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Efficient cell surface display of organophosphorous hydrolase using N-terminal domain of ice nucleation protein in *Escherichia coli*

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Abstract−Recombinant *Escherichia coli* systems expressing organophosphorous hydrolase (OPH) have been used for detoxifying toxic organophosphate compounds. However, a whole cell biocatalyst system has an intrinsic problem due to substrate diffusion limitation by its cell membrane. As a strategy for reducing this diffusion barrier limitation to enhance whole cell biocatalytic activity, we engineered *E. coli* cells to target OPH on cell surface using ice nucleation protein (InaK) as a surface targeting motif, especially N-terminal domain of InaK (InaK-N). The whole cell OPH activities of the cells expressing InaK/OPH fusion constructs were higher (~2.5-fold for InaK-N and ~5.7-fold for combined N- and C-terminal domain of InaK (InaK-NC)) than that of the cells expressing cytosolic OPH. Interestingly, the membrane targeting efficiency of the cells expressing InaK-N/OPH fusion proteins was ~2.2-fold higher compared to the cells expressing InaK-NC/OPH even though both whole cell and total cell lysate OPH activities were lower. Therefore, we found that the small size N-terminal domain of InaK is more efficient for targeting OPH on the cell surface, and the surface display of OPH using N-terminal InaK domain can reduce the mass-transfer problem in whole cell bioconversion system.

Key words: Cell Surface Display, Ice Nucleation Protein, N-Terminal Domain, Organophosphorus Hydrolase, *Escherichia coli*, Whole Cell Biocatalyst

INTRODUCTION

Organophosphate compounds are widely used in many pesticides (Paraoxon, Parathion, Coumaphos, and Diazinon) and chemical nerve agents (Sarin and Soman) [1]. Organophosphorus hydrolase (OPH) from *Pseudomonas diminuta* [2] or *Flavobacterium* sp. [3] is a homodimeric organophosphotriesterase that can degrade a broad spectrum of toxic organophosphates [4]. This enzyme can hydrolyze various phosphorus-ester bonds including P-O, P-F, P-CN, and P-S bonds [5]. The hydrolytic mechanism involves the addition of an activated water molecule at the phosphorus center [6]. The application of OPH for bioremediation is of great interest due to its high turnover rate.

Whole recombinant *Escherichia coli* expressing OPH can be used to degrade a variety of organophosphate compounds [7]. The ability of *E. coli* to grow much higher densities than *P. diminuta* and *Flavobacterium* enables the development of large-scale detoxification processes [8]. However, whole cell biocatalytic systems have fundamental problems on substrate diffusion limitations due to their cell membrane structures. Therefore, several techniques have been attempted to reduce these membrane barrier problems such as display on cell surface [9] and periplasmic secretion [10,11].

Bacterial cell surface display of heterologous proteins can be useful in procedures such as development of live vaccines and multiple antigen antisera [12], construction and screening of protein libraries [13,14], whole cell bioconversion and biocatalysis [15], and development of environmental bioadsorbents [16]. To enhance whole cell OPH activity, various display systems have been developed with different anchoring motifs [17]. Previously, we demonstrated that a truncated ice nucleation protein (INP) fragment consisting of only the N-terminal domain (InaK-N) that might serve as a potential anchoring motif could target green fluorescent protein (GFP), which is a widely used reporter protein [18], on the *E. coli* cell surface [19]. In this research, using InaK-N anchoring motif, we displayed OPH on the cell surface to overcome mass transfer problems and, consequently, to enhance whole cell bioconversion efficiency. We also compared the whole cell activities and surface targeting efficiencies with truncated InaK consisting of the N- and C-terminal domains (InaK-NC) that is usually used as an anchoring motif.

MATERIALS AND METHODS

1. Bacterial Strains, Plasmids, and Culture Condition

E. coli TOP10 [F- mcrA Δ(mrr-hsdRMS-mcrBC)Φ80lacZΔM15 ΔlacX74 deoR *recA1 araD139 Δ*(*ara-leu*)*7697 galU galK rpsL* (*StrR*) *endA1 nupG*] (Invitrogen) was used for constructing recombinant plasmids. *E. coli* W3110 [$F^-\lambda^-$*mcrAmcrB IN*(*ornD-ornE*)] was used as a host for surface display of InaK. Plasmids pINPN-OPH and

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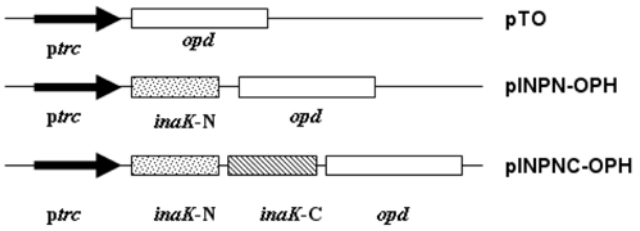

Fig. 1. Gene maps of recombinant plasmids harboring truncated *inaK*/*opd* fusion constructs. Plasmid pTrcHisC was used as a parent vector for constructing these fusions. Abbreviations: P*trc*, *trc* promoter; *inaK*, ice nucleation protein gene; *inaK*-N, N-terminal domain of *inaK*; *inaK*-C, C-terminal domain of *inaK*; *opd*, organophosphrous hydrolase gene.

pINPNC-OPH bearing *inaK* from *Pseudomonas syringae* KCTC 1832 and *opd* gene from *Flavobacterium* sp. ATCC27551 were used for expressing truncated InaK and OPH fusion hybrids (Fig. 1). Plasmid pTO [20] that carries *opd* gene for expression of cytosolic OPH, was also used as a negative control.

Recombinant strains bearing plasmids were inoculated in M9 medium (12.8 g/L $Na_2HPO_4 \cdot 7H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 3 mg/L $CaCl_2$, 1 mM $MgSO_4$) containing 0.5% (w/v) glucose and 50 μg/mL of ampicillin at the final concentration. Cells were cultured in 250-mL Erlenmeyer flasks with a 50 mL working volume at 250 rpm and 37 °C. When the cultures were grown to the optical density of 0.6 (at 600 nm, OD_{600}), 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) and 0.5 mM $CoCl_2$ (Sigma) were added to culture broth for induction of recombinant protein expression. After IPTG induction, cells were grown at 250 rpm and 25 °C for 24 h.

2. Analytical Assays for Cell Density and OPH Activity

Cell density (OD_{600}) was measured at 600 nm on a UV/VIS spectrophotometer (UV-1601PC; Shimadzu). After 24 h culture upon IPTG induction, cells were harvested and diluted to unit cell density (OD_{600}=1) with PBS buffer (pH 7.5). OPH activity was measured by following the increase in absorbance of *p*-nitrophenol from the hydrolysis of substrate (1 mM Paraoxon (Sigma)) at 400 nm (ε_{400}=17,000 M^{-1} cm^{-1}) by using a UV/VIS spectrophotometer. One unit of OPH activity was defined as mmoles Paraoxon hydrolyzed per min [21].

3. Cell Fractionation

Cells harboring *inaK-opd* hybrids were induced with 0.1 mM IPTG and cultured at 25 °C for 24 h. Harvested cells were diluted to set as unit cell density (OD_{600}=1), resuspended in PBS buffer containing 1 mM EDTA and lysozyme at 10 μg/mL, and incubated for 2 h at room temperature. The cell suspension was treated with an ultrasound sonication at 30 sec×2 cycles and saved for whole cell lysate fraction. To obtain total membrane fraction, whole cell lysate was pelleted by centrifugation at 39,000 rpm for 1 h using an ultracentrifuge (Optima™ LE-80K; Beckman). Equal volume of each fractionated sample was saved for OPH activity assays.

RESULTS AND DISCUSSION

E. coli W3110 cells harboring recombinant plasmids pTO, pINPN-OPH and pINPNC-OPH that encode OPH (cytosolic expression control), InaK-N/OPH (cell surface displayed OPH using N-terminal INP) and InaK-NC/OPH (cell surface displayed OPH using N- and C-terminal INP), respectively, were grown in M9 media for

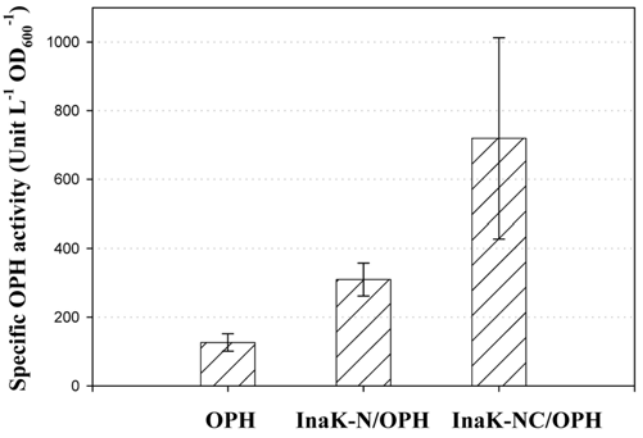

Fig. 2. Specific whole cell OPH activities of the cells harboring control cytosolic OPH, InaK-N/OPH, and InaK-NC/OPH fusions. Cells were grown in M9 media at 25 °C for 24 h upon 0.1 mM IPTG induction.

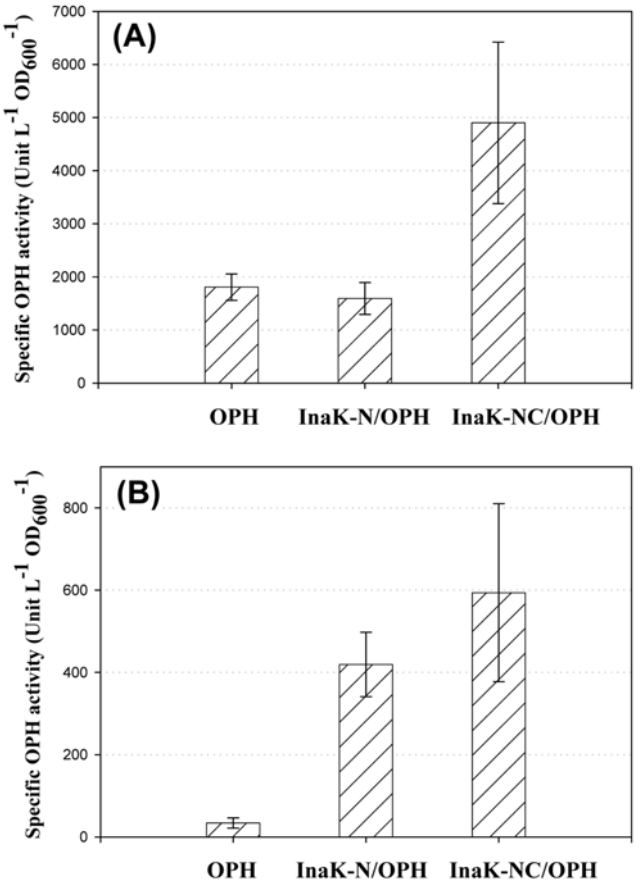

Fig. 3. Specific OPH activities in (A) total cell lysate and (B) total membrane fractions of the cells harboring control cytosolic OPH, InaK-N/OPH, and InaK-NC/OPH fusions. Cells were grown in M9 media at 25 °C for 24 h upon 0.1 mM IPTG induction.

24 h under 0.1 mM IPTG induction. Specific expression levels of each OPH sample were determined by monitoring OPH activity based on unit cell density (OD_{600}=1).

Each OPH activity of whole cell (intact cell), total cell lysate, and total membrane fraction was assayed after cell fractionation. The whole cell OPH activities of cells expressing InaK-N/OPH and InaK-NC/OPH fusion proteins were significantly enhanced ~2.5- and ~5.7-folds, respectively, compared to expressing cytosolic OPH (Fig. 2). This result might show that surface displaying of OPH can increase the whole cell OPH activity by reducing mass-transfer limitation of substrate. The cells expressing the InaK-NC/OPH fusion protein showed the highest whole cell OPH activity, which was ~2.3-fold greater than the cells expressing InaK-N/OPH fusion protein (Fig. 2). This higher whole cell OPH activity for the case of InaK-NC/OPH was from higher (~3-fold) total OPH expression levels (total cell lysate activity) (Fig. 3A). Even though the OPH activity of total cell lysate fraction for the case of cytosolic OPH was slightly higher than that of InaK-N/OPH (Fig. 3A), OPH activity in total membrane fraction was much higher in InaK-N/OPH compared to cytosolic OPH (Fig. 3B). Thus, we confirmed that N-terminal domain of INP targeted OPH on surface of *E. coli* cells. When we calculated the percentages of whole cell OPH activity per total (cell lysate) OPH activity (from Figs. 2 and 3A), the cells expressing InaK-N/OPH and InaK-NC/OPH have much higher values (~20% and ~15%, respectively) compared to cytosolic OPH (~7%). Based on these results, we can conclude that cell surface display strategy reduced cellular diffusion limitations in whole cell biocatalytic system.

When we calculated the percentages of total (cell lysate) OPH activity per total membrane OPH activity (from Figs. 3A and 3B), the cells expressing InaK-N/OPH and InaK-NC/OPH showed ~26% and ~12% higher values, respectively, compared to the control cells expressing cytosolic OPH (only ~2%) (Fig. 4). This result indicated correct localization of InaK/OPH fusion proteins on the outer membrane of host cells. Interestingly, membrane targeting efficiency for the cells expressing InaK-N/OPH fusion proteins was ~2.2-fold greater than the cells expressing InaK-NC/OPH fusion proteins (Fig. 4). Therefore, we confirmed that the single N-terminal domain of INP can successfully and more efficiently direct translocation of OPH to the cell surface of *E. coli* that is consistent with our previous study on GFP [19]. However, OPH activity of total membrane fraction was ~0.7-fold smaller for the InaK-N/OPH construct compared to the InaK-NC/OPH fusion protein (Fig. 3B). This result might be due to different total expression levels of OPH because total OPH activity of the cells expressing InaK-NC/OPH fusion was much higher than the cells expressing InaK-N/OPH fusion protein. Even though employment of N-terminal domain showed lower whole cell OPH activity than the case of both N- and C-terminal domain, we might successfully use sole N-terminal domain of INP for display of foreign target protein on cell surface due to its merits such as higher surface targeting efficiency and shorter length as a targeting motif.

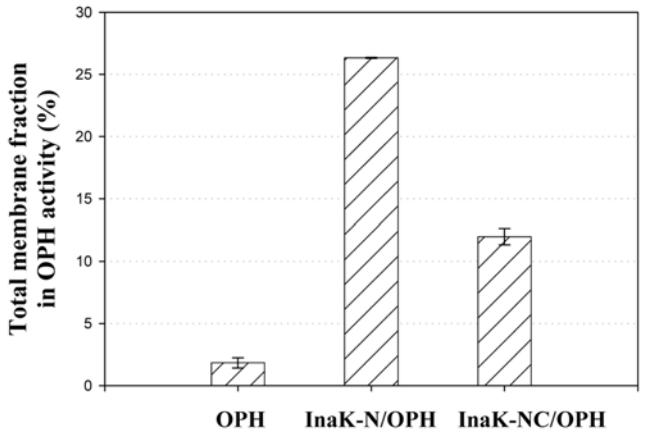

Fig. 4. Percentage of total membrane fractions in OPH activities of the cells harboring control cytosolic OPH, InaK-N/OPH, and InaK-NC/OPH fusions. Cells were grown in M9 media at 25 °C for 24 h upon 0.1 mM IPTG induction. Analyses were based on unit cell density (OD_{600}=1).

CONCLUSIONS

The whole cell OPH activities of the cells expressing InaK/OPH fusion constructs were ~2.5~5.7 times higher than that of the cells expressing cytosolic OPH, demonstrating the surface display of OPH reduced the diffusion limitation that is an intrinsic problem for the whole cell biocatalysis system. The membrane targeting efficiency of the cells expressing InaK-N/OPH fusion protein was ~2.2-fold higher compared to the cells expressing InaK-NC/OPH, and the percentage of whole cell OPH activity per total expression amount was also higher even though its whole cell OPH activity was lower. Therefore, we demonstrated that truncated ice nucleation protein InaK containing only the N-terminal domain can be successfully employed as a cell surface display motif, and the surface display of OPH can overcome the mass-transfer problems in whole cell bioconversion system, resulting in significantly enhanced whole cell biocatalytic activity.

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