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Review Article

Diisopropyl-fluorophosphatase as a Catalytic Bioscavenger

Hossein Allahyari[1], Ali Mohammad Latifi[1*]

Abstract

Organophosphorus chemicals, used as pesticides and warfare nerve agent, are highly toxic compounds that inhibit acetylcholine esterase enzyme rapidly. A novel effective treatment for nerve gas poising is using of hydrolytic enzymes to degradation of these agents. Diisopropyl-fluorophosphatase (DFPase) from *Loligo vulgaris* is highly stable and robust biocatalyst for the hydrolysis of various chemical warfare agents such as sarin, soman, tabun. Unfortunately, wild-type DFPase prefers less toxic isomers of these agents leading to slower detoxification. Also, due to non-human origin of the enzyme, immunological reactions occur when it is injected into body. In order to using DFPase as in vivo detoxifying agent, some manipulations to augment of its efficiency and to decrease of immunogenic problems are needed. Modifications such as PEGylation is one of the possible solutions to conquer these problems. Engineering of the enzyme for creating of new efficient variants is an interesting research field which leads to occurrence of novel and prominent bioscavenger, and delivery of these functional molecules to circulation in order to enzymatic hydrolysis of toxic agents would be the final object of research efforts.

Keywords: DFPase, Bioscavenger, Organophosphorus Compounds, Nerve Agents

1. Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

*** Corresponding Author**
Ali Mohammad Latifi
Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
E-mail: amlatifi290@gmail.com

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Introduction

Organophosphorus (OP) compounds, such as Sarin (GB), Tabun (GA), Soman (GD), and VX (Figure 1) are used as pesticides, insecticides, and chemical nerve agents. Organophosphates are highly toxic because they rapidly inactivate acetylcholinesterase (AChE), an enzyme required for nerve function, by forming a covalent adduct with an active site serine residue, leading to overstimulation of the nervous system and subsequently to respiratory failure and death [1]. Chemical retrieve of AChE can be done by administering "reactivators" such as oximes or other, but this approach has not effective well. Furthermore, covalent adducts of AchE encounter "aging", spontaneous de-alkylation, which makes adduct formation irreversible [2]. Therefore, current protocols for the prevention and treatment of organophosphate poisoning are largely ineffective, and so new strategies are desperately needed. A relatively new detoxification strategy for reducing the blood concentration of OP compounds is using molecules as selective scavenger [3].

Bioscavengers

Development of bioscavengers commenced from 25 years ago. They are divided in three categories such as stoichiometric, pseudo-catalytic, or catalytic. Stoichiometric bioscavengers are specific molecules that irreversibly bind to OPs in a mole-to-mole ratio (Fig. 2A). Pseudo-catalytic bioscavengers are stoichiometric bioscavengers in combination with a reactivator (Fig. 2B). Catalytic bioscavengers are OP-degrading enzymes with a turnover (Fig. 2C), so that administration of a small dose of a catalytic bioscavenger is thought to provide better protection than large doses of costly stoichiometric bioscavengers [4]. A biological scavenger, bioscavenger, should have some characteristics. For example, it is fast acting and catalyzes hydrolysis of specific substrates. It will be ideal to any bioscavenger having a good stability in blood circulation, and not evoke an immune response due to antigenic challenge to the immune system [5].

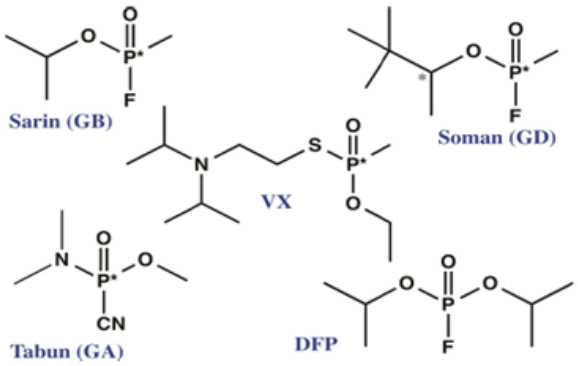

Figure 1. Structure of some Organophosphorus (OP) compounds. An asterisk denotes a chiral center [2].

Also, if an enzyme is to be an effective catalyst for the hydrolysis of organophosphate nerve agents, the rate constant (kcat/Km, a measure of the speed of a chemical reaction) for the process must exceed 10^7 M^{-1} min^{-1} [6]. Another consideration to take into account is that the G-type nerve agents are chiral molecules, they form isomers known as enantiomers that are mirror images of each other. Only one of the two enantiomers (the SP enantio-

mer) is toxic. Frustratingly, OP degrading enzymes primarily catalyze the non-toxic isomer (the RP enantiomer) of nerve agents, and the kcat/Km for the hydrolysis of the SP enantiomer is about hundred M^{-1} min^{-1} [7]. Optimization of these enzymes to improve their catalytic efficiency and other characteristics, is one of the interesting research fields using protein engineering.

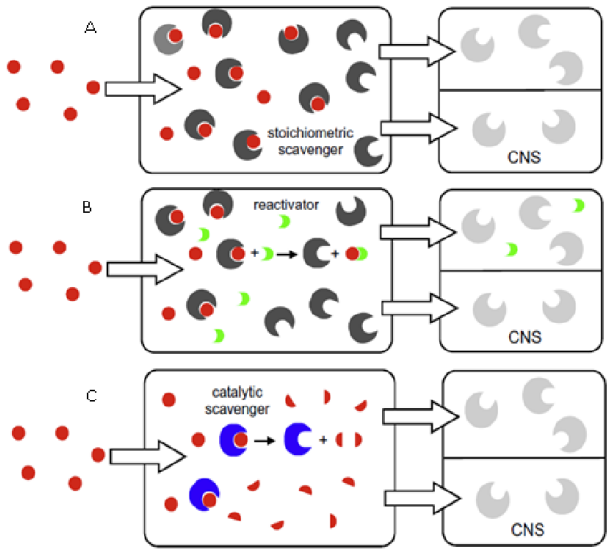

Figure 2. Types of bioscavengers. (A) Stoichiometric bioscavengers irreversibly bind to OP in a mole-to-mole ratio that prevent peripheral and central hAChE inhibition. (B) Pesuedo-catalytic bioscavengers are stoichiometric bioscavengers in combination with a reactivator that potentially increases protection efficacy of them. (C) Catalytic bioscavengers are OP-degrading enzymes with turn over, so that small dose of these provide better protection than other types of bioscavengers [4].

Molecular structure of DFPase

The diisopropyl-fluorophosphatases are a group of enzymes classified into Mazur-type DFPase and squid-type DFPases based on their size, structure, substrate specificity and stimulation by Mn^{2+} [8-13]. Because of their ability to hydrolyze the fluoride containing organophosphate compound diisopropyl-fluorophosphate (DFP), this type of phosphotriesterases (PTE) are known as DFPase. Squid-type DFPase was originally discovered in *Loligo vulgaris* head axon and nervous system and due to have appropriate enzymatic characteristics, it had been subject of biotechnological researches [14]. DFPase has a monomeric structure, it contains 314 amino acids in a 35 KDa polypeptide chain that assumes β-propeller structure with six propeller blades (Fig. 3). Each propeller is made of four pleated β-sheets.

DFPase comprises two calcium ions in its structure (Fig. 4A). The more deeply buried calcium has been identified as the high-affinity Ca^{+} and it is responsible to the structural stability of enzyme whereas, the more solvent exposed calcium, known as the low-affinity Ca^{+}, plays a role in the hydrolytic reaction [15]. The importance of the active site-bound ion for catalysis was highlighted by the elimination of the ion that destroyed enzyme activity [16, 17]. These metal ions are bound to the enzyme by amino acid side chains (Fig. 4B). Structural and catalytic types are ligated to the protein by Asp232, His274 and by Asp229, Glu21, Asn120, Asn175 side chains, respectively. Additionally, three water molecules are ligated to each of calcium ions [15].

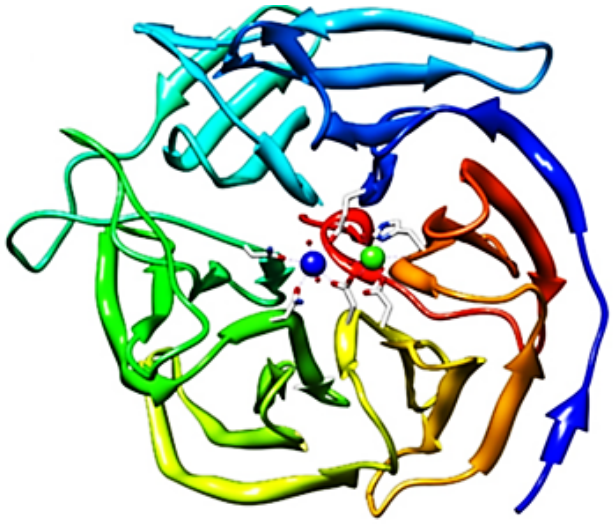

Figure 3. Top down view of DFPase showing the six bladed β-propeller structure and two metal ions [15].

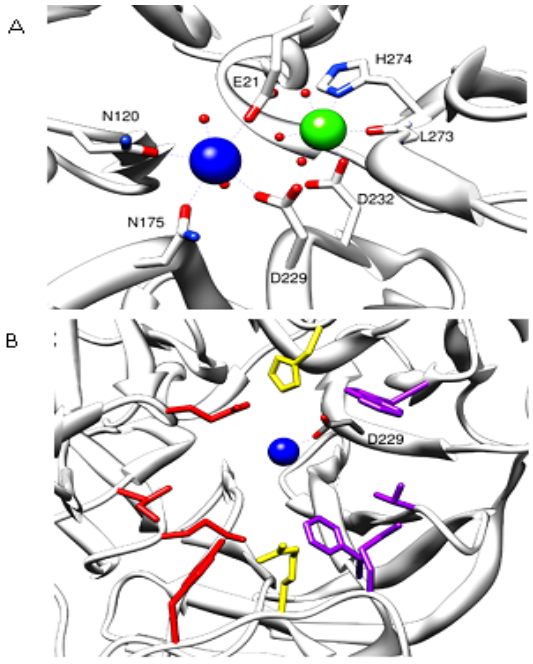

Figure 4. (A) Metal center ligation of DFPase. Catalytic and structural calcium is shown in blue and green, respectively (B) Substrate binding site in DFPase. Side pocket residues Tyr144, Met90, Ile72 and Glu37 are shown in red. The central cleft residues Arg146 and His287 are shown in yellow. Purple residues Trp244, Thr195, Phe173 and Met148 are from second side pocket. Catalytic aspartate is labeled [15].

Function and action mechanism of DFPase

Despite that molecular conformation of DFPase is similar to other PTEs such as PON1, it has not hydrolytic activity against compounds with P-O or P-S leaving group bonds, except for a few GD simulant containing P-O bonds [8, 18-22]. In addition, DFPase is only member of PTEs that has not efficient hydrolytic activity for lactones or esters. At present, the physiological function of the enzyme is not recognized and no natural substrate for DFPase has been identified.

Although DFPase can detoxify G-type nerve agents as GA, GB, GD and GF (Cyclosarin) but its catalytic efficiency for these nerve agents is less than that for DFP, one-tenth for GA and one-third for the others [23, 24]. Additionally, though DFPase has the ability to hydrolyze these agents efficiently, but its catalytic efficiency toward the more toxic stereoisomer (SP enantiomer) is too low to be as a bioscavenger [25, 26]. But in spite of these limitations, DFPase has some advantages such as high stability to both pH and temperature conditions that presented it as a suitable candidate to have biological scavenging property against compounds with P-F and P-CN bonds [24]. Native DFPase is highly stable at 6°C and 50°C with half-life of 290 and 3 days respectively. Other OP hydrolytic enzymes such as phosphotriesterase (half-life of 1.5 h at 50°C) and organophosphorus acid anhydrolase (half-life of 100 min at 37°C) have less thermostability than DFPase [27, 28].

To design improved variants of OP hydrolyzing enzymes, it will be helpful to comprehend the biochemical mechanisms of these detoxifying molecules with diverse substrates [15]. The proposed mechanism of DFPase is illustrated in Figure 5. The presently accepted mechanism for DFPase presumes upon binding of the substrate by the catalytic calcium and active site residues, then fluoride is released after inline nucleophilic attack by Asp229 [2]. Hydrolysis of the acyl enzyme intermediate by a conserved water sequesters the phosphate moiety from the active site.

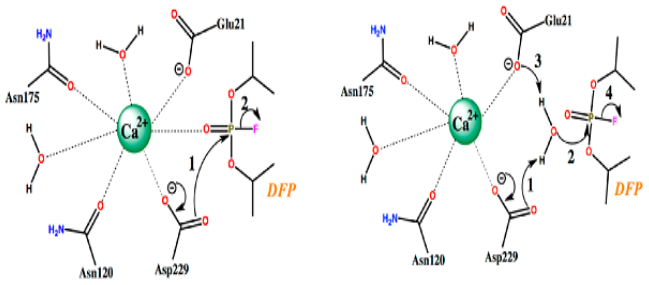

Figure 5. (Left) Proposed mechanism for phosphoenzyme intermediate formation involving Asp229 as the nucleophile. (i) Asp229 attacks the phosphorus center of DFP to form a pentavalent intermediate and (ii) the P−F bond dissociates to form a tetrahedral phosphoenzyme intermediate. Hydrolysis of the phosphoenzyme intermediate is not shown. (Right) Proposed mechanism for hydrolysis involving an activated water as the nucleophile. (i) Asp229 abstracts a proton from a water molecule either stepwise or in concert as (ii) water attacks the phosphorus center, (iii) Glu21 abstracts a proton either stepwise or in concert as (4) water forms a bond with phosphorus, and (iv) the P−F bond dissociates [2].

As mentioned earlier, DFPase is a vigorous and stable enzyme that efficiently hydrolyses DFP and other toxic warfare agents. As an ideal bioscavenger for detoxifying the AChE inhibitors in bloodstream before they are allowed to reach and supress their target, DFPase and its relatives have some problems that must be solved earlier. Since 1990s, some attempts were done to resolve them [2-5,18, 22, 29-31]. Various protein engineering methods, for example rational design of DFPase active site and directed evolution of catalytic activity of same enzymes toward toxic stereoisomerare implicated by several research groups [7, 17, 32-36]. Indeed, random mutagenesis methods are successfully being used to reverse the stereoselectivity of mammalian PON1 [37]. The final goal is to creating an enzyme that has enantioselectivity for the more toxic stereoisomer while its total activity is retained. Yet, many aspects of enzymatic mechanisms of DFPase in OP compound catalysis is not known completely [38]. For example, exact inter-molecular interactions in active site of this enzyme is not determined to the full. Wymore and colleagues investigated the mechanisms of two major substrates hydrolysis by DFPase with quantum simulation. They reported that hydrolysis mechanism of DFP and sarin by DFPase is different [2]. They proposed that an intervening water molecule activated by Asp229 residue has major role in catalysis of sarin hydrolysis while in DFP hydrolysis, Asp229 residue acts as a nucleophilic molecule. Their findings may lead to improved strategies for re-engineering of DFPase for more efficient degradation of OP compounds. Melzer and co-workers constructed two quadruple mutants of DFPase with a reversed enantioselectivity for G-type nerve agents for first time using rational design techniques. These mutants also have exhibit improved enzymatic activity against substrates in their study [7]. They reported that the created variants of DFPase could be used for practical rapid OP detoxification, as *in vivo* bioscavenger.

Immunogenicity and half-life

To avoid immunological issues, it is ideal to use human OP nerve agent hydrolytic enzymes as an *in vivo* bioscavenger [31]. The intense research about any human protein that has OP nerve agent hydrolysis potential is underway [5, 18, 39]. For example, human prolidase from erythrocytes or liver is one of the hopeful cases that needs to be re-engineered to reach a favorable level of function [40]. Despite the advantage of human origin of these proteins, some notable disadvantages, such as low catalytic efficiency, also exist that have limited their applications *in vivo* and *in vitro* [41]. Due to easy expression of some proteins in prokaryote hosts, it seems that the solving of immunological challenges of non-human enzymes is also one of the promising methods that helps to use them as in vivo scavenger [27, 42-44]. Another problem associated with non-human enzymes as bioscavenger, is quick clearance of them from the circulation. Covalent attachment of polyethylene glycol (PEG) chains to enzymes is a prominent solution to overcome these issues. PEGylation enlarges the hydrodynamic radius of proteins and shields its surface. PEG modification increases the stability against proteases, reduces immunogenicity, and delays renal excretion

significantly leading to prolonged half-life, reduced side effects, and increased pharmacological efficiency [45-47]. Melzer and co-workers demonstrated that DFPase can be coupled with multiple PEG chains without severe decrease in enzymatic function [48]. They found that PEGylated wild type and mutant DFPases have a different protective effects. Natural DFPase only in high doses could save tested animals while the mutant variants keep animal alive without or with very mild symptoms. It should be noted, the PEGylation protocol used in this study induced antibody response against enzyme and in order to diminish immune reaction, this process need to be optimized. Despite what mentioned earlier, this is a clear proof of principle that a DFPase mutant engineered for preference of the more toxic stereoisomers of the nerve agents and with an improved substrate affinity can be used as a potential *in vivo* bioscavenger against poisoning with OP nerve agents.

As noted above, among various protective approaches that have been developed to lessen the toxic effects of OP compounds, use of hydrolytic enzymes to degrade these chemicals is an effective approach [8, 12, 49]. An enzyme-based bioscavengers consist of several groups that act as a different manner. Catalytic bioscavengers are type of enzymes that behave as catalyst for OP degradation process, one molecule of enzyme can inactivate thousands of new OP molecules in the blood circulation before they can reach and block AChE. Application of enzymes with human origin for *in vivo* OP detoxification will be ideal, if we can resolve the specificity, stability and efficiency issues of these candidates. Using of non-human sources such as living organism in intense environments to prepare favorable catalytic bioscavengers is another helping option and engineering these proteins that catalyze hydrolysis of chemical warfare agents is an outstanding prophylactic medicare to reduce their toxic effect. The most important requirements about these foreign enzymes are immune system induction against repeated injection of these proteins and very low affinity to more toxic isomers of OP chemicals comparing the less toxic ones [44, 45].

DFPase as an enzyme that destroys all G-types of nerve agents and has stability against high temperatures and intensive pH conditions, is prominent nominee for *in vitro* scavenging of OP nerve agents. Entering of DFPase into circulation as a catalytic bioscavenger, needs to creation some modifications in enzyme structure and function. Structural manipulations must preserve bioscavengers against quick clearance from *in vivo*. Also, these handlings should diminish immune responses to bioscavengers. Adding PEG moieties to these proteins is one of the most effective solutions. Surface modification using PEG conjugates has shown advantageous results and reported no evidence of cytotoxicity associated with PEGylation [3]. PEGylation not only can increase half-life of DFPase in the blood, but also can decrease the immune reaction to the enzyme. Although PEGylation process about DFPase require some optimizations, the reported results show the promising way [48]. Based on published data, it is clearly demonstrated that several PEG chains are joined to DFPase without severe decrease in its enzymatic activity. *In vivo* studies of PEGylated wild type DFPasein exposed animals with nerve agent showed that merely high amounts of enzyme can save treated animals. The possible explanation of this occurrence refer to selectivity of wild type DFPase for less toxic stereoisomers of these agents. While more promising results are assumed from PEGylated DFPase mutant that prefer more toxic stereoisomers for hydrolysis. So, genetic engineering methods can help to creation and substitution of some amino acids that have effective role in structure and function of the enzymes. Upon to reported results [7, 16], using rational design, the DFPase mutants have been constructed that have high affinity against more toxic isomers and even displayed increased enzymatic activity against its substrate.

Although, administration of PEGylated DFPase leads to evoke moderate antibody response, some reasons can be given. For example, high amounts of enzyme used for treatment is one of them that needs to be optimized. Also, using of mixture of PEG conjugated and un-conjugated enzymes can be another possible explanation for concluded outcome. As mentioned above, the findings such as mentioned above clearly display importance of enzymatic enginering efforts to improve the activity and selectivity of catalytic bioscavengers such as DFPase. Considering promising results, it seems further optimization is required for DFPase modification as a bioscavenger. Despite the problems associated to DFPase induced immune responses and catalytic efficiency, there are some obvious proofs for DFPase that is going to use as effective catalytic bioscavenger.

Conclusion

The organophosphates include an extremely wide group of structurally related compounds. The toxicity of these chemicals has stimulated a search for enzymes that are able to catalyze the hydrolysis reaction of them. It is reasonably amazing to discover enzymes in nature that can detoxify these comparatively synthetic compounds. It is more surprising than finding of them, application of these enzyme as bioscavenger to remove poison effect in affected person. Nowadays, none of candidates gratify the necessaries for an ideal bioscavenger.

Nominee bioscavenger proteins generally function either by stoichiometrically capturing and isolating the OP toxicant or by catalytically hydrolyzing the chemical into biologically inactive forms. The stoichiometric bioscavengers, in spite of exceptional affinity for almost all of G and V agents, have restricted capacity to bind more than one molecule of OP agents. Use of catalytic bioscavengers can be the best option to defeat restriction of binding capacity. Because of non-human origin being of these catalytic enzymes, some challenges including catalytic efficiency, immunogenicity and circulation half-life must be optimized.

DFPase could be one of the best candidates to play the bioscavenger role for catalytically hydrolysis of OP compounds. It has good stability in harsh conditions and can detoxify broad range of chemicals agents. Adjustment of immunogenicity properties of DFPase resolves the most important challenge of this enzyme in catalytic bioscavenger development.

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