Biochemical Engineering Journal 94 (2015) 134–144

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Review

Protein engineering of representative hydrolytic enzymes for remediation of organophosphates

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ARTICLE INFO

Article history:
Received 18 July 2014
Received in revised form
30 September 2014
Accepted 13 November 2014
Available online 21 November 2014

Keywords:
Bioremediation
Directed evolution
Nerve agent
Organophosphates
Prophylactic
Rational design

ABSTRACT

Organophosphorus (OP) pesticides are highly effective insecticides used worldwide due to their low cost and ease of application. OP compounds also form the core of G-series and V-series neurotoxic warfare agents. The potential lethality of these compounds has led to research initiatives that identify and evolve promising OP degradation enzymes through protein engineering. Traits commonly targeted in these studies include enzymatic catalysis, stability, stereoselectivity, and substrate preference. Evolved OP degradation enzymes can then be used in applications that include enzymatic remediation of contaminated agricultural and industrial sites, development of biosensors for environmental monitoring, and the prevention of OP poisoning through biocatalytic prophylaxis. This review will discuss the recent protein engineering strategies and achievements made in the development of evolved broad-spectrum OP degradation enzymes. The potential applications of these enzymes and new avenues for acquiring novel templates for future studies will also be discussed.

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1. Introduction

Organophosphorus (OP) insecticides and nerve agents irreversibly bind the enzyme acetylcholinesterase (AChE). As a result of this AChE inhibition, the neurotransmitter acetylcholine accumulates at toxic levels causing abnormal nerve function at muscarinic and nicotinic receptors of the central nervous system and neuromuscular junctions of the body. The severity of exposure is dependent upon the dosage, route of entry, and nature of the compound(s) involved, but symptoms typically include uncontrollable

Abbreviations: AChE, acetylcholinesterase; DFP, diisopropylfluorophosphate; HuBChE, human butyrylcholinesterase; MBL, metallo-β-lactamase; MPH, methyl parathion hydrolase; OP, organphosphorus; OPAA, organophosphorus anhydrolase; *opd*, organophosphorus degradation; OPH, organophosphorus hydrolase; PLL, phosphotriesterase-like lactonase; PON1, paraoxonase-1; PTEp, hosphotriesterase.

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http://dx.doi.org/10.1016/j.bej.2014.11.010
1369-703X/

muscle contractions, convulsions, and brain seizures that can lead to respiratory failure if untreated [1]. OP poisoning is common in the developing world and it is believed that millions are poisoned yearly with over 200,000 fatalities occurring worldwide. Many of these fatalities are intentional, making OP poisoning one of the most common forms of suicide in developing countries [2,3].

OP insecticides are often rapidly degraded from exposure to sunlight, water and microbial hydrolysis in the soil [4]. However, persistent overuse can and does lead to the accumulation of OP insecticides in our food, topsoil or wastewater runoff. This presents a significant threat to beneficial flora, aquatic life, agricultural workers, and consumers [5,6]. OPs also form the core of several very toxic chemical warfare nerve agents. G-series nerve agents including tubun (GA), sarin (GB), soman (GD) and cyclosarin (GF) are volatile liquids that are non-persistent in the environment and will degrade rapidly over time. V-series compounds such as VX and its associated Russian and Chinese analogs are non-volatile, persist far longer when released into the environment, and can be 10 fold as toxic as G-series nerve agents [7]. All OP compounds have either an R_P or S_P chiral configuration depending on the orientation of the chemical subgroups around the central phosphorus atom with the S_P configuration often representing the more toxic isomer [8–12].

The identification of microbial organisms that possess the capacity to degrade OP compounds has resulted in several promising biotechnology platforms with significant applications. These applications include environmental monitoring and remediation, destruction of nerve agent stockpiles, and acting as bioscavengers in medical prophylactics for prevention and treatment of OP poisoning. Nevertheless, there are major hurdles to clear before any lab-based initiatives can be deployed effectively. Degradative formulations used in field decontamination must be stable under harsh environmental conditions, while effectively maintaining their functionality in the presence of different chemicals, inert stabilizers, and supplementary additives [13,14]. Enzymatic bioscavengers must have stable half-lives, possess broad substrate ranges, and confer full protection at low dosages [15,16]. Enzymes considered for post-exposure treatment of acute OP poisoning must work very rapidly and avoid triggering an immunogenic response in the patient [16,17]. Most importantly, both environmental and medical applications require enzymes that are effective against racemic mixtures as well as the individual stereoisomers of the compound(s) present [8–12]. To address such concerns researchers have utilized a combination of directed evolution and rational design strategies to improve upon desirable traits of several well-characterized OP degrading enzymes for the accelerated remediation of key OP insecticides and nerve agents (Fig. 1).

Directed evolution is an approach to protein engineering that artificially introduces random mutations with the goal of creating improvements to desirable protein traits. A major advantage of directed evolution methodology is that foreknowledge of the protein's structure and catalytic mechanisms is unnecessary. Simple strategies may utilize chemical mutagens such as hydroxylamine to introduce mutations to plasmid DNA *in vitro* or bacterial mutator strains like *Escherichia coli* XL-1 Red, which are deficient in DNA repair pathways [18,19]. More complex methods include gene shuffling, in which DNA from the gene of interest is extracted from related organisms, fragmented, and allowed to re-anneal at random to form new recombinants, as well as error-prone PCR, a modification to standard PCR reactions designed to further disrupt the fidelity of Taq polymerase [19–21]. Error-prone PCR is a particularly powerful technique for controlling the overall level of mutation in a protein. Current error-prone PCR protocols call for every four rounds of amplified template to be serially diluted into a fresh PCR reaction, allowing the researcher to generate separate pools of protein variants with increasing numbers of mutations [21].

Regardless of the strategy employed, directed evolution studies always use high-throughput selective screening methods to resolve beneficial mutations. Evolved variants can then be iteratively cycled through subsequent rounds of mutation and screening to generate additional desirable mutations. While effective, this screening process is laborious and experiments may require many mutagenic cycles comprised of hundreds to thousands of screened clones for discovery of one appropriately evolved variant [18–21].

Rational design approaches strictly utilize site-directed mutagenesis to alter a specific site or sites on the protein of interest [19,22,23]. Strategies are generally both structurally and computationally driven. Studies often incorporate software packages such as *MODELLER*, *ROSETTA* and *ORBIT* to model and compare related protein structures as well as to predict and assess potential substitutions at mutagenic hotspots. Advanced protein-substrate docking models are then combined with low-throughput screening techniques that utilize small pools of mutagenic PCR primers that have been spiked with stabilizing mutations to efficiently generate variants with larger numbers of synergistic mutations [22–24]. While rational design is still limited in actual practice, these strategies will continue to benefit from every protein structure that is made freely available to other researchers [19,25,26].

Alternatively, if only pieces of structural or catalytic data for a protein are known, a semi-rational approach combining both directed evolution and rational aspects can be used to great effect. Many semi-rational approaches employ both saturation and combinatorial site-directed mutagenesis. Saturation mutagenesis can involve either a single critical residue or several residues that affect the desired enzymatic trait(s). These sites are mutagenized to include all possible substitutions and can be done individually or in tandem with other catalytic sites to effectively combine the net effects of each mutation [27,28]. Two popular modifications of this principle include iterative saturation mutagenesis and combinatorial active site testing. Iterative saturation mutagenesis extends the concept of saturation to a series of defined multisites, usually composed of 1–3 residues, which have been rationally selected across the protein of interest. Each residue positioned within these multisites is exhaustively mutagenized and screened before moving to the next multisite. Combinatorial active site testing uses structural information to identify pairs of residues with side chains that extend into the protein's active site. Defined active site pairs are then saturated and screened for enhanced enzymatic catalysis. Applied individually or in tandem, these two powerful techniques provide a comprehensive look at mutagenic hotspots on the protein of interest allowing the researcher to select for the best possible substitution or combination of substitutions possible [29,30].

This review will discuss recent protein engineering studies and potential applications of broad-spectrum OP metalloenzymes including bacterial phosphotriesterases (PTEs), serum paraoxonase-1 (PON1), and microbial prolidase enzymes that represent the best OP degradation enzymes yet characterized (Table 1).

2. Bacterial PTEs are the gold standard in enzymatic remediation of OP nerve agents

The best described of all genes involved in OP metabolism are the OP degradation (*opd*) gene(s) and associated PTE enzymes that they encode. These enzymes are often referred to as organophosphorus hydrolases (OPH) in the literature. The first *opd* gene was found on large plasmids isolated from *Sphingobium fuliginis* ATCC 27551 (formerly *Flavobacterium* sp. ATCC 27551) [13,37] and *Brevundimonas diminuta* GM (formerly *Pseudomonas diminuta* GM) [13,38,39]. This gene is part of a large, transposable catabolic operon capable of horizontal movement to other soil bacteria [40–42]. A close homolog of the *opd* gene, designated *opd*A, was found in an Australian strain of