



Rational design of organophosphorus hydrolase with high catalytic efficiency for detoxifying a V-type nerve agent



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ABSTRACT

V-type nerve agents, known as VX, are organophosphate (OP) compounds, and show extremely toxic effects on human and animals by causing cholinergic overstimulation of synapses. The bacterial organophosphorus hydrolase (OPH) has attracted much attention for detoxifying V-type agents through hydrolysis of the P–S bond. However, low catalytic efficiency of OPH has limited the practical use of the enzyme. Here we present rational design of OPH with high catalytic efficiency for a V-type nerve agent. Based on the model structure of the enzyme and substrate docking simulation, we predicted the key residues that appear to enhance the access of the substrate to the active site of the enzyme, and constructed numerous OPH mutants. Of them, double mutant, L271/Y309A, was shown to exhibit a 150-fold higher catalytic efficiency for VX than the wild-type.

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1. Introduction

V-type warfare nerve agents are organophosphate (OP) compounds, and inhibit acetylcholinesterase (AChE), thereby disrupting hydrolysis of the neurotransmitter, acetylcholine (ACh) [1,2]. Excess level of ACh continuously stimulates muscle contraction, causing various types of symptoms including dyspnea, and dimming of vision and eye pain vomiting, abdominal pain, convulsions, respiratory failure, paralysis, and death [3]. Of many V-type agents, VX (O-ethyl-S-diisopropyl amino methyl methylphosphonothiolate, Fig. 1A) is the most well-known, showing LD₅₀ (lethal dose, 50%) of 10 mg for human [4]. V-type agents are known to be stable and less volatile, and they can persist for 2–6 days in the environment [5]. Because of their extreme toxicity and environmental durability, efficient detoxification of V-type agents would be of great significance.

Many methods have been attempted for detoxification including bleaching, alkaline hydrolysis and incineration, but they require harsh conditions and discharge toxic byproducts [6]. The OP-degrading enzymes has attracted much attention as promising

candidates for detoxification of nerve agents owing to their fast hydrolysis rate compared to chemical methods [2,7,8]. Of them, bacterial organophosphorus hydrolase (OPH) has been extensively studied for detoxifying V-type agents because of its distinct hydrolyzing activity toward P–S bond of organophosphate compounds such as demeton, malathion, acephate, phosalone, and VX [9,10]. However, the catalytic efficiency and soluble expression level of OPH in bacteria were shown to be very low. To improve the catalytic efficiency of OPH, random and directed evolution approaches have been attempted [3,11–17]. Interestingly, co-expression of chaperones led to an increase in soluble expression level of OPH [18]. Nonetheless, despite the extensive studies, the catalytic efficiency of OPH for hydrolyzing P–S bond of OPs including V-type agents remain to be further improved.

Here we present the rational design of OPH to improve the catalytic efficiency for a V-type nerve agent. Based on the model structure of OPH and substrate docking simulation, we predicted the key residues that appear to enhance the access of the substrate to the active site of the enzyme, and constructed the variants by site-directed mutagenesis. The resulting OPH variants were tested in terms of catalytic efficiency for an analogue substrate, and five single and double mutants were selected. Of them, double mutant, L271/Y309A, was shown to have a 150-fold increased catalytic efficiency for a V-type nerve agent, VX, compared to the wild-type. Interestingly, Y309A exhibited high soluble expression level in *Escherichia coli* even without chaperones and folding partners.

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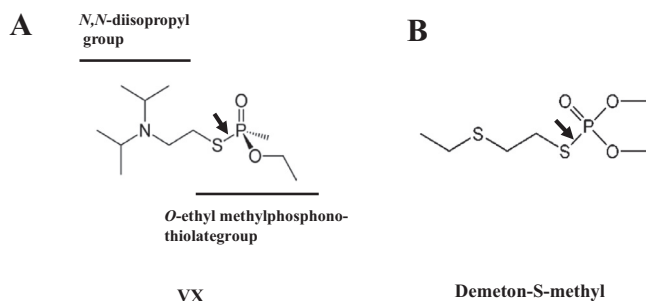


Fig. 1. Organophosphorus compounds used in this study. (A) VX, (B) Demeton-S-methyl. Arrows represent cleavage site of OPH.

2. Materials and methods

2.1. Materials

E. coli XL1-Blue and BL21 (DE3) cells were purchased from Real Biotech Corporation (Taipei, Taiwan). Expression plasmids, pMAL-c2x and pET28a, were obtained from New England Biolabs (Beverly, MA, USA) and Novagen (Madison, WI), respectively. Oligonucleotides were synthesized by Bioneer (Daejeon, Korea). Demeton-S-methyl used as an analogue for a V-type nerve agent (Fig. 1B) and antibiotics were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Luria–Bertani (LB) media were from Duchefa Biochemie (Haarlem, Netherlands). The restriction endonucleases, T4 DNA ligase, PCR polymerases, and other PCR reagents were purchased from Takara Bio (Shiga, Japan). All other reagents were of analytic grade.

2.2. Docking simulation and structural analysis

The structure of a starting template of OPH was constructed by a homology modeling using MODELLER [19]. Alignment was trivial as the mutant contains no insertion or deletion. PDB entry 1EYW [20] was used as the sole template. As a result, 64 models were produced, and the substrate was docked to each model structure. Ideal geometry of VX was obtained from PubChem (CID 39793). Docking was performed using in-house software. The objective function included Lennard-Jones (LJ) potential for vdW energies and restraint terms. Parameters for the 6–12 LJ potential were adopted from the Gromos54a7 force field [21]. The phosphorus was selected as the root atom, which was randomly placed in a 5 Å-wide cubic space with uniform probability. The cube was placed at the spot corresponding to the phosphorus of the substrate analogue in PDB entry 1EYW [20]. Restraints were imposed on the phosphorus so that removing it from the zincs would lead to a steeply increasing penalty. Specifically, it was confined within 4 Å range of Zn_α and 3.5 Å from Zn_β and farther separation resulted in a penalty which was proportional to the fourth power of the excess distance. Location (by the root atom), orientation and torsional angles of the substrate were optimized by an algorithm based on iterative hill-climbing. A pose was encoded by a 14-D vector and was modified by random vectors from 14-spherical normal distribution. The first 3 elements representing the location of the root atom relative to the origin were divided by 10 to decrease its sensitivity to random perturbation. A group of poses were simultaneously evolved. Optimization followed a series of phases. Population size, magnitude of perturbation (temperature), and stop condition were predetermined for each phase. The stop condition was a prescribed number of consecutive and/or accumulative failures to improve total energy. After each step, the magnitude of the random vector was scaled down, poses were sorted by

total energy, and some worst poses were dropped. The protein 3D structures were visualized by PyMOL [22].

2.3. Construction of OPH variants

E. coli XL1-Blue and BL21 (DE3) were used as the host cell for cloning and expression of OPH mutants. The gene coding for OPH from *Flavobacterium* sp. was provided by GenoFocus (Daejeon, Korea). As a starting template, the variant containing A80V, I106V, F132D, K185R, D208G, H257W, I274N, S308L, and R319S was constructed from wild-type OPH according to previous study [3]. All variants were amplified by overlapping PCR method. The amplified PCR products were introduced into the pMAL-c2X plasmid using the *Eco*RI, *Hind*III and T4 DNA ligase, and were transformed into *E. coli* XL1-Blue competent cells harboring pGro7 having GroEL and GroES chaperones [18]. To test the soluble expression levels of variants, designated variants were cloned into pET28a plasmid with *Eco*RI and *Sall* followed by transformation of *E. coli* BL21 (DE3).

2.4. Growth, expression and preparation of OPH variants

The transformed cells were cultured in LB media containing carbenicillin (100 μg mL⁻¹), chloramphenicol (25 μg mL⁻¹), L-arabinose (1 mM) and ZnCl₂ (0.2 mM). When the OD₆₀₀ reached 0.4, IPTG was added to a final concentration of 0.5 mM, followed by further incubation at 30 °C during 12 h. The MBP-fused enzymes were purified using a MBP Excellose[®] SPIN Kit (Bioprogen, Daejeon, Korea) according to the manufacturer's instructions. The concentration of purified enzyme was determined by Bradford assays [23]. *E. coli* BL21 (DE3) cells with OPH variants were grown at 37 °C in LB medium containing kanamycin (100 μg mL⁻¹). When the OD₆₀₀ reached 0.4, IPTG was added to a final concentration of 0.5 mM, followed by further incubation at 30 °C for 12 h.

2.5. Enzyme assay

The relative activities of OPH mutants for nerve agent analogue were measured by Ellman assay [3,24]. The reaction mixture (200 μL) contained 50 mM Tris–HCl (pH 7.0), 100 mM NaCl, 0.2 mM ZnCl₂, purified enzyme (100 μL of 0.1 mg mL⁻¹), Demeton-S-methyl (final 1 μM), and 0.5 mM DTNB [Ellman's reagent, 5',5'-dithiobis (2-nitrobenzoic acid)]. The kinetic constants (*K_m* and *k_{cat}*) for the wild-type and mutants for Demeton-S-methyl were determined by monitoring the formation of 2-nitro-5-thiobenzoate at 412 nm. The reaction was initiated by the addition of enzyme. Due to the extreme toxicity of real nerve agent VX, the assay was performed in a chemical surety materiel laboratory at the Armed Forces CBR Defense Command (Korea). The reaction mixture consisted of 0.2 mM VX in buffer (pH 8.5, 50 mM Ammonium carbonate, 0.1 mM CoCl₂) and the variants of OPH. The reaction was carried out at room temperature, and reaction products were analyzed using gas chromatography, Agilent 6890 GC (Agilent, USA). The initial reaction rates were determined at different substrate concentrations, and the *K_m* and *k_{cat}* values were obtained from the Lineweaver–Burk plot.

3. Results

3.1. Construction of a starting OPH

Previous studies confirmed several amino acid residues that increased the catalytic activity of OPH from *Flavobacterium* sp. toward various OPs and its soluble expression. Changes of three residues, K185R, D208G and R319S, were shown to enhance

soluble expression of OPH [25], while A80V and I274N resulted in a slight increase in the hydrolysis rate [26]. With additional four mutations, namely I106V, F132D, H257W and S308L, the catalytic activity of OPH for Demeton-S-methyl and VX increased up to 35.5-fold [3]. Based on these findings, we constructed a starting OPH by introducing nine mutations into OPH from *Flavobacterium* sp. using site-directed mutagenesis, and the resulting mutant was designated 9M. In addition, the 30-residue signal peptide of the N-terminus was removed for the expression of OPH in a fully matured form [27]. Although the soluble expression of 9M was significantly improved compared to the wild-type, we introduced chaperone proteins of GroEL-GroES into the expression system [18] to further increase the soluble expression, and fused matose binding protein (MBP) as a fusion tag at the C-terminus of OPH variants.

3.2. Structural analysis of the substrate binding pocket of OPH

To get some insight into the beneficial mutations for increasing the catalytic efficiency of OPH toward a V-type agent, we performed docking simulation of VX onto the substrate binding pocket of the 9M model structure. As shown in Fig. 2A, VX was completely resided in the substrate binding pocket of the enzyme. While *O*-ethyl methylphosphonothiolate group was fit to the active site of the enzyme (Fig. 1A), bulky *N,N*-diisopropyl group is located in the entrance of the substrate binding pocket (Fig. 2A and B). Considering the possible steric hindrance by a bulky *N,N*-diisopropyl group to the enzyme, the access of VX to the active site of the enzyme would depend on the entrance region of the substrate binding pocket. Choline-like *N*-dialkyl group is the characteristic moiety of V-type agents [28]. Therefore, we reasoned that enlarging the entrance of the substrate binding pocket would enhance the access of V-type agents to the substrate binding pocket of the enzyme, and accordingly the catalytic efficiency. With this hypothesis, we selected the residues around the entrance (Fig. 2B and Table 1). Four residues (D132, T173, L271 and Y309) which are located at the outer rim of the entrance, were predicted to interact with *N*-dialkyl group. Additional four residues, W131, H201, D233 and H254, were selected from inside of the entrance region, which also possibly contact with *N*-dialkyl group. Some of these residues, including W131, H201, L271 and Y309, were shown to position around the substrate analogue in OPH [20]. In an attempt to enhance the structural flexibility around the entrance region, two residues, T172 and P256, were also selected.

Table 1
Selected residues for enlarging the entrance of substrate binding pocket of OPH.

Group	Residues	Rationale
Group1	D132, T173, L271, Y309	Widening the outer rim of entrance
Group2	W131, H201, D233, H254	Expanding the inside of entrance
Group3	T172, P256	Increasing structural flexibility of entrance

3.3. Construction and activity assay of OPH variants

To examine the effects of the selected residues, we changed each of the residues to Ala to enlarge the entrance of the substrate binding pocket. We determined the catalytic efficiencies of the resulting mutants toward Demeton-S-methyl as an analogue of V-type agents by Ellman assay (Fig. 3A). Because Ellman assay specifically tracks the release of free thiol [24], we could measure not only hydrolytic activity of the variants but also their specificity toward the P–S bond of substrate. Three variants, T172A, L271A and Y309A, were shown to exhibit increased activities for Demeton-S-methyl compared to 9M. This result suggests that enlarging the entrance of substrate binding pocket can increase the catalytic activity of OPH, mainly by enhancing accessibility of substrate to the active site. To investigate the combinatorial effect of these mutations, double or triple mutants were constructed, and their activities were determined (Fig. 3B). Two double mutants, T172/Y309A and L271/Y309A, showed more than 2-fold increased activities compared to 9M, whereas other double mutant (T172/L271A) and triple mutant (T172/L271/Y309A) exhibited decreased activities (Fig. 3B). This result suggests that combination of T172A and L271A affects negatively on the hydrolysis activity of OPH. Based on the results, we finally selected three single and two double mutants, T172A, L271A, Y309A, T172/Y309A and L271/Y309A, to examine the catalytic efficiency for a real nerve agent VX.

3.4. Catalytic efficiency of the mutants for VX

We determined the catalytic activities of five selected mutants for a real nerve agent, VX (Fig. 4A). Of tested single mutants showing enhanced hydrolysis for the analogue, single mutant Y309A showed a slightly increased activity toward VX compared to 9M (Fig. 4A). The catalytic activity of double mutant L271/Y309A was about 1.5-fold higher than 9M (Fig. 4A). To compare the catalytic

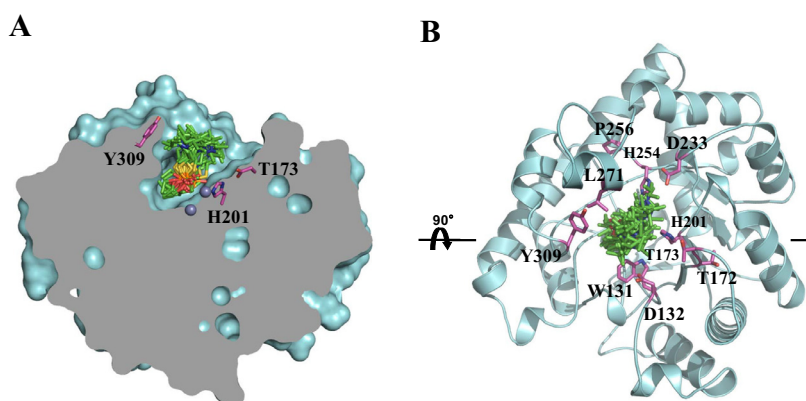


Fig. 2. Docking simulation results of VX on the model structure of OPH. (A) Cross-section of the substrate binding pocket bound with VX. Two zinc ions in catalytic site are shown in gray sphere. (B) Cartoon representation of 9M homology model bound with VX. The view is rotated by 90° from (A) as indicated. Black bar indicates the plane of cross-section in (A). Selected residues for mutagenesis are shown in stick representation (magenta). 20 redundant modes of VX from docking simulation were shown in stick representation (green, yellow and orange). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

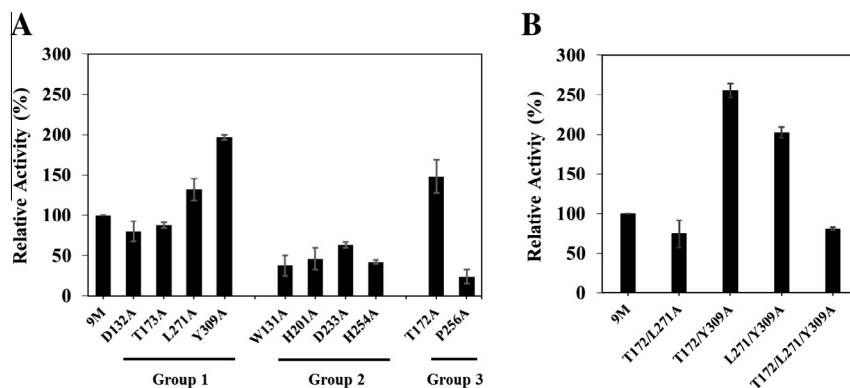


Fig. 3. Mutational effects on hydrolysis of analogue substrate. (A) Relative activities of single variants for Demeton-S-methyl. Groups indicated under bar graph are same as in Table 2. (B) Relative activities of double and triple mutants toward Demeton-S-methyl. The activity represents average and standard deviations in triplicate experiments.

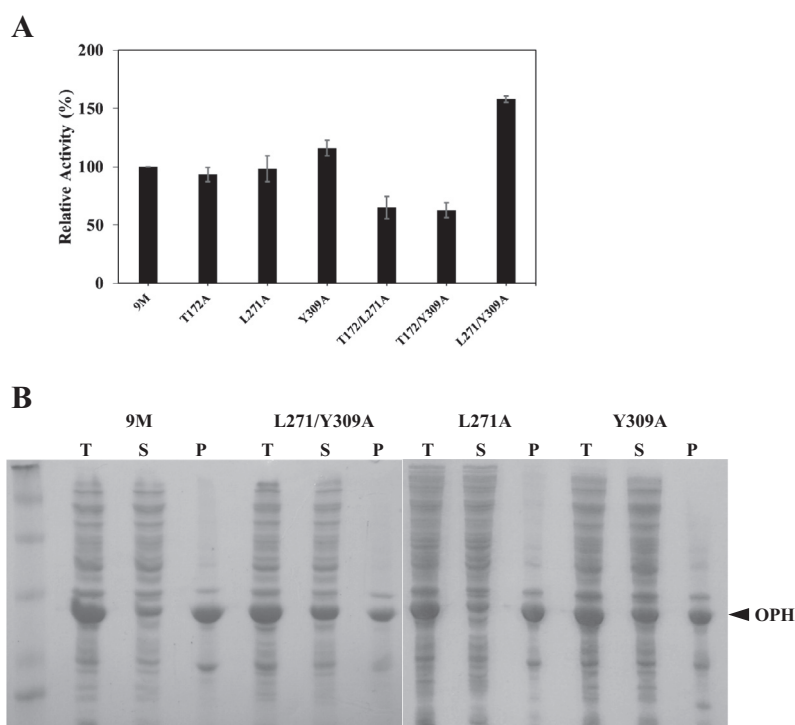


Fig. 4. Effects of the selected residues on hydrolysis of VX and soluble expression of OPH. (A) Relative activities of single, double and triple mutants toward a V-type agent, VX. (B) Soluble expression levels of the OPH mutants. T, S and P represent total lysate, soluble fraction, and pellet, respectively. The activity represents average and standard deviations in triplicate experiments.

efficiency, we determined kinetic constants (K_m and k_{cat}) of double mutant L271/Y309A and 9M (Table 2). The double mutant showed a 5-fold higher catalytic efficiency than 9M. As the catalytic rate of 9M was 30-fold increased compared to wild-type OPH, the catalytic efficiency of the double mutant corresponds to a 150-fold increase compared to the wild-type.

To check the possible effect of the two residues on the soluble expression, we examined the soluble expression levels of single and double mutants of L271A and Y309A in the absence of chaperones and MBP in the expression system. As shown in Fig. 4B, the OPH variant containing the Y309A mutation showed a significant

increase in soluble expression compared to 9M, whereas single mutant, L271A, exhibited a similar soluble expression. The soluble fraction of Y309A was estimated to be about 50% of the expressed enzyme, and double mutant also showed a similar level (Fig. 4B). This result indicates that the Y309A mutation can increase the soluble expression level of OPH even without chaperones and MBP.

4. Discussion

We have demonstrated that the catalytic efficiency of OPH for a real nerve agent, VX, was significantly enhanced by a rational design approach. Docking simulations based on the model structure of OPH enabled the prediction of the residues that appeared to enhance the catalytic efficiency by enlarging the entrance of the substrate binding site of the enzyme. Considering the kinetic constants of the mutant, the prediction was shown to be well coincident with the catalytic behavior of the mutant. The double

Table 2
Kinetic parameters of the OPH mutants for VX.

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} M^{-1}$)	Fold increased
9M	0.0007	0.15	4.86	
L271/Y309A	0.15	5.87	24.9	5.1

mutant, L271/Y309A, showed a significant increase in both k_{cat} and K_m when compared to 9M. This result indicates that enlarging the entrance facilitates the access of the substrate to the active site and accordingly the turnover number (k_{cat}). At the same time, the dissociation of the substrate also became easier, and the substrate concentration required for a half-maximal velocity (K_m) increased. This result strongly supports that the present approach to enlarge the entrance of substrate binding site is effective for enhancing the catalytic efficiency of OPH toward a V-type agent with a bulky N-dialkyl group. Real nerve agents are extremely toxic and dangerous, and prediction of the key residues using docking simulation is likely to be useful for rationally designing OPH with high catalytic efficiency for a real nerve agent. Low soluble expression level of OPH limited the practical application of the enzyme, and several approaches including random, directed evolution, and chaperones have been attempted [18,25]. Interestingly, the Y309A mutation led to a significant increase in soluble expression level compared to 9M even without chaperones and MBP. Mutational analysis revealed that two residues, L271A and Y309A, play a crucial role in an increase of both catalytic efficiency and soluble expression of OPH.

Current methods to detoxify V-type nerve agents have severe drawbacks. In other words, major approaches including bleach treatment, alkaline hydrolysis, and incineration usually produce toxic byproducts and require harsh conditions. Thus, the development of enzyme-based method has been of great significance. However, the restrictions and safety concerns by extreme toxicity of V-type agents have hampered the design of related enzymes with high catalytic efficiency. In this regard, structure-based rational design and test of the mutants using an analogue of high correlation with real nerve agents can be effectively used for developing the enzyme-based detoxification process for nerve agents.

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