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Enhanced Activity and Stability of Organophosphorus Hydrolase via Interaction with an Amphiphilic Polymer

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Abstract

A simple approach to enhancing the activity and stability of organophosphorous hydrolase (OPH) is developed based on interactions between the hydrophobic poly(propylene oxide) (PPO) block of amphiphilic Pluronics and the enzyme. This strategy provides an efficient route to new formulations for decontaminating organophosphate neurotoxins.

Neurotoxic organophosphate (OP) compounds are widely used as pesticides and, unfortunately, chemical warfare agents. Bioremediation can be accomplished through hydrolysis of the phosphate ester bond with genetically engineered OP degrading enzymes, whole cells containing such enzymes, or enzymes anchored on cell surfaces.¹ Due to higher activity and ease of modification, cell-free enzymes are often more attractive to utilize. Organophosphorus hydrolase (OPH) is one of the most active and widely studied enzymes for detoxification of a variety of OP compounds.^{1b, 1d, 2} However, the enzymes have a relatively short shelf-life and lose their activity in the presence of residual organic solvents^{2b} or high temperatures that are encountered in practical usage. To improve the enzyme stability, various post-processing approaches have been investigated, such as immobilizing enzymes on substrates,³ integrating enzymes into firefighting foams,⁴ embedding OP enzymes into hydrogels,⁵ covalently conjugating polymers onto OP enzymes^{3a, 6} and electrostatically complexing OPH with polyelectrolytes.⁷ Additionally, enhanced OP degrading activity has been observed when OPH is immobilized on functionalized mesoporous silica through ionic interactions.⁸

An important challenge in OP bioremediation is not only enhancing the stability of vulnerable enzymes, but also the preparation of surfactant formulations that can remove

hydrophobic OPs from surfaces to enable degradation by waterborne enzymes. Therefore, it is desirable to prepare stable formulations of enzymes and surfactants for large area decontamination. Co-addition of various types of polymers as additives has been utilized for the stability of protein/enzyme solutions.⁹ Among those, amphiphilic polymers can be useful as surfactant formulations, satisfying the purpose of OP bioremediation. Herein, we demonstrate that amphiphilic poly(ethylene oxide-*b*-propylene oxide-*b*-ethylene oxide) (PEO-PPO-PEO) triblock copolymers, known as Pluronics, can physically associate with OPH (E.C. 3.1.8.1; Fig. 1a), and lead to improvements in both the stability and activity of the enzyme due to interactions between the hydrophobic block of Pluronic and hydrophobic amino acids in OPH. This approach of simply blending the enzyme with inexpensive, non-toxic, biocompatible, and commercially available Pluronic provides an efficient formulation for OP detoxification with long pot life.

Although surfactant molecules typically reduce the activity of enzymes, the addition of Pluronic F127 to OPH resulted in an activity increase under many practically relevant conditions. OPH activity was monitored using the absorbance of *p*-nitrophenol, the product of the paraoxon hydrolysis. Because of the finite mixing time involved in the measurement, the activity was compared using k_{cat}/K_M ,^{1b, 11} the enzymatic efficiency, in lieu of the traditional activity measurement. Similar kinetic parameters were obtained for fresh OPH (k_{cat} : 2900 s⁻¹, K_M : 0.08 mM; less than a week after cell lysis), comparable to the reports from the literature.^{11–12} To study the pot life under practical usage conditions, a low concentration of OPH, 0.2 µg/mL was used for storage and diluted to 0.05 µg/mL immediately before the kinetic tests. In the absence of Pluronic, OPH lost half of its efficiency in two hours at RT (Fig. 1b and Fig. S1), and less than 10% of the original efficiency remains after 4 days at 4°C (Fig. S2). The activity drops similarly from 96±7 mM/min to 42±10 mM/min after two hours at RT. Enzyme half-life depends strongly on testing conditions such pH, temperature, and concentration, leading to reported half-lives ranging from hours to months.^{5b, 6a, 11, 13} At diluted concentrations used in our study, BSA is typically required as stabilizer to prolong OPH pot life,¹⁴ indicating the instability of OPH in our testing conditions.¹⁵ Increasing the enzyme:polymer molar ratio slowed the activity loss: at a 1:10 molar ratio, more than 85% of the initial activity remained after two hours. Under these testing conditions, the enzyme is fully soluble (Fig. S3), the paraoxon concentration (equal or less than 0.1 mM) is below the solubility limit (~3 mM), and the Pluronic F127 concentration (1 nM–100 µM) is below the critical micelle concentration (CMC; 550µM in water).¹⁶ Therefore, solubilization or micellization effects by the Pluronic are unlikely to be related to the enhancement, and it is hypothesized that the polymer interacts directly with the enzyme to increase stability. Surprisingly, at 1:1,000 and 1:100,000 molar ratios, the initial OP degrading activities were enhanced by 14% and 40% compared to the initial activity of OPH alone, and after two hours 104% and 120% remained, respectively. On the other hand, PEO alone (green diamond in Fig. 1a) creates a minimal effect, indicating that the PPO block is critical to the enhancement of the OPH pot life and activity.

Using a suite of analytical techniques, the PPO block of the Pluronic is shown to interact with hydrophobic residues of OPH. MALDI-TOF was used to identify a possible binding

between the OPH enzyme and the polymer. In the absence of Pluronic, the spectrum revealed a major peak at 39,064 g/mol as well as peaks at 77,813 g/mol (blue curve in Fig. 2a), corresponding to m/z and $2m/z$ for OPH monomers and dimers. After the addition of Pluronic at a 1:3 molar ratio of enzyme to polymer, a new peak at 51,254 g/mol was observed (* in Fig. 2a) which can be attributed to the adduct of OPH monomer and Pluronic F127 (MW: 12,600 g/mol), suggesting that Pluronic interacts with OPH.

NMR showed a shift in peak positions that are consistent with hydrophobic interactions between OPH and the PPO block of the Pluronic. The ^1H NMR spectrum of the Pluronic block copolymer showed a peak at ~1 ppm that denotes the $-\text{CH}_3$ pendant groups of the hydrophobic PPO segment (Fig. S4). This peak (Fig. 2b) upshifts by 0.01 ppm when the enzyme is mixed with the polymer at a 1:1 ratio. An upfield shift of the same magnitude in the ^1H NMR spectrum has been reported for hydrophobic interactions between Pluronic (particularly PPO, $-\text{CH}_3$ groups) and the poorly water-soluble hydrophobic drug, paeonol,¹⁷ while 2D NOESY strongly correlates PPO with an enzyme peak (Fig. S5). Therefore, the observed shift is consistent with hydrophobic interactions between OPH and Pluronic.

The Raman spectrum at the same molar ratio used in MALDI-TOF showed a notable difference at 1004 cm^{-1} , indicating an interaction between Pluronic and Phenylalanine (Phe) in OPH. After normalization of both spectra to the 1650 cm^{-1} amide band (Fig. S6), the 1004 cm^{-1} band from the symmetric ring breathing of Phe¹⁸ was reduced by 12.4% in the presence of Pluronic (* in Fig. 2c), clearly indicating interaction of the polymer with Phe residues, likely those located on the solvent-accessible surface of OPH. Among 32 Phe residues in the OPH dimer, 14 residues are located on the surface, related to the 1004 cm^{-1} Raman signal, while the others are buried inside the structure (Table S1); therefore, only 44% of the Phe residues are available for interaction with the polymer. Limitations on the enzyme:polymer molar ratio may further reduce differences in the Raman signal, or preferential interaction with specific Phe residues could account for the observed magnitude. While interactions with Phe are most easily detected due to high Raman sensitivity for this amino acid, interactions with other hydrophobic residues cannot be precluded (15.8% reduction in the Trp fluorescence intensity of OPH/Pluronic mixture compared to OPH alone was also observed; Fig. S8). By combining all the spectroscopy results, it is concluded that the nonpolar PPO block of the Pluronic can interact with the hydrophobic amino acids on the outer surface of OPH, bringing the two macromolecules into close proximity.

These hydrophobic interactions between Pluronic and OPH were directly related to the enhanced enzyme stability by examining three different destabilizing conditions: organic solvents, high temperatures and long storage times. If the OPH/polymer complexes are essential to maintain the OPH activity, addition of polymers to the enzymes before exposure to destabilizing conditions would theoretically provide a better OP degrading activity than addition afterwards. To test this hypothesis, the OPH activity was measured in each of the three destabilizing conditions with different OPH-containing samples (Fig. 3). During measurements, OPH and polymers were mixed at 1:1000 molar ratio because of the immediate enhanced effects shown during the OPH pot life testing (Fig. 1b).

Detoxification of hydrophobic OP compounds by the OPH enzyme can be performed by utilizing water-compatible organic solvents,^{4b, 5b, 6a, 13} such as MeOH, which helps to solubilize the OPs at the cost of quenching the catalytic site of OPH (Fig. S9). In the presence of 10% (v/v) MeOH, the OP degrading efficiency was impressively suppressed to 2% of its value compared to the enzyme in methanol-free buffer ($k_{\text{cat}}/K_M = 18600 \text{ s}^{-1}\text{mM}^{-1}$), whereas 31% of the OP degrading efficiency remained when OPH was premixed with Pluronic before exposure to MeOH (Fig. 3a). The effect on activity follows a similar trend, dropping from $170 \pm 20 \text{ mM/min}$ in buffer to $9 \pm 2 \text{ mM/min}$ after exposure to MeOH or $83.9 \pm 0.1 \text{ mM/min}$ when premixed with Pluronic. When Pluronic was added to OPH after MeOH exposure (the molar ratio of methyl groups between Pluronic and MeOH is $1 : 7 \times 10^5$), the activity recovers to 10% of the original, much worse than for premixed OPH/Pluronic. This result further confirms that OPH/polymer complexes more effectively detoxify paraoxon in the presence of MeOH than OPH alone. Pre-constructed OPH/polymer complexes can effectively mitigate MeOH quenching, confirming the hypothesis of enhanced OPH activity due to the OPH/Pluronic complexes.

During storage or use, OPH can be exposed to high temperatures which can aggregate enzymes (Fig. S10). Heating pre-constructed OPH/Pluronic complexes showed that Pluronic can effectively stabilize OPH in high heat conditions (Fig. 3b). After exposure to 70°C , OPH retained 25% of its activity after 10 min and 2% of its activity after an hour (Fig. S11). However, when the polymer is premixed with OPH, 85% of its activity remained after 10 minutes at the elevated temperature. Addition of the polymer to the enzyme after the heat exposure resulted in minimal recovery of activity. This indicates that OPH/Pluronic complexes can slow the aggregation at high temperatures, acting like a shielding factor – a phenomenon related to the mitigation of protein aggregation by protein-Pluronic associations.^{9b}

To understand whether OPH/polymer mixtures enhance the OPH stability during long-term storage, OPH ($0.2 \mu\text{g/mL}$) and OPH/Pluronic samples were prepared and stored in solution as well as in a lyophilized form. For direct comparison, all samples were stored at 4°C . The OP degrading activity of OPH in both samples (solution and lyophilized) dramatically dropped within a week ($>95\%$; Fig. S12). Although lyophilization caused an initial drop in the activity ($\sim 15\%$), OPH/polymer mixtures maintained 49% of the original activity after 25 days (Fig. 3c). Pluronic added to already lyophilized OPH only restored the activity back to 14 % of the original value. Therefore, pre-constructed OPH/Pluronic complexes can protect OPH against denaturation during lyophilization and can slow down the OPH denaturation process.

The ability of PPO-containing block copolymers to enhance OPH stability and detoxification activity is general for a variety of molecular structures. Other types of Pluronics (triblock and diblock copolymers) were investigated by blending with OPH at 1:1000 molar ratio (Fig. S13). The initial activity of OPH/polymers complexes increased 10% – 40% compared to the activity of OPH alone, with 10% decrease or less over 2 hours at RT. Statistically significant differences in the activity, as a function of the PPO weight % or the architecture (diblock vs. triblock), were not observed compared to Pluronic F127 ($p\text{-value} > 0.05$).

Although a detailed mechanism for how the PPO block of Pluronic can enhance the catalytic activity of OPH is not elucidated, physicochemical characterization suggests that the hydrophobic PPO blocks of Pluronic interact with hydrophobic amino acids of OPH, resulting in an increased activity and pot life for dilute OPH concentrations, MeOH inhibition, enhanced stability against high temperature, and extended shelf life. This simple approach of constructing enzyme/polymer complexes can improve the solubilization and detoxification of a wide variety of OPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) The surface of OPH dimer is illustrated by hydrophobicity (color bar)¹⁰ and hydrophilicity (blue) (PDB ID: 3E3H). Yellow windows point out OP catalytic sites. (b) Hydrolysis of paraoxon by OPH with Pluronic F127 and PEO(PEG) polymers (green diamond). See standard deviation of data points in Fig. S1.

Figure 2.

MALDI-TOF (a), ¹H NMR (b) and Raman (c) spectra of OPH, Pluronic and OPH/Pluronic.

The inset in (a) shows the molar mass of OPH monomer by SDS-PAGE.

Figure 3.

Enhanced OP degrading efficiency by OPH/Pluronic mixtures in 10% MeOH (a), at RT after 10 minutes at 70°C while in solution (b) and after resolubilizing lyophilized samples stored for 25 days at 4°C (c).