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Decorating outer membrane vesicles with organophosphorus hydrolase and cellulose binding domain for organophosphate pesticide degradation

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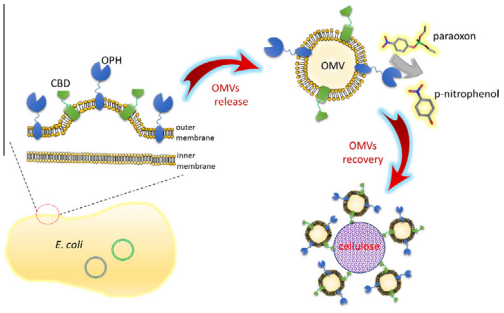
HIGHLIGHTS

- An OPH and a CBD were appended to OMVs via genetic fusion.
- The engineered OMVs exhibited an enhanced activity toward paraoxon degradation.
- Recovery of the engineered OMVs could be achieved easily via cellulose pull-down.
- OPHs tethered on OMVs revealed improved temperature, pH and long-term stabilities.

GRAPHICAL ABSTRACT



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ABSTRACT

Outer membrane vesicles (OMVs) are nanoscale spheres naturally released from Gram-negative bacteria. They contain a diverse array of proteins and lipopolysaccharide but do not replicate, which increases their safety profile and renders them attractive for environmental applications. Herein, an efficient and reusable biocatalyst for enhanced degradation of organophosphate pesticides was developed. Organophosphorus hydrolase (OPH) was tethered onto OMVs via a genetically fused ice nucleation protein (INP) to form OMV-based biocatalysts. To accomplish quick purification and easy recovery of the engineered OMV using cellulose, a cellulose binding module (CBM) was collaterally tethered on the OMV. The OPH-decorated OMVs exhibited an enhanced degradation rate when assayed with paraoxon as a substrate. In addition, the thermal stability and pH tolerance were also enhanced remarkably. Furthermore, the resulting biocatalysts could still retain more than 80% activity even after 15 cycles of recovery and reuse, demonstrating their potential use in bio-catalytic decontamination of organophosphate compounds.

1. Introduction

Due to intensive industrialization and manufacturing activities, leaks and inappropriate disposal of organic compounds have resulted in the contamination of environment. Bioremediation, which refers to the use of biological systems such as bacteria, fungi and enzymes to degrade pollutants, has been widely recognized as a powerful tool for the treatment of environmental contaminants [1]. In most cases, bioremediation usually exploits mixed cultures of microorganisms in either natural or engineered environments, wherein the specific enzymes produced by the microorganisms and their mechanistic actions involved in pollutant biodegradation are not yet fully established in some systems [2].

With recent advances in molecular biology, mechanisms of pollutant degradation can be determined with such ease and accuracy that enzymes and microbial systems can be characterized and utilized more efficiently [3]. Compared to the utilization of microbial

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systems in pollutant degradation, enzymatic systems are more efficient and attractive due to the fact that it can degrade the pollutant of interest with an increased tolerance, higher rate and specificity, resulting in less by-products and biomass formation [4]. For example, enzymes that can hydrolyze organophosphorus compounds have been identified and characterized from different microbial species thus, exploiting these enzymes for effective detection and biodegradation of organophosphate pesticide seems to be an emerging approach [5,4,6]. However, major limitations for enzymatic bioremediation are the high cost, and tedious work required for enzyme extraction and purification [7,8]. Enzyme immobilization is a process that makes the enzymes more resistant to harsh environments and enables the enzymes to be recovered and recycled by attaching the enzyme to a solid support (e.g. polymers, metals, nanomaterials) [9,10]. Furthermore, enzyme activities after immobilization are sometimes reduced due to enzyme conformational change, mass transfer limitation, or detachment of enzymes from supports [11]. For example, an elegant study on enzymatic bioremediation of organophosphorus insecticides by recombinant organophosphorus hydrolase showed significant reduction in enzyme activity after immobilization [12]. Therefore, an efficient enzyme immobilization technology that can provide strong interaction between supports and enzymes without compromising the activity of enzymes is highly desired.

Outer membrane vesicles (OMVs) are protein, lipid, and polysaccharide-based vesicles derived from the outer membrane of many gram-negative and gram-positive bacteria as part of their natural growth cycle [13]. They are produced when small portions of the outer membrane bulge away from the cell, pinch off, and then release. Although it is still poorly understood why certain membrane proteins are preferentially enriched in OMVs while others are excluded, it has been demonstrated that *Escherichia coli* (and other gram-negative bacteria) can be engineered to incorporate heterologously expressed proteins into either the membrane or the lumen fraction of OMVs [14,15]. Engineered OMVs are particularly attractive for enzyme immobilization applications owing to their nanoscale size, ease and low cost of production via fermentation and their extreme stability at ambient conditions [16]. However, the recovery of OMVs requires the utilization of an ultracentrifugation step, which is cost, energy and labor intensive, especially for environmental applications. Therefore, a simple and cost-effective method for OMV recovery is considered a necessity.

Cellulose binding domains (CBDs) are proteins that can bind rapidly, tightly, and specifically to cellulose in a wide range of pH and temperatures [17]. Therefore, they are commonly used in biotechnology as a fusion partner for immobilizing proteins on cellulose [18]. Cellulose is a naturally abundant, inexpensive, and chemically inert material with inherently low binding characteristics [19]. The unique properties of CBDs, in addition to the low cost of cellulose, may not only eliminate the aforementioned problems in the recovery of OMVs, but also enable the commercial application of OMV-based enzymatic bioremediation. Herein, we investigated the possibility of targeting an organophosphorus hydrolase (OPH) and a CBD to the surface of OMVs using two complementary surface anchors for quick and simple recovery of the engineered OMVs using cellulose. In addition, we also demonstrated the potential use of the OPH-and-CBD-decorated OMVs in decontaminating an organophosphate pesticide, paraoxon.

2. Materials and methods

2.1. Strains and plasmids

E. coli strains JM109 and JC8031 were used in this study for plasmid construction and OMV production, respectively. To display the OPH on the surface of OMVs, plasmid pVLT33-INPOPH6 encoding a fusion protein that contains an ice nucleation protein (INP) and the OPH was constructed by PCR amplification of the corresponding DNA fragment from pINCOP [20] by using the forward primer FEcorI3INP: 5′-gggggaattcaggaaacgatgaatatcg-3′ and the reverse primer ROPHHis6HindIII: 5′-ccccaagctttcagtggtggtggtggtg gtgtacgcccaaggtcg-3′. The PCR product was then digested with *Eco*RI and *Hin*dIII and inserted into linearized pVLT33 [21]. Plasmid pUCBD [22] that has been described elsewhere was used for surface display of the CBD on OMVs. A control *E. coli* strain, JC8031 harboring pVLT33 and pUC18 plasmids, was developed for comparison.

2.2. Protein expression and OMVs collection

The recombinant *E. coli* was pre-cultured in 3 mL of LB medium supplemented with appropriate antibiotics at 37 °C for 12 h. Then, the cells were transferred into 100 mL of LB medium containing 10 mmol/L of $CoCl_2$ and appropriate antibiotics, and then incubated at 37 °C with continuous shaking at 250 rpm in the dark before the optical density (OD_{600nm}) reaches 0.8. The cells were then cooled to 30 °C, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μmol/L. After 4 h of induction, cells were harvested by centrifugation at 5000 g for 10 min at 4 °C. The cell-free supernatant was collected and passed through a 0.45 μm pore-size vacuum filter (Millipore). Vesicles were collected by centrifugation at 50,000 g for 2 h and resuspended in 50 mmol/L of Tris-HCl buffer (pH 8) supplemented with 10 mmol/L of $CoCl_2$.

2.3. Protein characterization

Proteins on OMVs were characterized via SDS-PAGE using Laemmli method (also known as glycine-based buffer system). SDS-PAGE gels were blotted onto a polyvinylidene difluoride membrane. OPHs on OMVs were probed by a primary anti-His tag mouse monoclonal antibody (Genetex) and labeled with a secondary AP-conjugated goat anti-mouse IgG antibody (Jackson Immuno). The expression of CBM on OMVs was characterized by assaying the binding ability of OMVs on microcrystalline cellulose Avicel (Sigma-Aldrich). Briefly, 5 μg of OMVs were mixed with Avicel in 50 mmol/L of Tris-HCl (pH 8) containing 10 mmol/L of $CaCl_2$ for 10 min. After centrifugation at 5000 rpm for 3 min, the OMV-bound Avicel was precipitated and washed three times with ice-cold Tris-HCl (pH 8). The pellets were collected and then tested for their activities on paraoxon (Sigma-Aldrich) degradation. The amount of OPH on Avicel was quantitatively determined via densitometry after Western blot analysis.

2.4. Immunofluorescence microscopy

The display of OPH enzyme on the *E. coli* surface was verified via immunofluorescence microscopy. Briefly, *E. coli* cells displaying OPH were harvested by centrifugation. Collected cells were resuspended in 250 μL of PBS containing 1 g/L bovine serum albumin and 0.5 μg of anti-His6 immunoglobulin G (IgG; Genetex) for 4 h with occasional mixing. Then, the probed cells were pelleted, washed with PBS, and resuspended in PBS plus 1 g/L bovine serum albumin and 0.5 μg anti-mouse IgG conjugated with Alexa 488 (Thermo scientific) for labeling. After incubation for 2 h, the labeled cells were pelleted, washed twice with PBS, and resuspended in PBS to an OD_{600nm} of 1. Five microliter of the labeled suspensions were spotted onto glass slides and were further analyzed under a fluorescence microscope (Olympus IX73, USA).