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Designer oleosomes as efficient biocatalysts for enhanced degradation of organophosphate nerve agents

Sheng-Hsiang Leng, Cai-En Yang, Shen-Long Tsai *

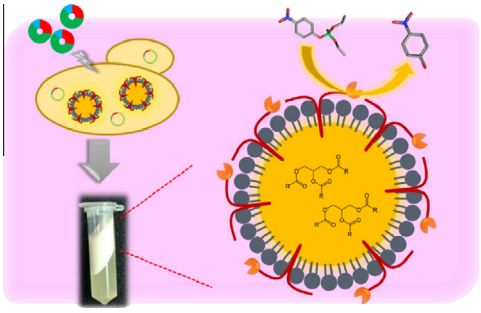
Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei 10607, Taiwan

HIGHLIGHTS

- A green approach to prepare efficient biocatalysts *in vivo* for enhanced degradation of organophosphate nerve agents.
- Organophosphorus hydrolase (OPH) was genetically fused with oleosins to form oleosome-based biocatalysts.
- The developed biocatalysts exhibited a 5-fold increase in the rate of paraoxon degradation compared to native OPH.
- Thermal stability, solvent tolerance and reusability of the biocatalysts were remarkably enhanced.
- The biocatalysts exhibited great potentials in decontamination of organophosphate neurotoxins in environmental samples.

GRAPHICAL ABSTRACT

Designer oleosomes produced by a genetically engineered oleaginous yeast reveal great potentials in biocatalytic decontamination of organophosphate neurotoxins.



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ABSTRACT

Engineering enzymes with higher activities, stabilities and reusabilities has been constantly pursued for applications of enzymatic catalysis in industrial, pharmaceutical and environmental processes. We report here a novel approach to prepare efficient biocatalysts *in vivo* for enhanced degradation of organophosphate nerve agents. Organophosphorus hydrolase (OPH) was genetically fused with oleosins to form oleosome-based biocatalysts. Quick purification of intact OPH-associated oleosomes can be achieved by using a flotation centrifugation method due to the high triacylglycerol (TAG) content in the oleosomes, making it easy for further protein recovery and reuse. The newly developed biocatalysts exhibited a 5-fold increase in the degradation rate against paraoxon compared to free OPH. In addition, the thermal stability and solvent tolerance were also enhanced remarkably. More importantly, the resulting biocatalysts remained intact and active even after repeated recycling, suggesting their potential use for bio-catalytic decontamination of organophosphate neurotoxins.

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1. Introduction

Organophosphorus compounds are widely used as pesticides, insecticides, and even chemical warfare agents. Due to their high toxicity and a lack of safety precautions, their widespread contamination of the environment and the ever-present threat of bioterrorism continue to be a concern today [1,2]. Current methods for their disposal, including chemical treatment, incineration, and landfills, are problematic due to the secondary risk of exposure and, in some cases, economically restrictive [3–5]. Therefore, there is an urgent need for safe,

* Corresponding author. Tel.: +886 22737 6628; fax: +886 22737 6644.
E-mail address: stsai@mail.ntust.edu.tw (S.-L. Tsai).

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economical, and reliable methods for detoxification and remediation of these compounds. Significant research efforts have been emphasized on the use of biotechnology for the targeted biodegradation of these agents [6,7]. Compared to the potential disadvantage of conventional methods, biological methods are more attractive because they are far less disruptive and more cost-effective.

Biotechnological methods of detoxifying organophosphates can be divided into two categories: those that use microorganisms as catalysts, and those that use enzymes as catalysts. Generally, microorganisms are the most efficient catalytic agents. However, this method has several major drawbacks, namely, poor tolerance to xenobiotics, resistance to mass transport of the substrate and product across the cell membranes, and potential drug resistance developed in microorganisms via horizontal gene transfer [8,9]. Alternatively, when properly harnessed, enzymatic approaches have much to offer for biodegradation of these organophosphate compounds [10,11]. Among the many enzymes that are currently under investigation, organophosphorus hydrolase (OPH), an enzyme that hydrolyses a wide range of organophosphates, has drawn significant attention [12]. However, the use of OPH for organophosphates degradation suffers from the limited stability, the inability for simple recovery and reuse, and the poor stability in non-ideal environmental settings. Even though OPH immobilization has been shown to overcome some of these problems [13,14], many of current immobilization approaches are not ideal because of the long preparation times, complex multistep procedures, harsh reaction conditions, and loss of activity during immobilization. Therefore, a new approach that is simple and environmental friendly is urgently needed in order to expanding its possible applications.

Oleosomes are spherical organelles consist of a triacylglycerol (TAG) core encapsulated by a monolayer of phospholipids in which a unique protein, oleosin, is embedded to stabilize the structure and prevent coalescence of oil in plant cell [15]. Due to the high TAG content, oleosomes have been employed as a carrier molecule for the expression and purification of recombinant pharmaceutical peptides and industrial enzymes in plant cell, *Saccharomyces cerevisiae* and *Escherichia coli* [16,17]. However, the slow growth and low heterologous protein expression level associated with plants, the very low level intracellular oil accumulation associated with *S. cerevisiae* and the inclusion body formation of oleosin fusion proteins in *E. coli* have rendered such applications less practical. Therefore, an ideal host that provides a fast growth rate, a high expression level, and a rich oil content is desired in order to achieve maximum benefits from this elegant system.

Yarrowia lipolytica, a well-known oleaginous yeast that naturally accumulates lipids to more than 20% of their dry cell weight (DCW), has already been exploited for the production of commercially useful lipids due to its short doubling time, high expression level, and simple scale-up [18]. In addition, it has been endorsed by the U.S. FDA (Food and Drug Administration) as "Generally Recognized as Safe" (GRAS) for numerous processes [19], making this microorganism an ideal host for large-scale production of oleosome-associated proteins. Herein, we report a bioinspired green approach that employs recombinant DNA technology to allow the production, immobilization, and purification of OPH decorated oleosome biocatalysts in a single step. To demonstrate its potential use in decontaminating organophosphate neurotoxins in the environment, the biocatalytic activity, the thermo-stability, the solvent tolerance and the reusability were examined and compared with native OPH.

2. Materials and methods

2.1. Strains and vectors

E. coli strain JM109 and BL21 (DE3) were used in this study for plasmids construction and free OPH expression, respectively. Additionally, the oleaginous yeast *Y. lipolytica* strain Polg (Yeastern Biotech Co. Ltd.) was also utilized for the expression of the designer oleosomes (oil bodies displaying OPH). The *E. coli–Y. lipolytica* shuttle vector pYLEX1 (Yeastern Biotech Co. Ltd.) was utilized for the oleosin–OPH fusion construction. The plasmids pINCOP [20] and pET-Ols-Xa-GFP [17] containing the OPH, and oleosin genes, respectively, were used as templates for PCR amplification.

2.2. Plasmids construction and transformation

The plasmid pYOlsOPHHis$_6$ encoding the oleosin–OPH fusion protein was constructed in two steps. First, the OPH gene was PCR amplified from pINCOP by using forward primer FBamH1Hind3OPH: 5′-*gggggatccggtggaaagcttatgcaaacgagaagggttgtgctcaag*-3′ (*Bam*HI and *Hin*dIII sites underlined) and reverse primer ROPH-His6: 5′-*cccggtaccttagtggtggtggtggtggtgtacgcccaaggtcggtgacag*-3′ (*Kpn*I site and His$_6$ tag underlined) The PCR product was then digested with *Bam*HI and *Kpn*I and inserted into pYLEX1 to create plasmid pYLEX-OPH. Subsequently, the oleosin gene was PCR amplified from pET-Ols-Xa-GFP using forward primer FBamH1Hind3Ols: 5′-*gggggatccatggctgagcattatggtcaacaacagc*-3′ (*Bam*HI site underlined) and reverse primer RBamH1OlsLinkerHind3: 5′-*cccaagcttactacctccacctccaacaggctgctgcgagaactg*-3′ (*Hin*dIII site underlined). The resulting PCR producer was digested with *Bam*HI and *Hin*dIII, and inserted into *Bam*HI-*Hin*dIII linearized pYLEX-OPH plasmid to generate pYOlsOPHHis$_6$.

The plasmid pETOPH encoding the OPH gene for free enzyme expression was constructed as follows. The OPH gene by was first PCR amplified with plasmid pINCOP using the primer pair FNde1OPH: 5′-*gggcatatgcaaacgagaagggttgtgctcaagtc*-3′ (*Nde*I site underlined) and RXho1OPH: 5′-*ccctcgagtacgcccaaggtcggtgacag*-3′ (*Xho*I site underlined). The amplified fragment was ligated into the *Nde*I and *Xho*I sits of the plasmid pET24a(+) to form pETOPH. *E. coli* transformation was performed following the standard methods [21]. *Y. lipolytica* transformation was carried out according to the optimized one-step *Y. lipolytica* transformation method [22].

2.3. Protein expression in Y. lipolytica and its recovery

The recombinant *Y. lipolytica* was pre-cultured in 3 mL of YPD medium at 30 °C for 12 h. Then, the cells were transferred into 50 mL of YNBO medium [23] containing 10 mM of $CoCl_2$ and incubated at 30 °C for 6 days with continuous shaking at 250 rpm in dark. The cells were harvested by centrifugation at 5000*g* for 5 min, washed three times with 10 mL 1X PBS buffer, and were finally re-suspended in 4 mL of 1X PBS buffer. Thereafter, the suspended cells were divided into four aliquots and ~400 mg of glass beads were added into each aliquot. The cell and glass bead mixtures were vortexed at maximum speed for 1 min and then cooled in ice bath for another 1 min. This step was repeated several times to achieve full cell disruption.

The designer oleosomes were harvested by centrifugation at 15,000 rpm for 10 min. A two-phase system was formed after centrifugation, where the oleosomes was floated to the top of the samples as a white creamy layer. The designer oleosomes, collected from the white layer, were then whished with PBS and stored in ice.

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