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Engineering Improved Microbes and Enzymes for Biodegradation of Nerve Agents

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Introduction

The growing population in the twentieth century has necessitated higher yields in agriculture. To this end, pesticides have become an integral part of modern agriculture. The use of pesticides has resulted in higher crop yields, but at the same time released hazardous chemicals into the environment. Due to their low cost, efficacy and wide spread availability, organophosphate (OP) compounds have been one of the most widely used classes of pesticides throughout the world for industrial, agricultural as well as home use.

The effectiveness of OP compounds as pesticides and insecticides also makes them hazardous to humans and to the environment. Organophosphates and their family of compounds are potent neurotoxins that share structural similarities to chemical warfare agents such as sarin, soman and VX. Organophosphates act as cholinesterase inhibitors and in turn disrupt neurotransmission in insects as well as mammals. Classical symptoms of OP exposure include salivation, lacrimination, urination and defecation. Exposure to OP compounds can cause fatigue, dizziness, vomiting, paralysis and even death (Casarett and Doull, 1996).

As a result of their toxicity and wide spread usage, there is a need to treat the large amounts of wastes from unused pesticide concentrates, agricultural runoff, accidental spillage, and from cleaning of spray equipment and storage tanks. In addition to OP pesticides, disposal of huge amounts of OP compounds in the form of chemical weapons must be handled. The Chemical Weapons Convention (CWC) disarmament agreement required the immediate ceasing of development, production and stockpiling of chemical weapons. Further, the ratification by the United States of the CWC on April 30, 1997 required the destruction of all chemical weapons stockpiles by April 30, 2007. Clearly there is a need for rapid and efficient methods by which to dispose of these compounds.

Organophosphorus hydrolase (OPH) isolated from soil microorganisms has been shown to degrade these pesticides effectively (Dumas et al., 1989). However, the use of OPH for detoxification has always been limited by the high cost associated with purification. Whole cell detoxification is more cost effective; however, it is limited by the transport barrier of organophosphates across the cell membrane. Surface expression of OPH has been used to circumvent the transport limitations imposed by the cell membrane (Fig. 1). Whole cells expressing OPH on the cell surface degraded parathion and paraoxon 7-fold faster compared to whole cells expressing OPH intracellularly (Richins et al., 1997). The resulting live

biocatalysts were also considerably more stable and robust than purified OPHs, retaining 100% activity over a period of one month when maintained at 37°C (Chen and Mulchandani, 1998). Immobilization of these novel biocatalysts by physical adsorption onto solid supports provides an attractive means for pesticide detoxification in place of immobilized OPH (Mulchandani et al., 1999). However, a gradual cell detachment from the support reduced the effectiveness of the immobilized-cell system for long-term operation. A significant improvement, both in terms of economics and technology could be achieved with reversible and specific adhesion to the support.

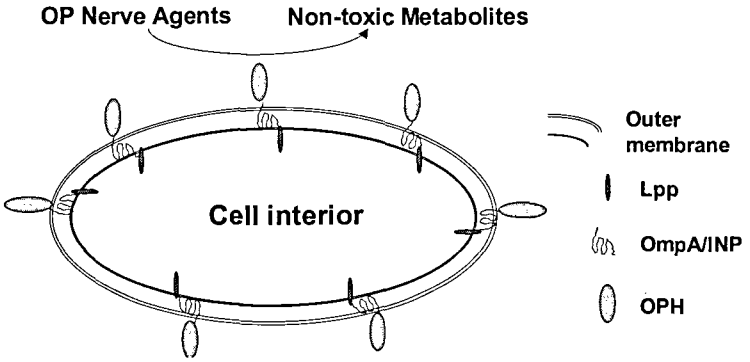

Fig. 1 Cell surface expressed OPH

Specific adhesion of whole cells to cellulosic materials with high affinity has been demonstrated by anchoring the cellulose-binding domain (CBD) from *Cellulomonas fimi* on the surface of *E. coli* (Francisco et al., 1993). This was exploited to enable very strong attachment of the organophosphate-degrading cells to cellulose supports for long-term usage (Wang et al., 2002). Two different surface anchors (Lpp-OmpA and INPNC) were employed to target OPH and CBD onto the cell surface, respectively, in order to minimize direct competition of the same translocation machinery. Whole-cell immobilization with surface-anchored CBD was very specific, forming essentially a monolayer of cells onto different supports as shown by electron micrographs. Immobilized cells degraded paraoxon rapidly and retained almost 100% efficiency over a period of 45 days. This is also the first reported genetic co-immobilization of two functional moieties onto the surface of *E. coli*.

Although the enzymatic hydrolysis of organophosphates such as parathion and methyl parathion reduces the toxicity by nearly 120-fold, the hydrolyzed product, *p*-nitrophenol (PNP), is still considered a priority pollutant by the U.S. EPA. A novel approach was developed to enable the simultaneous degradation of organophosphates and PNP by anchoring OPH on the surface of a native PNP degrader, *Moraxella* sp (Shimazu et al., 2001). The result is a single microorganism that is endowed with the capability to rapidly degrade organophosphate pesticides and PNP. This is also the first report on the functional expression of enzymes on the surface of Gram-negative bacteria other than *E. coli*.

Although OPH hydrolyzes a wide range of organophosphates, the effectiveness of hydrolysis varies dramatically. For example, some highly used organophosphorus insecticides such as methyl parathion, chlorpyrifos and diazinon are hydrolyzed 30-1000 times slower than the preferred substrate, paraoxon.

Sequential cycles of DNA shuffling and screening were used to "fine tune" and enhance the activity of OPH towards poorly degraded substrates. Because of inaccessibility of these pesticides across the cell membrane, OPH variants were displayed on the surface of *E. coli* using the truncated ice-nucleation protein in order to isolate novel enzymes with truly improved substrate specificities (Cho et al., 2002). Two rounds of DNA shuffling and screening were carried out and several improved variants were isolated. One variant 22A11, in particular, hydrolyzes methyl parathion 25-fold faster than the wild type. Similarly, directed evolution was used to improve the catalytic efficiency for a virtually non-hydrolyzable substrate, chlorpyrifos, by more than 750-fold, resulting in an enzyme that degrades this substrate as fast as paraoxon (Cho et al., 2004). Because of the success we achieved with directed evolution of OPH for improved hydrolysis of methyl parathion, this method can be extended in creating other OPH variants with improved activity against poorly degraded nerve agents such as sarin and soman.

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