

## Immobilization of organophosphate hydrolase on biocompatible gelatin pads and its use in removal of organophosphate compounds and nerve agents

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Bacterial organophosphate hydrolases (OPH) have been shown to hydrolyze structurally diverse group of organophosphate (OP) compounds and nerve agents. Due to broad substrate range and unusual catalytic properties, the OPH has successfully been used to develop eco-friendly strategies for detection and decontamination of OP compounds. However, their usage has failed to gain necessary acceptance, due to short half-life of the enzyme and loss of activity during process development. In the present study, we report a simple procedure for immobilization of OPH on biocompatible gelatin pads. The covalent coupling of OPH using glutaraldehyde spacer has been found to dramatically improve the enzyme stability. There is no apparent loss of OPH activity in OPH-gelatin pads stored at room temperature for more than six months. As revealed by a number of kinetic parameters, the catalytic properties of immobilized enzyme are found to be comparable to the free enzyme. Further, the OPH-gelatin pads effectively eliminate OP insecticide methyl parathion and nerve agent sarin.

**Keywords:** Organophosphates, Organophosphorus hydrolase (OPH), Enzyme immobilization, Detoxification, Sarin, Methyl parathion, Gelatin

The neurotoxic organophosphates (OP) include chemical warfare agents, insecticides and plasticizers. The residues of OP compounds, which contribute about 70% of the total insecticides<sup>1</sup>, have been detected in various components of the environment. Due to serious health problems associated with consumption of OP contaminated food<sup>2</sup>, a number of attempts have been made to develop eco-friendly enzyme-based technologies for detection and decontamination of OP compounds<sup>3-5</sup>. Of the various enzymes known to act on OP compounds, organophosphate hydrolase (OPH) isolated from *Pseudomonas diminuta*<sup>6</sup> and *Flavobacterium* sp. ATCC 27551<sup>7</sup> has been extensively studied. The OPH, known to hydrolyze P-O and P-F bonds found in structurally diverse groups of OP compounds is coded by a highly conserved plasmid borne organophosphorus degrading (*opd*) gene<sup>8-10</sup>.

The OPH group of enzymes due to their wide substrate range<sup>11-15</sup> and  $K_{cat}$  values reaching diffusion limit are one of the most preferred enzymes for developing bioremediation tools used for detection and decontamination of OP-compounds and nerve agents<sup>13,16-19</sup>. Although the OPH has been used in combination with acetylcholine esterase (AChE) to develop a number of technologies aiming for the rapid detection and decontamination of OP compounds<sup>20-27</sup>, these technologies have a number of disadvantages as in most of the cases substantial amounts of OPH activity is lost during the process of immobilization. Further, due to short half-life, the OPH containing matrices have been found to be unsuitable for prolonged storage. In addition, the matrices used for immobilization are not compatible for use on human subjects that come in contact with OP compounds.

Despite a clear ban, studies on Gulf war veterans have shown the exposure to the OP compounds<sup>28,29</sup>. Such reports together with large stockpiles of nerve agents waiting to be destroyed according to Chemical Weapons Convention Treaty further justifies the need for developing biocompatible and eco-friendly

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technologies for removing OP residues from human subjects and environment. Here, we report immobilization of OPH on gelatin pads using a homo-bi-functional cross-linker. The stability and kinetic properties of biocompatible OPH-gelatin pads have also been studied using nerve agent sarin and methyl parathion as assay substrate.

## Materials and Methods

### Materials

Biochemicals used in the study were procured from Sigma Aldrich, USA. HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] was purchased from GE Health Care, USA. Most of the routinely used chemicals were purchased from local companies.

### Expression and purification of OPH

In our previous study, we reported expression of OPH with C-terminal His-tag in *E. coli* as soluble cytoplasmic active protein<sup>30</sup>. In the present study, we describe large scale single step procedure for purification of recombinant OPH from *E. coli*. As the pPHLNS400 coded OPH contains C-terminal His-tag, large-scale purification of OPH was done essentially by affinity chromatography using Ni-NTA column. Five liters of *E. coli* DH5 $\alpha$  (pPHLNS400) culture grown in YTG medium (yeast extract, 24 g/L, tryptone, 12 g/L, glycerol, 0.4% and 100 mM potassium phosphate buffer, pH 7.0) containing 1 mM CoCl<sub>2</sub> and 50  $\mu$ g/ml of ampicillin was grown at 30°C for 12-15 h. An additional amount of ampicillin (50  $\mu$ g/ml) was added before incubating the culture for an additional 25 h (total time: approximately 40 h). After incubation, the cells were harvested at 8000 rpm for 10 min at 4°C and cell pellet obtained was resuspended in 4-5 vols (w/v) of 10 mM potassium phosphate buffer (pH 6.5-6.9) amended with 50  $\mu$ M CoCl<sub>2</sub>.

The cell suspension was sonicated (Vibra-Cell VCX 750 Sonicator, USA) by applying 15 s on and 45 s off cycle for 30 min at 38% pulse power. The lysate was centrifuged at 15000 rpm for 45 min and the particulate-free supernatant was collected. Contaminating nucleic acids were removed by adding streptomycin sulphate (SS) solution (10%) in 10 mM Tris buffer (pH 7.0) to bring to a final concentration of 1%. The precipitated nucleic acids were eliminated by centrifuging the contents for 15 min at 15000 rpm. The clear supernatant was then loaded on to Ni-Sepharose matrix equilibrated with sodium phosphate

buffer (50 mM, pH 7.2; 300 mM of NaCl and 50  $\mu$ M of CoCl<sub>2</sub>). Fast protein liquid chromatography (FPLC, AKTA Basic, GE Health Care, USA) was performed at a flow rate of 1 ml/min. The protein bound to the Ni-column was eluted by using a linear gradient of 50-500 mM of imidazole in phosphate buffer (50 mM, pH 7.2; 300 mM of NaCl, 50  $\mu$ M CoCl<sub>2</sub>) at a flow rate of 1 ml/min. The eluted fractions were analyzed for purity on a 12.5% SDS-PAGE gel.

### Enzyme assay and protein estimation

The 100 ng of OPH was mixed with 1 ml of 50 mM Tris glycine buffer (pH 9.0) containing 50  $\mu$ M CoCl<sub>2</sub> and 0.5 mM methyl parathion and activity was assayed by measuring formation of *p*-nitrophenol<sup>31</sup> at 410 nm (410 nm,  $\epsilon_{410} = 16,500 \text{ M}^{-1}\text{cm}^{-1}$  for *p*-nitrophenol) for 5 min at 37°C. Protein concentration was determined by Bradford Method<sup>32</sup>. Specific activity was expressed as micromoles of *p*-nitrophenol produced/min/mg of protein. One IU of enzyme was given as amount of OPH required to hydrolyze 1  $\mu$ mol of methylparathion/min<sup>7</sup>.

### Immobilization of OPH on gelatin pads

#### Preparation of gelatin pads

The principle of immobilizing OPH on gelatin pads is shown in Fig. 1C. Pure gelatin (5 g) was dissolved in 100 ml of water and heated to 70°C for 30 min. The solution was cooled rapidly to 40°C and immediately formalin was added to a final concentration of 0.07% and the contents were left for 30 min to facilitate gelatin cross-linking (intra cross-linking). Above reaction mixture was foamed using a high speed homogenizer and the foam obtained was then passed into a container (10 cm  $\times$  8 cm  $\times$  8 mm) before freezing for 10 h at -20°C. The frozen foam was lyophilized to remove the excess moisture and the resultant foam was then tested for its insolubility before using it for OPH immobilization (Fig. 1b).

#### Covalent cross-linking of OPH with gelatin foam

A total of 5 gelatin foams were prepared as described above. These insoluble gelatin foams (10 cm  $\times$  8 cm  $\times$  8 mm) were soaked in 1 L of phosphate buffer (10 mM, pH 7.4) for 30 min and thoroughly washed for several rounds before equilibrating in the same buffer. The equilibrated gelatin pads were soaked in glutaraldehyde solution (0.05% in 1 L of phosphate buffer) for 4 h. While the pads were soaked in glutaraldehyde solution for every 30 min they were squeezed and left in the same solution to facilitate better interaction between

glutaraldehyde spacer and gelatin pads. The foam pads were washed 3-times with phosphate buffer (10 mM, pH 7.4) to remove excess glutaraldehyde by squeezing and by adding fresh phosphate buffer (10 mM, pH 7.4). The equilibrated pads were finally suspended in 50 ml of OPH (1 mg/ml) and incubated for 1 h at room temperature to facilitate interactions between OPH and glutaraldehyde spacer attached to gelatin pads.

After incubation, the pads were manually squeezed to remove the buffer and uncoupled OPH from the pads. These pads were then reincubated in the phosphate buffer (10 mM, pH 7.4) to facilitate coupling of OPH to the free glutaraldehyde spacers. The process was repeated till the quantity of OPH in the buffer remained constant. Finally, the gelatin foam was left in OPH solution for another 36 h to obtain better coupling efficiency. After completion of coupling reaction, the OPH-gelatin pads were suspended in 40 ml of mannitol (1.4 mg/ml) containing bovine serum albumin (BSA 0.1 mg/ml) as stabilizing agents and subjected to lyophilization for 24 h. After lyophilization, the OPH-gelatin pads were divided into two batches. One batch was left at room temperature and the second batch was kept at 4°C until further use.

#### *Determination of OPH load on gelatin pad*

The OPH density on gelatin pad was estimated by assaying OPH activity per mg of gelatin pad. After performing cross reaction, the pads were removed from the reaction buffer and extensively washed by repeated squeezing in phosphate buffer (10 mM, pH 7.4) to remove OPH absorbed to the gelatin pads. The squeezing process was continued till no OPH activity was observed in the squeezed buffer. A small portion of pad was taken to determine specific OPH activity in OPH-gelatin pads.

#### *Catalytic properties of gelatin immobilized OPH*

To find out the efficiency of coupling and catalytic activity, 5 mg of OPH immobilized gelatin foam was used to perform the activity assay. Initially, foam was transferred in 2.5 ml of phosphate buffer (10 mM, pH 7.4) and vortexed for 2 min to separate the uncoupled and free enzyme (this step was repeated thrice). After each step, foam was manually squeezed and squeezed buffer was collected to perform OPH assay. Absence of OPH activity in squeezed buffer was taken as an indication for elimination of free OPH from gelatin pads. At this stage, a portion of OPH-gelatin pad was taken to calculate the enzyme load on matrix by

estimating the specific activity of OPH. After assaying the enzyme activity the pads were squeezed and thoroughly washed before reusing them for determination of OPH specific activity. This process was repeated for nearly 15-times to know the reusability of OPH immobilized foam. Assay protocol described earlier was followed, except that the reaction volume was increased to 5 ml to facilitate immersion of OPH-gelatin bits in the assay buffer.

#### *Determination of sarin hydrolysis by immobilized-OPH*

The hydrolysis of sarin was determined by nuclear magnetic resonance (NMR) spectroscopy. The reaction tubes were prepared in triplicates and subjected to NMR analysis using 400 MHz, Bruker instrument. Magnetic nuclei of  $^{19}\text{F}$  were used to detect product formation by chemical shift. The rate of hydrolysis of sarin was calculated by estimating the formation of hydrogen fluoride (HF) formed during OPH-mediated hydrolysis. Further, the disappearance of sarin was also monitored at 0, 10 and 60 min. Hexafluoroacetone (2  $\mu\text{l}$ ) in 98  $\mu\text{l}$  of DMSO was used as standard and all the experiments were carried out at  $37 \pm 1^\circ\text{C}$ . Studies on degradation of sarin were initiated in a reaction tube containing 2 ml HEPES buffer (100 mM, pH 7.5) with 7.27  $\mu\text{l}$  of sarin (25.95 mM) and 40 mg of OPH immobilized gelatin pad. In control tube, only gelatin pad was added to the reaction mixture.

## **Results and Discussion**

OP nerve agents pose a silent global threat, as they may be used as chemical warfare agents. Although a number of chemical and biological methods are in use for the decontamination of nerve agents, the biological decontamination process is considered superior than the chemical process due to its ability to eliminate OPs without causing further damage to the environment<sup>33</sup>. The discovery of bacterial OPH in 1980's was a breakthrough in the search for the green processes for decontamination of OP compounds. A number of bioremediation tools involving immobilized OPH have been made available to decontaminate OP compounds<sup>34,35</sup>. However, they suffer from a number of limitations, the foremost being the loss of the enzyme activity during immobilization; in certain cases, nearly 50% of the enzyme activity is lost during the process of immobilization<sup>37</sup>. Even after immobilization, the stability of immobilized enzyme was a serious limitation. When compared with available methods,

OPH-gelatin pads were found to be highly stable and required no special conditions for storing up to six months.

#### Rapid single step OPH purification

Although many reports are available on the heterologous expression of OPH in various expression systems<sup>37-41</sup>, but most of the methods are either cumbersome or take longer time to purify the expressed protein. To overcome this, we have earlier reported the expression of OPH with a C-terminal His-tag that can be used as affinity tag for purification of OPH<sup>30</sup>. The culture conditions reported in this study reduced the formation of inclusion bodies of the expressed protein. Further, after obtaining the clear particulate-free supernatant from the lysate prepared from the induced culture it was directly applied to the affinity matrix and after thorough washing with phosphate buffer (50 mM, pH 7.2; 300 mM of NaCl, 50  $\mu$ M CoCl<sub>2</sub>), the bound OPH was collected by increasing the concentration of imidazole (50-500 mM as a linear gradient) in the phosphate buffer (50 mM, pH 7.2; 300 mM of NaCl, 50  $\mu$ M CoCl<sub>2</sub>). The protein obtained was pure (Fig. 1A) and the yield was about 10 mg of protein from 1 L culture which corresponds to nearly 2000 IU/mg of OPH. The purification process was completed in 12 h, as compared to the earlier procedures taking almost 5 days to get pure OPH in sufficient quantities. Thus, due to its simplicity, the procedure can be easily adapted for scale-up process.

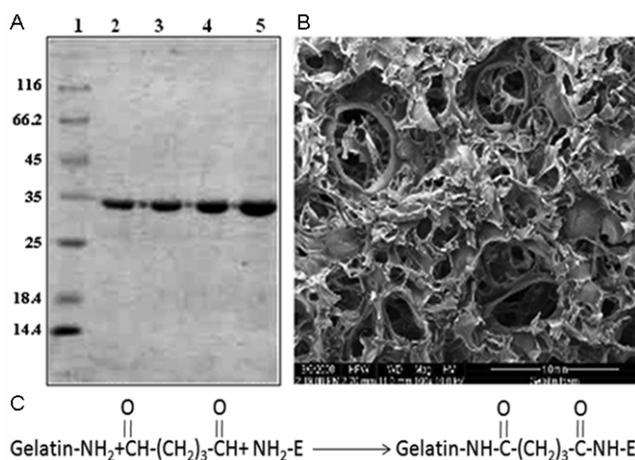


Fig. 1—(A): SDS PAGE showing the purification of OPH-6His expressed in *E. coli* DH5 $\alpha$ . [Lane 1, standard protein molecular weight markers and lanes 2-5, OPH present in fractions collected from Ni-Sepharose affinity column; (B): Scanning electron micrograph of the gelatin foam; and (C): Reaction mechanism explaining covalent coupling of OPH using glutaraldehyde, a homo bi-functional cross-linker]

#### Immobilization and stability of OPH on gelatin pads

In the present study, gelatin was used as supporting matrix for immobilization of OPH. Out of 50 mg (100,000 IU) of protein (OPH) used, 40 mg (80,000 IU) of OPH was found to be either absorbed or covalently-linked to the matrix, while the remaining OPH (20,000 IU) was found in the suspended solution. Out of this bound OPH, 99.5% (79,600 IU) was adsorbed to the matrix, while the remaining 0.5% (400 IU) was covalently linked to the matrix. This 0.5% corresponds to 0.2 mg of OPH was covalently immobilized on 5400 mg of gelatin pad. When calculated for mg of matrix, 37 ng of OPH was present on 1 mg of gelatin pad.

The 40 mg of OPH gelatin pad hydrolyzed 25.95 mM of sarin in 10 min. The amount of OPH present on 40 mg of gelatin was found to be 1.4814  $\mu$ g of protein corresponding to 2.9628 IU of OPH which indicated that 1.75  $\mu$ M of sarin was hydrolyzed per second per  $\mu$ g of immobilized OPH.

#### Kinetic properties of immobilized OPH

After evaluating methyl parathion hydrolysis (Figs 1, 2), we extended our studies to find out the effect of immobilization of OPH in hydrolyzing the sarin. Sarin hydrolysis rate was monitored through NMR either by using <sup>31</sup>P and <sup>19</sup>F nuclei. But the use of <sup>19</sup>F nuclei was found to be advantageous, as it offered higher sensitivity than <sup>31</sup>P. The rate of hydrolysis of sarin was monitored by observing the formation of HF. In a typical reaction mixture, complete hydrolysis of sarin was observed in 10 min. In agreement of this observation, no peak in NMR was observed at 59 and 62 ppm, rather a peak at 120 ppm was noticed indicating complete hydrolysis

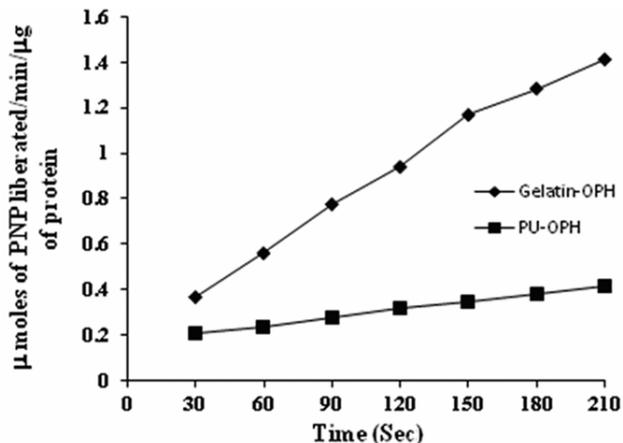


Fig. 2—Specific activity obtained for OPH-gelatin (♦) and OPH-PU (■)

of sarin added to the reaction tube. In a control reaction where only gelatin foam was used, the characteristic peak at 120 ppm, indicating the hydrolysis of sarin was not observed even after 60 min of incubation. However, only two peaks at 59 and 62 ppm which are specific for parent compound sarin were observed.

In order to find out the relative performance of OPH-gelatin pads, we made OPH-polyurethane foam (PUF) pads, following the procedures described elsewhere<sup>42</sup> and the OPH specific activities obtained with these two immobilized systems were compared (Fig. 2). The data presented in this study clearly indicated that OPH immobilized on gelatin pads was more active than the OPH immobilized on PUF. Though the reason for such reduction in OPH activity in PUF was unexplainable with the present data, but decreased OPH activity might be to some extent due to the harsh chemical procedures followed during the course of immobilizing OPH on to PUF<sup>42</sup>.

Further, portion of OPH gelatin pads stored at room temperature was taken periodically and specific activity was calculated and the results were plotted (Fig. 3). No significant loss in OPH activity was observed till 180 days, but the activity started decreasing beyond that. The pads stored under aseptic and cold conditions retained OPH activity beyond 1 yr (data not shown). Thus, in stability and performance OPH-gelatin pads were superior to other reported immobilized systems (Table 1). The unusual stability might be due to the fact that gelatin being itself a protein is shown to stabilize the immobilized protein<sup>43</sup>. Further, additives such as BSA and mannitol used in the present study might have enhanced the stability of immobilized OPH. In addition, as the OPH gelatin pads are biocompatible they can be directly placed on the wounds of soldiers and other human subjects who come in contact with OP compounds. Moreover, gelatin being a biological material and is also used in a number of surgical applications cause no harm to the human subjects

The kinetic properties of the OPH immobilized on gelatin were found to be comparable with those the OPH immobilized on other matrices (Table 1). Further, the gelatin-OPH pads successfully eliminated nerve agent such as sarin, when added to the reaction mixture. However, when phosphate buffer was used in the reaction mixture, with time most of the added OPH got precipitated, affecting hydrolysis of sarin. No sarin hydrolysis was observed even after the fresh enzyme was added to the reaction mixture. This might

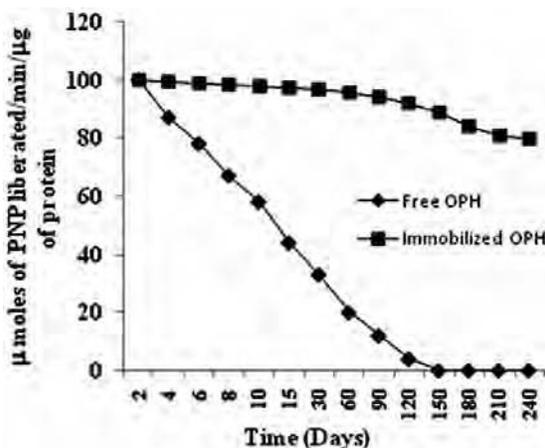


Fig. 3—Stability of free (♦) and immobilized OPH (■) stored at room temperature [The OPH activity ( $\mu\text{M}$  of PNP liberated/min/ $\mu\text{g}$  of protein) was measured using methyl parathion as assay substrate]

Table 1—Comparison of OPH activity and stability of immobilized OPH on various matrices

Matrix	Stability	$K_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_{\text{m}}$ ( $\mu\text{M}^{-1}$ )	$K_{\text{cat}}/K_{\text{m}}$ ( $\text{M}^{-1}\text{s}^{-1}$ )	Reference
Gelatin	180 days at RT, >360 days at 4°C	*100 #33 × 10 <sup>4</sup>	*200 #10	*5 × 10 <sup>5</sup> #33 × 10 <sup>6</sup>	This work
Carbonyl diimidazole support	66.9 days	NM	NM	NM	23
PUF	146 days	NM	NM	NM	42
Glass beads	180 days	NM	38 × 10 <sup>6</sup>	NM	46

NM, Not measured, RT, Room temperature, \* and # represent values obtained using methylparathion and sarin as assay substrates respectively.

be due to a reduction of pH of the reaction mixture as a consequence of formation of two moles of acid (isopropyl methylphosphonic acid and HF) from one mole of sarin. This situation was avoided by using 100 mM HEPES as assay buffer, which is known to provide better buffering conditions. In 100 mM HEPES buffer, sarin added to the reaction mixture was completely hydrolyzed and no precipitation of the enzyme was observed. Although reports are available showing nerve agent hydrolysis using both chemical and enzymatic methods<sup>44</sup>, most of them are confined to the usage of free OPH<sup>45</sup>. In fact, this is the first report, where hydrolysis of nerve agents is shown using an immobilized OPH.

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