro, yet no activity against Pro-Leu, Pro-Gly, and the substrates for aminopeptidase P [1]. Accordingly, it is reasonable to believe that *E. coli* prolidase may have the activity against organophosphorous compounds like OPAA. From this point of view, we expressed and characterized the recombinant *E. coli* prolidase with organophosphorous compounds by cloning the *pepQ* gene from *E. coli* BL21.

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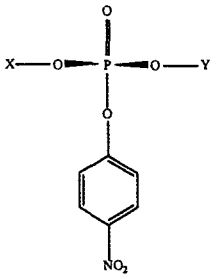
MATERIALS AND METHODS

Cloning of *Escherichia coli* pepQ Gene

The chromosomal DNA of *E. coli* BL21 was purified with a commercial kit (Promega) and used as a template for amplification of its *pepQ* gene by polymerase chain reaction (PCR). The primers for *pepQ* gene were designed on the basis of GenBank data (X54687). Open reading frame of pepQ gene was first amplified by PCR, with primers (5'- ATG GAA TCA CTG GCC TCG AT-3' and 5'-TTA TTC TTC AAT CGC TAA CA -3'), and then the PCR fragment was reamplified with the following primers: Primer 1: 5'-GGA ATT AAG CTT AAG GAG ATA TAC ATA TGG AAT CAC TGG CCT CG -3', Primer 2: 5'-CCG GAA TTC TTA TTC TTC AAT CGC TAA CA -3'. Primer 1 included *Hind*III and ribosomal binding site while primer 2 included *Eco*RI site. PCR was performed under the conditions of 95°C for 30s, 45°C for 1 min, 72°C for 3 min with 30 cycles. PCR fragments were

Table 1. Organophosphorous compounds used for characterization of *E. coli* prolidase



Substrate	X	Y
R_p-II	CH_2CH_3	CH_3
S_p-II	CH_3	CH_2CH_3
R_p-III	$CH(CH_3)_2$	CH_3
S_p-III	CH_3	$CH(CH_3)_2$
R_p-IV	C_6H_5	CH_3
S_p-IV	CH_3	C_6H_5
R_p-VI	$CH(CH_3)_2$	CH_2CH_3
S_p-VI	CH_2CH_3	$CH(CH_3)_2$
R_p-VII	C_6H_5	CH_2CH_3
S_p-VII	CH_2CH_3	C_6H_5
R_p-IX	C_6H_5	$CH(CH_3)_2$
S_p-IX	$CH(CH_3)_2$	C_6H_5

digested with *Hind*III and *Eco*RI after purification with a commercial kit (Promega) and cloned into a pBluescript SK^+ vector.

DNA Sequencing

DNA sequences were analyzed by use of an Automated DNA sequencer in Gene Technology Laboratory at Texas A&M University. DNA and protein sequences were compared using a multiple sequence alignment program (CLUSTALW) from Biology Workbench (the University of California San Diego).

Purification of Prolidase

Cells were cultured in Terrific Broth (TB) media containing tryptone 12 g/L, yeast extract 24 g/L, glycerol 4 mL dissolved in 89 mM phosphate buffer (pH 7.0) in the presence of ampicillin (50 mg/L). Cells were harve-sted by centrifugation and suspended in 50 mM HEPES buffer (pH 8.0) containing 0.1 mM $MnCl_2 \cdot 2H_2O$. Cell walls were disrupted by ultra-sonication for 30 min. An equal volume of 1% protamin sulfate solution was added to the supernatant and centrifuged. The supernatant was treated with ammonium sulfate (60% saturation). After centrifugation for 20 min at 10,000 × g, the protein precipitates were suspended in 50 mM HEPES (pH 8.0) and loaded to a Ultrogel AcA 44 (Life Technologies, USA) gel column (8 × 200 cm). Proteins were eluted with 50 mM HEPES buffer (pH 8.0) containing 0.1 mM $MnCl_2 \cdot 2H_2O$. Active fractions were concentrated through a membrane filter (MW cutoff 10,000) and loaded to HPLC anion exchange column (HiLoad Q-Sepharose) or HPLC gel column (HiLoad Sephadex 200) for further purification.

The recombinant prolidase showed activity against paraoxon (Sigma), so that the enzyme activity was measured by monitoring the appearance of *p*-nitrophenol from paraoxon at 400 nm (extinction coefficient of paraoxon, $1.7 \times 10^4\ M^{-1}cm^{-1}$) using a microplate reader (Spectra-max plus, Molecular Devices, USA).

Kinetic Characterization and Data Analysis

The catalytic activity for the *E. coli* prolidase with various organophosphorous compounds (Table 1) that were synthesized in Texas A&M University was measured by monitoring the appearance of *p*-nitrophenol at 400 nm. Because of the limited solubility of some of the organophosphorous compounds, a portion of methanol was added to make 10% methanol/buffer solution with 50 mM HEPES buffer (pH 8.0) containing 0.1 mM $MnCl_2 \cdot 2H_2O$. Enzymatic reactions were performed with various concentrations of substrates (0.01 – 5 mM) in 1 mL cuvettes at 25°C for 5 min. The kinetic parameters (V_{max} and K_m) were determined using a Sigma Plot curve fitting program.

RESULTS AND DISCUSSION

Cloning and Expressing of *pepQ* Gene

According to protein database, there were two kinds of *E. coli* prolidase, 646 and 443 amino acids. PCR primers were designed on the basis of the larger size of *pepQ* gene (GenBank accession no. X54687). The amplified DNA fragment from *E. coli* BL 21 chromosome showed about 2,000 bases and cloned into pBluescript vector (data not shown). The recombinant pBluescript vector was transformed to the host strain of *E. coli* BL21. It was expected from 646 amino acids that the expressed prolidase has about 73 kDa molecular mass, but the purified prolidase from recombinant *E. coli* BL21 has about 50 kDa in SDS-PAGE (Fig. 1). To understand this difference, the cloned pepQ gene was fully sequenced. It was found that there was a stop codon appeared at 1330 nucleotides, resulting in making 443 amino acids (data not shown).

Interestingly, these DNA sequences matched well with those of *E. coli* K12 prolidase (GenBank accession no. P21165), suggesting that *E. coli* prolidase of 646 amino acids was possibly a mutant derived from *E. coli* prolidase of 443 amino acids. When the amino acids of *E. coli* prolidase were compared with those of structurally similar protein, *E. coli* aminopeptidase P, the amino acids between 444-646 were not likely to contribute the active

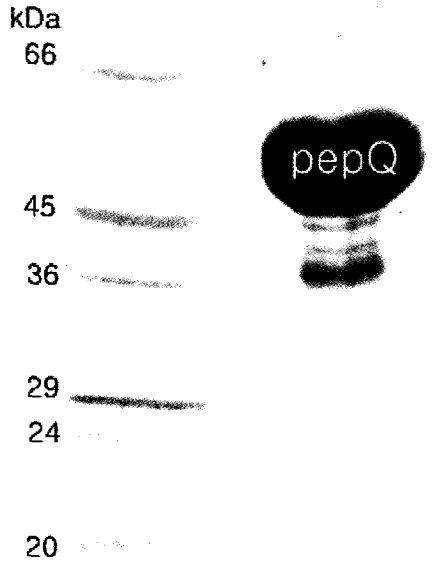


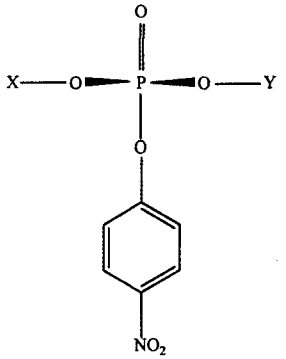
Fig. 1. Recombinant PepQ protein (50 kDa) purified by gel chromatography.

sites at all (data not shown). Although 24 nucleotides were different between pepQ genes of *E. coli* BL21 and *E. coli* K12, their translated amino acid residues were exactly the same (data not shown). The recombinant molecular mass of *E. coli* BL21 prolidase was calculated to be 50,176 Dalton (pI 5.60). This smaller size of prolidase also has 50% homology with OPAA-2 enzyme. The activity of *E. coli* prolidase depended upon Mn^{2+} rather than Co^{2+} or Zn^{2+} (data not shown).

Kinetic Characterization of *E. coli* Prolidase

The *E. coli* prolidase showed activities against various organophosphorous compounds, although its activity was two to three orders of magnitude less than that of the well-known organophosphorous hydrolase, phosphotriesterase (Table 2). Phosphotriesterase degraded the S_p-enantiomers of organophosphorous compounds faster than the R_p-enantiomers [9-11]. The recombinant *E. coli* prolidase also showed stereochemical selectivity against certain racemic mixtures such as methyl ethyl *p*-nitrophenyl phosphate and methyl phenyl *p*-nitrophenyl phosphate. The catalytic efficiency (k_{cat}/K_m) of *E. coli* prolidase against the S_p-enantiomer was about two orders of magnitude better than that against the R_p-enantiomer

Table 2. Kinetic values of *E. coli* prolidase against various organophosphorous compounds



Substrate	X	Y	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	Phosphotriesterase[a] k_{cat}/K_m ($M^{-1}s^{-1}$)
R_p-II	CH_2CH_3	CH_3	1.5	11.9	7.9×10^3	3.2×10^7
S_p-II	CH_3	CH_2CH_3	2.1	420.6	2.0×10^5	3.4×10^7
R_p-III	$CH(CH_3)_2$	CH_3	1.7	24.1	1.3×10^4	8.5×10^5
S_p-III	CH_3	$CH(CH_3)_2$	1.9	40.4	2.0×10^4	2.7×10^7
R_p-IV	C_6H_5	CH_3	1.3	55.2	4.2×10^4	1.0×10^6
S_p-IV	CH_3	C_6H_5	0.3	1184.9	3.8×10^6	9.3×10^7
R_p-VI	$CH(CH_3)_2$	CH_2CH_3	0.5	7.0	1.3×10^4	4.1×10^6
S_p-VI	CH_2CH_3	$CH(CH_3)_2$	2.7	46.3	1.7×10^4	4.2×10^7
R_p-VII	C_6H_5	CH_2CH_3	0.14	4.5	3.2×10^4	3.7×10^6
S_p-VII	CH_2CH_3	C_6H_5	0.5	19.8	3.8×10^4	7.6×10^7
R_p-IX	C_6H_5	$CH(CH_3)_2$	0.2	4.3	2.1×10^4	5.2×10^6
S_p-IX	$CH(CH_3)_2$	C_6H_5	0.7	11.8	1.6×10^4	1.8×10^8

[a] The values were obtained from Chen-Goodspeed *et al.* [11].

(Table 2). Stereochemical selectivity of *E. coli* prolidase was not observed to other type of racemic mixtures such as methyl isopropyl *p*-nitrophenyl phosphate, ethyl isopropyl *p*-nitrophenyl phosphate, ethyl phenyl *p*-nitrophenyl phosphate, and isopropyl phenyl *p*-nitrophenyl phosphate.

In conclusion, we showed that *E. coli* prolidase could decontaminate highly toxic organophosphorus compounds. This result corroborates the previous report that organophosphorous acid anhydrolase was a type of X-Pro dipeptidase [12]. *E. coli* prolidase activity was dependent on the presence of manganese. Prolidase of *Aspergillus nidulans* also required manganese as a cofactor [13], whereas prolidases of *Lactobacillus bulgaricus* and *Pyrococcus furious* were dependent on zinc and cobalt, respectively [14,15]. The activities of these microbial prolidases against organophosphorous compounds, how-ever, were not known.

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