Short Communication

Immobilization of organophosphorus hydrolase for the degradation of organophosphorus nerve agents

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Organophosphorus hydrolase (OPH) (EC 3.1.8.1) was first reported by Sethunathan and Yoshida (1973) as an enzyme that was capable of degrading organophosphorus pesticides such as parathion or diazinon, and was produced by *Flavobacterium* sp. that was later identified as *Sphingobium fuliginis* (Kawahara et al., 2010). The enzyme was cloned and has been biochemically studied to degrade pesticides and also other organophosphorus compounds such as nerve agents (Dumas et al., 1989; Hoskin et al., 1995; Lai et al., 1995; Mulbry et al., 1986; Vanhooke et al., 1996). Since the efficient decontamination of organophosphorus nerve agents, such as *O*-isopropyl methylphosphonofluoridate (sarin) and *O*-ethyl-*S*-(2-diisopropylaminoethyl) methylphosphonothiolate (VX), has been greatly needed as a countermeasure against chemical terrorisms such as the sarin gas attacks in Japan in 1994 and 1995 (Seto et al., 2000), many groups, including ourselves, have developed genetically manipulated OPH to degrade nerve agents (Gopal et al., 2000; Hill et al., 2003; Jeong et al., 2014; Nakayama et al., 2014; Reeves et al., 2008). Organophosphorus nerve agents are relatively unstable in alkaline conditions, and, therefore, strong alkaline liquids have been used for the decontamination. Among the nerve agents, VX is relatively resistant to alkaline degradation (Seto, 2011), and a stronger reagent, hypochlorite, is needed for its decontamination. However, alkalis and hypochlorite can damage the environment, and human skin, and should be avoided in decontamination in the case of chemical terrorism in a crowded city. Compared with a chemical treatment, enzymatic degradation is gentler and causes less damage to the environment.

In our previous study (Ohmori et al., 2013), we estab-

lished the purification method of OPH by adding His-tag at the N-terminal of OPH, and applying the enzyme to a Ni-chelating His-tag affinity column. In the study, OPH bound to a Ni-chelating resin was considered to be an immobilized enzyme, but the enzyme could be easily eluted when the eluent contained salts that affect the affinity between His-tag and Ni²⁺. In order to use OPH for practical decontamination onsite, the enzyme should be stable and easy to handle, and a stably immobilized enzyme would be the best to fulfill these requirements, because we can use immobilized enzyme as a reusable bioreactor to treat polluted water in a large scale. In the present study, we have examined several immobilization methods, and found that a gel of calcium alginate is the most effective material for the immobilization.

Organophosphorus compounds used in this study are described below. *O,O*-Diethyl *p*-nitrophenylphosphate (paraoxon) was purchased from Sigma Aldrich (St. Louis, MO, USA). Sarin, *O*-ethyl *N,N*-dimethylamino-phosphonocyanidate (tabun), *O*-pinacolyl methylphosphonofluoridate (soman), *O*-cyclohexyl methylphosphonofluoridate (cyclohexylsarin), VX, and *O*-isobutyl-*S*-(2-diethylaminoethyl) methylphosphonothiolate (RVX) were synthesized in the laboratory of the National Research Institute of Police Science. All the nerve agents were handled carefully with protective clothing in a fume hood, and destroyed with sodium hypochlorite at the end of each experiment. Chemical warfare agent synthesis and usage were conducted with the approval of the Ministry of Economy, Trade, and Industry of Japan.

Escherichia coli DH5 α harboring pTV118N-E/H-OPH with one mutation to substitute Tyr to His at position 254

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Table 1. Specific activities of hydrolysis of immobilized OPH preparations toward paraoxon.

Carrier for immobilization	Total protein* (mg/ml resin)	Specific activity* (U/mg protein)
Purified OPH		
Ni-chelating His-bind resin	0.45	0.57
CNBr-activated Sepharose 4B	0.23	0.07
Porous silica IB-S60P	0.11	0.74
Crude OPH		
Porous silica IB-S60P	4.83	0.11
Calcium alginate gel	4.80	0.01**

*The experiments were repeated twice, and representative results are shown.

**The activity was not properly measured because *p*-nitrophenol was trapped in the calcium alginate gel and only a small amount was eluted.

Table 2. Effect of sonication for the recovery of VX and RVX from OPH-immobilized porous silica.

Preparation of porous silica IB-S60P	Residual nerve agent (%)*	
	VX	RVX
Without immobilization (Control)	16.6 ± 2.3	10.5 ± 1.3
Immobilization of crude OPH	7.9 ± 0.4	4.8 ± 0.4
Immobilization of purified OPH	7.7 ± 1.5	4.6 ± 0.5

*An aliquot of the porous silica with or without OPH was mixed with a reaction mixture (0.4 ml) containing 50 µg/ml of VX or RVX, and incubated at 37°C for 20 min. After incubation, the porous silica was sonicated to extract residual VX or RVX. All assays were performed in triplicate, and the mean values ± standard deviations are shown.

Table 3. Degradation of VX and RVX by OPH-immobilized calcium alginate gel.

Preparation	Residual nerve agent (%)*	
	VX	RVX
Calcium alginate gel without OPH	81.8 ± 3.2	84.2 ± 3.9
OPH-immobilized calcium alginate gel	52.8 ± 4.5	53.3 ± 4.6

*An aliquot of the calcium alginate gel with or without OPH was mixed with a reaction mixture (0.25 ml) containing 100 µg/ml of VX or RVX, and incubated at 37°C for 8 h. After incubation, the calcium alginate gel was sonicated to remove residual VX or RVX. All assays were performed in tetraplicate, and the mean values ± standard deviations are shown.

from the N-terminal (the strain KGU0255) (Nakayama et al., 2014) was used in this study. The cells of KGU0255 were suspended in 20 mM Tris-HCl buffer (pH 8.0) and disrupted by sonication (Ultrasonic Homogenizer Model UH-300, SMT Ltd, Tokyo, Japan) for 10 min. The cell debris and membrane fraction were removed by centrifugation at 40,000 × *g* for 10 min, and the supernatant was used as a crude OPH preparation. The OPH was purified according to the method described previously (Ohmori et al., 2013) if necessary. The OPH preparations were incubated with one mM CoCl₂ at 4°C overnight for activation directly, or after it was immobilized, and used for the activity assay.

Paraoxon was used as a substrate for the usual assay of

A **B** **Fig. 1.** Degradation of nerve agents by immobilized OPH preparations.

An aliquot of immobilized OPH was mixed with a reaction mixture (0.4 ml) containing 50 µg/ml of nerve agents, and incubated at 37°C for 20 min. A, Covalently immobilized to Sepharose 4B; B, Immobilized to porous silica IB-S60P.

the enzyme activity. Paraoxon (4 mM) dissolved in 50 mM Tris-HCl (pH 9.0) was added to a Co²⁺-activated OPH solution or applied to the OPH-immobilized column. The change in absorbance at 410 nm of the solution, or the column eluate, was measured and the concentration of the product *p*-nitrophenol was calculated as described elsewhere (Funhoff et al., 2001; Ohmori et al., 2013). The protein concentration was determined according to the method of Bradford (1976). The activity was calculated and expressed in units of µmole of *p*-nitrophenol released per minute.

For the degradation assay for nerve agents, an aliquot of Co²⁺-activated OPH solution, or immobilized OPH, was mixed with a reaction mixture containing one of the nerve agents (Nakayama et al., 2014). In the case of immobilized OPH, the immobilized resin was mixed with the solution of the nerve agent, and incubated under the conditions described in the footnotes of Tables 2 and 3. After the immobilized OPH was removed from the reaction mixture, one ml of methylene chloride that contained 10 µg/ml of nonadecane as an internal standard was added to the solution and the residual nerve agent was extracted by shaking. An aliquot of the methylene chloride layer was injected into an Agilent 6890 gas chromatography (GC)/5973 mass spectrometry (MS) (Agilent, Santa Clara, CA, USA) with the analytical conditions described previously (Ohmori et al., 2013). The residual nerve agent concentrations were calculated from the peak areas on mass chromatograms of GC-MS. All assays were performed in triplicate or in tetraplicate.

The immobilization of OPH to Sepharose 4B was per-

formed according to the manufacturer's instruction using CNBr-activated Sepharose 4B (GE Healthcare Japan, Tokyo, Japan). Porous silica IB-S60P and other polymers were purchased from Chiral Vision (Leiden, The Netherlands) as Immobead Enzyme Carrier Kit (IMMO-10). The porous silica gel (0.25 g) was washed with ethanol and water, mixed with 1 ml of OPH solution, and incubated overnight for adsorption. Sodium alginate (Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to the OPH solution to the final concentration of 2%, and the mixture was dropped into a solution of 2% calcium chloride for gelation.

The immobilized preparations of OPH were packed in small plastic columns (approx. one ml), and the activities toward paraoxon were compared with that of the OPH-adsorbed Ni-chelating resin, prepared as in the previous study (Ohmori et al., 2013). The total adsorbed protein calculated from the difference of the applied and eluted amounts of protein, and the specific activity to hydrolyze paraoxon are shown in Table 1. The specific activity of the OPH-adsorbed porous silica was comparable to that of the Ni-chelating resin-adsorbed OPH. On the other hand, the covalently bonded OPH-Sepharose 4B preparation showed a much lower specific activity. In addition to porous silica, OPH was adsorbed to polyacrylic acid or polystyrene provided by the same kit of Chiral Vision, but the adsorbed OPH showed only weak activity to hydrolyze paraoxon. These results indicated that the porous silica was a suitable carrier for the immobilization. The activity of OPH-immobilized calcium alginate gel was also examined using crude OPH (Table 1). The gel turned yellow immediately after the incubation, suggesting that the activity was rather high, but most of the hydrolysis product, *p*-nitrophenol, was trapped in the gel, and the activity could not be correctly determined.

Degradative activities of immobilized OPH preparations toward nerve agents were compared in a batch manner because of the poor elution rate of nerve agents through the packed column. As shown in Fig. 1A, OPH-Sepharose 4B exhibited weak degradative activities toward sarin and cyclohexylsarin, and apparently no activity toward soman and VX. Compared with OPH-Sepharose 4B, OPH-IB-S60P was expected to show a stronger activity toward nerve agents. However, most of the nerve agents were found to be adsorbed by the porous silica, or partially degraded non-enzymatically, and the intact nerve agents could not be detected efficiently in the supernatant, or were completely lost in the case of VX (Fig. 1B). Porous silica from another source was also used for immobilization, but the adsorption ratio of nerve agents seemed to be even higher (data not shown).

To avoid the adsorption of VX by the porous silica, amino compounds such as glycine, arginine, triethylamine, or bovine serum albumin were used for pretreatment, but they failed to block the adsorption to the silica. After the trials of other methods, sonication before extraction with methylene chloride was found to be most effective. As shown in Table 2, the residual ratios of VX and RVX were shown to be reduced by the incubation with immobilized OPH, although the amounts of recovered VX and RVX in the control experiments were still very low.

In order to prove the enzymatic degradation, calcium alginate was used as a material for immobilization because the adsorption of nerve agents was found to be low in the preliminary experiments. As shown in Table 3, the amounts of both VX and RVX were reduced to approximately half by the incubation for 8 h with OPH-calcium alginate gel. The non-enzymatic reduction of the amounts of VX or RVX during these assays was less than 20% (Table 3), indicating clearly that these nerve agents were degraded by the immobilized OPH. The non-enzymatic reduction of these nerve agents was deduced to be the result of chemical degradation and not adsorption to the gel because a similar amount of reduction also occurred even without the gel. Crude enzyme was used in this experiment because purified enzyme is not applicable to the large-scale production of OPH.

In the present study, the efficiency of immobilization methods of OPH was examined for the materials of CNBr-activated Sepharose 4B, the porous silica, and the calcium alginate gel. The porous silica was proved to have a high ability to immobilize OPH without losing the activity to hydrolyze paraoxon, and had an advantage in stability compared with Ni-chelating His-bind resin. However, the nerve agents were found to be strongly adsorbed by the porous silica, and enzymatic degradation could not be clearly proved. In contrast to porous silica, the adsorption of VX or RVX by the gel of calcium alginate was low, and clear evidence of the enzymatic degradation toward VX and RVX could be obtained. Calcium alginate gel is easy to prepare and economically a suitable material for large-scale preparation. In addition, the reaction speed of OPH-calcium alginate gel could be improved by fining the particle of the gel during gelation. The results obtained in this study suggest the possible use of immobilized OPH for the decontamination of organophosphorus compounds, especially nerve agents, spread by accidents or by chemical terrorism. For the effective degradation of nerve agents in polluted water in a large scale, a bacterial strain or a production system producing higher amounts of OPH is required. These studies are in progress in our laboratories.

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