Bioremediation of Organophosphorus Pesticides by Surface-Expressed Carboxylesterase from Mosquito on *Escherichia Coli*

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The insecticide resistance-associated esterase, carboxylesterase B1 (CaE B1), from mosquito was used to degrade the organophosphorus compounds. To eradicate the need for enzyme purification and minimize the resistance to mass transport of the substrate and product across the cell membranes, the CaE B1 was displayed on the cell surface of *Escherichia coli* fused to the C-terminus of the ice nucleation protein (INP). The presence of CaE B1 on the bacterial cell surface was verified by SDS-PAGE, Western blotting analysis, and immunofluorescence microscopy. More than 50% of active CaE B1 is exported across the membrane and anchored onto the cell surface as determined by proteinase accessibility and cell fractionation experiments. In contrast, only a 6% drop in activity for proteinase K-treated cells was detected from *E.coli* cells containing pET-B1. From the degradation experiment, more than 80% of the malathion was degraded by whole cells containing plasmid pUC-NC-B1. Constitutive expression of CaE B1 on the surface using INPNC resulted in no cell lysis, and the suspended cultures also exhibited good stability. Because of their high biodegradation activity and superior stability, these "live biocatalysts" are promising for detoxification of organophosphorus pesticides.

Introduction

The high rate of population growth in the world requires high yields of crops from existing agricultural land. For high yields of food grains, the use of chemical pesticides has become indispensable. It is estimated that four million tons of pesticides are applied to crops annually worldwide for pest control, but less than 1% of the total applied pesticides get to the target pests (*1*). Organophosphorus compounds make up for about 70% of the pesticides used worldwide. High-level exposure to these neurotoxins can result in death, while repeated or prolonged exposure can cause delayed cholinergic toxicity and neurotoxicity (*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*). Therefore, there is an increasing interest in developing safe, convenient, and economically feasible methods for their degradation such as bioremediation.

Generally, microorganisms seem to be the most efficient bioremediation agents (*4*−*6*). However, insects are equally adept in surviving a wide variety of physical and biological conditions due to their amazing adaptability. The ability to survive the defenses of their hosts has required that insects evolve unique metabolic mechanisms for detoxification of a wide variety of compounds (*7*). Recently, many insecticide-resistance associated esterases have been purified and characterized from several insect species (*8*, *9*). Insect metabolic enzymes can also be used in bioremediation via standard recombinant technologies, either as single isolated gene products or more likely in combination with existing bioremediation organisms.

It was shown that the organophosphate resistance in *Culex* is mainly conferred by the overproduction of esterase B1, as a result of gene amplification. This gene has been cloned into *E. coli* pET(28a) (*10*, *11*), which expresses the carboxylesterse B1(CaE B1) effectively in a fermentor. The CaE B1 has also demonstrated a high degradation property for some of the organophosphorus (*10*, *12*).

However, practical applications of large-scale enzymatic degradation have always been limited by the cost of purification and stability of the enzyme. As a cost-effective approach, bioremediation by whole cells immobilized onto a solid support (such as in an immobilized-cell bioreactor) may be a desirable alternative. However, it still has drawbacks, namely, resistance to mass transport of the substrate and product across the cell membranes if the enzymes are located within the cell. A clever solution to the above-mentioned problem is to express the enzyme of interest on the outer surface of a cell. This strategy could potentially eradicate the need for enzyme purification, minimize the resistance to mass transport,

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and also result in a directed immobilization, which might be beneficial.

In the present study, we expressed the CaE B1 onto the surface of *E. coli* fused to the truncated version of an ice nucleation protein (INP) containing only the N- and C-terminal portions (INPNC). Proteinase accessibility assay, Western blotting analysis, and immunofluorescence microscopy were used to verify expression of CaE B1 on the surface of *E. coli*. Due to their high biodegradation activity and stability, these "live biocatalysts" are promising for detoxification of organophosphorus pesticides.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. *E. coli* strains XL1-Blue (*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *rel*A1, *lac* [F′, *pro*AB, *lac*I^{q}ZDM15, Tn10 (Tet^r)]) and DH5α (*sup*E44, Δ*lac*U169 (ψ80 *lac*ZΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*a1) were used in this study. For INPNC-CaE B1 expression, plasmid pPNCO33 (*13*) was used as the template for the truncated INP-INPNC. Plasmid pET-B1 (*10*) carrying a carboxylesterase B1 gene was used as the source of the CaE B1 gene and as a control for the production of native CaE B1 in the cytoplasm. Plasmid pUC 18 (Invitrogen) was used for constitutive expression of CaE B1 on the cell surface using the INPNC.

Strain-bearing plasmid pUC-NC-B1 was grown in Luria−Bertani (LB) media supplemented with 100 μg/mL ampicillin. Cells were grown in flasks with vigorous aeration (250 rpm) at either 20 or 30 °C. Strain-bearing plasmid pET-B1 was similarly cultured, except that it was supplemented with 100 μg/mL kanamycin.

Plasmid Construction. The truncated INPNC-CaE B1 fusion was constructed as follows. PCR was used to amplify the INPNC using the pPNCO33 as the template. Two oligonucleotides, 5′-GGGAATTCAGGAAACAATGAATATCGACAAAGCGTTGGTA-3′ and 5′-CCCTGCAGTTCTCGACCTCTATCCAGTC-3′, served as primers for the 5′ and 3′ end of the fragment, respectively. The resulting 900bp INPNC fragment was digested with *Eco*R I and Pst I and ligated into a similarly digested pUC 18, resulting in pUC-NC. The 1.6 kb fragment of the CaE B1 gene was amplified from the pET-B1 with primers 5′-CCCTGCAGCAATGGGCAGCAGCCATCATCATCATCAT-3′ and 5′-GGCTGCAGCGTCAAAACAGCTCATCATTC-3′, digested with Pst I, and inserted into the Pst I digested pUC-NC to generate pUC-NC-B1.

Cell Fractionation. The procedure of Francisco et al. (*14*) was used for the fractionation of inner and outer cell membranes. Bacterial cells were collected from the culture medium by centrifugation at 5000*g* (MIKRO 22R, Germany) for 10 min, washed once with 0.2 M phosphate buffer (pH 7.0), and resuspended in the same buffer containing 50 mg/L lysozyme for 20 min at 30 °C. The samples were then lysed using sonication, applying to each 10 mL volume 10 cycles of 15 s, alternating with 45 s cooling periods. Nucleic acids and other cell debris were then removed by centrifugation at 12 000*g* (MIKRO 22R, Germany) for 15 min and the supernatant ultracentrifuged at 115 000*g* (HITACHI CP70G, Japan) to separate the membrane and soluble fraction. The supernatant was collected as the soluble fraction, while the pellet representing the membrane fraction was resuspended in the same buffer.

Carboxylesterase B1 Assays. Lysate activity was assayed with β-naphthyl acetate (β-NA) as the substrate in 0.2 M phosphate buffer, pH 7.0. To substrate solutions pre-equilibrated at 37 °C was added 0.5 mL of diluted enzyme solution to a final volume of 3 mL. The samples were mixed, and the absorbance was measured in a spectrophotomer (Beckman Du-650) at 555 nm for β-NA (15). Whole-cell activity was assayed similarly, except that cells were added directly. Activities are expressed as micromoles of β-NA hydrolyzed per minute (U), per OD_{600} of whole cells.

Proteinase Accessibility Assay. Cells harboring pET-B1 and pUC-NC-B1 were centrifuged and resuspended in 1 mL of 15% sucrose, 15 mM Tris-HC1, and 0.1 mM EDTA, pH 7.0. Samples were incubated for 1 h with 5 μL of 20 mg/mL proteinase K at room temperature. To inhibit further proteinase K activity, 10 μM phenylmethylsulfonylfluoride was added after incubation. Proteinase K-treated and untreated cells were assayed for CaE B1 activity as described above.

Mouse Anti-CaE B1 Antibody. CaE B1 was purified from cells containing pET-B1 by metal chelation chromatography (data not shown) and used to produce anti-CaE B1 antibody. The purified enzyme was diluted in 0.2 M phosphate buffer, pH 7.0, and used for immunization. Purified CaE B1 was administrated to a 5-week-old mouse, followed by booster injections with the same antigen twice every 2 weeks. After about one and a half months supply of anti-CaE B1 serum was obtained and used without further purification.

SDS-PAGE and Western Blotting. Expression of recombinant proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Prepared protein samples were fractionated on 10% SDS-PAGE gel according to Leammli (*16*). For Western blotting, the gel was transferred to a nitrocellulose membrane, and the analysis was performed using a Novagen Western Reagents Kit. The membrane was blocked with 3% bovine serum albumin in TBS buffer, probed with mouse anti-CaE B1 serum at a 1:500 dilution, and reacted with alkaline phosphatase-conjugated goat anti-mouse IgG at a 1:5000 dilution for 1 h at 37 °C. After 5 washings with TBS (200mM Tris-HCl, pH 7.5, 150mM NaC1), antigen−antibody binding was detected.

Immunofluorescence Microscopy. Cells expressing INPNC-CaE B1 were harvested, washed, and resuspended to $OD_{600\ nm} = 0.5$ in 0.2 M phosphate buffer (pH 7.0) containing a 3% bovine serum albumin and 1:500 diluted mouse anti-CaE B1 serum. After overnight incubation at 4 °C, the cell−antibody complex was rinsed with phosphate buffer 5 times and probed with goat anti-mouse IgG FITC conjugate (1:1000) diluted in 0.2 M phosphate buffer (pH 7.0) containing a 3% bovine serum albumin for 2 h at 37 °C. After 5 washings with phosphate buffer solution, the presence of FITC was observed using a confocal laser microscope (Olympus IX71).

Degradation of Malathion by Whole Cells Containing Plasmid pUC-NC-B1. Cells harboring pUC-NC-B1 and pUC 18 (as a control) plasmids were grown in LB medium at 30 °C for 2 days, centrifuged at 5000*g* for 10 min, and resuspended in 40 mL of 0.2 M phosphate buffer, pH 7.0, to $OD_{600} = 1.0$. Malathion (10 μL, 0.01 M concentration) was added into each mixture, and the mixtures were incubated with vigorous aeration (200 rpm) at 30 °C. Every 2 h, 3 mL of each sample was removed, filtered through a Whatman No. 1 filter into a tube, and extracted 3 times with 1:3 petroleum ether in separatory funnels. The pooled extract was evaporated to dryness, and the residues were redissolved in 3 mL of

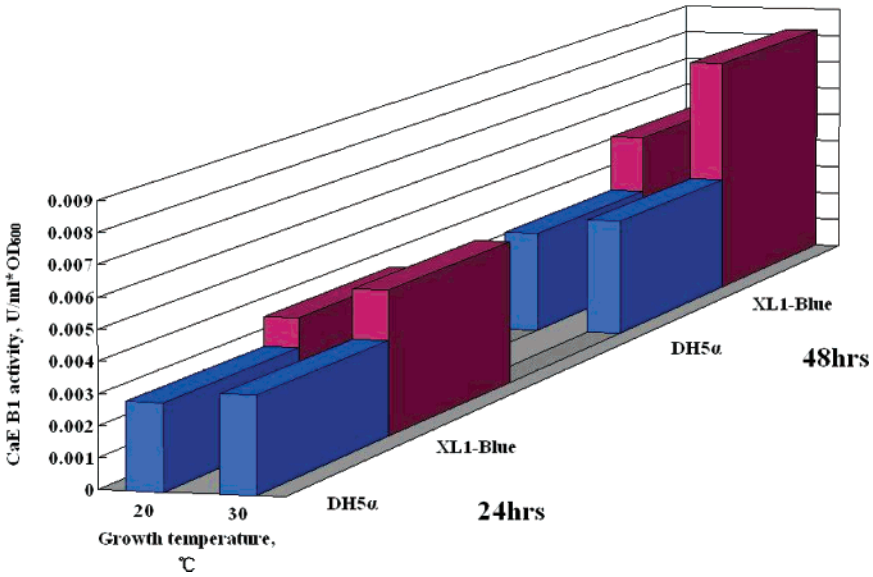

Figure 1. CaE activity of strains XL1-Blue and DH5α harboring plasmid pUC-NC-B1 at 20 and 30 °C. Cells were grown in LB medium, 48 h after incubation.

Table 1. Percentage of Surface-Expressed CaE B1 Calculated from Proteinase K Treatment, Whole-Cell versus Lysate Assays, and Membrane Fractionation Experiments

plasmid	% decrease in activity in proteinase K-treated cells	whole cell activity (as % of lystate activity)	% activity in membrane fraction
pUC-NC-B1	54	61	58
pET-B1	6	2	3

petroleum ether and analyzed for malathion using the method of Barlas (*17*).

Stability Study of Resting Culture. Cells containing plasmid pUC-NC-B1 were grown in 50 mL of LB medium for 2 days, harvested, washed once with 0.2 M phosphate buffer (pH 7.0), resuspended in 5 mL of the same buffer, and incubated at room temperature. Over the 2 week duration, 0.5 mL of each sample was removed every 2 days. Samples were centrifuged, resuspended in 1 mL of 0.2 M phosphate buffer (pH 7.0), and assayed for CaE B1 activity as described above.

Results

Construction of Expression Plasmids and Surface Expression of INPNC−CaE B1. To express CaE B1 on the cell surface, we chose to use INPNC as a membrane-anchoring motif. When INPNC is expressed on the cell surface, it aggregates into a multimer resistant to protease and remains stable in the stationary state. The expression vector pUC-NC-B1, based on pUC 18, was constructed for surface expression of INPNC-CaE B1. The recombinant plasmids were sequenced and contained the correct open reading frame (ORF) for the fusion.

To identify a superior host for anchoring CaE B1 onto the surface, two commonly used *E. coli* strains, XL1-Blue and DH5α, were chosen to investigate the CaE B1 expression and activity. Figure 1 shows the resulting CaE B1 activity from these recombinant strains carrying plasmid pUC-NC-B1 cultured at 30 °C after 1 and 2 days. CaE B1 activity was significantly higher in second-day cultures, and it was noticeably lower in strain DH5α in both the first- and second-day cultures. Moreover, incubation at 20 °C resulted in lower activity for both host cells.

Although CaE B1 activity was successfully achieved with either host cells, these results demonstrated that the XL1-Blue may be more efficient in targeting proteins, and only the properties of this cell line were further investigated.

Surface Localization of CaE B1. Proteinase accessibility experiments were carried out to ascertain the presence of enzymatically active CaE B1 on the cell surface. Since protease K cannot readily diffuse across the cell membrane, degradation should only occur with proteins exposed on the surface. As shown in Table 1, more than 50% reduction in the CaE B1 activity was observed in *E. coli* (pUC-NC-B1) cells after 1 h incubation. In contrast, only a 6% drop in activity for proteinase K-treated cells was detected from *E. coli* cells containing pET-B1, suggesting that the majority of CaE B1 was exposed on the cell surface.

The percentage of CaE B1 on the cell surface was also estimated by measuring CaE B1 activity in the membrane and soluble cell fractions. Over 50% of the activity was detected in the membrane fractions (Table 1). Moreover, more than 60% CaE B1 activity was present on the cell surface as judging from the activity ratio between whole cells and cell lysates. This contrasts markedly with only about 2% CaE B1 activity in the cells expressing CaE B1 from pET-B1.

Expression of INPNC-CaE B1 was also clearly confirmed by SDS-PAGE, Western blotting with mouse anti-CaE B1 sera, and immunofluorescence microscopy. From the Western blotting (Figure 2), proteins of approximately 95 kDa were detected from the membrane fraction and lysate. A lower molecular weight band of about 60 kDa was detected from the lysate of cells containing pET-B1. It is clearly indicated that INPNC-CaE B1 fusions were successfully produced in the membrane fraction. To clearly demonstrate the surface localization of CaE B1, immunofluorescence microscopy was used. *E. coli* cells were probed with mouse anti-CaE B1 serum as a primary antibody and then fluorescently stained with FITC-labeled goat anti-mouse IgG antibody. As shown in

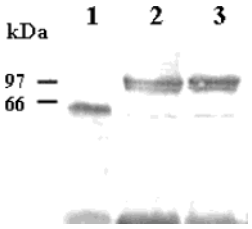

Figure 2. Western blot analyses of CaE B1 and INPNC−CaE B1 fusion proteins. 1, lysate of cells containing pET-B1; 2, lysate of cells containing pUC-NC-B1; 3, membrane fraction of cells containing pUC-NC-B1. Mobility of molecular size markers is shown on the left.

fluorescence micrographs (Figure 3b), cells harboring pUC-NC-B1 were brightly stained. Control cells carrying plasmid pUC 18 were not stained at all with the FITC-labeled goat anti-mouse IgG antibody (Figure 3a). These results confirm the surface localization of the INPNC-CaE B1 fusion proteins on the cell surfaces of *E. coli* XL1-Blue.

Degradation of Malathion. To confirm the ability of the engineered cells to degrade malathion, batch degradation experiments were conducted. As shown in Figure 4, about 65% of the malathion was degraded after 2 h and almost 90% of malathion was detoxified in 4 h by whole cells expressing CaE B1 on the surface. In contrast, more than 70% of the malathion remained in the control cultures expressing CaE B1 intracellularly, even at the end of the 10 h incubation period. The low degradation rate of whole cells expressing CaE B1 intracellularly may arise from a substantial diffusional barrier for malathion to cross the cell membrane.

Stability of Cultures Expressing INPNC−CaE B1 Fusion Proteins. Because constitutive expression of outer membrane protein can result in membrane destabilization and cell lysis, the stability of suspended cultures was monitored. It was demonstrated that whole-cell activity of XL1-Blue harboring the pUC-NC-B1 remained at essentially the original level during the two-week period (Figure 5). This observation is consistent with the results of other reports using the INP system for the surface expression of proteins (*18*−*20*).

Discussion

Recent detailed studies of the mechanisms of insecticide resistance have identified many insect genes used to degrade pesticides in the environment. The concept of utilizing the insect gene sequences for bioremediation has been patented by Roe's laboratory (*21*). Oakeshott used a gene, encoding Drosophila esterase-6 to decontaminate wastewaters from irrigation operations and cleanup of surface-contaminated fruit and vegetables (*22*). In this work, the CaE B1 was used to degrade malathion, a pesticide belonging to the family of organophosphorus nerve agents. Additionally, unlike other organophosphate-degrading enzymes such as organosphosphorus hydrolase and organophosphorus acid anhydrolases (*21*), it is not necessary to provide metal ions as the coenzyme for achieving high CaE B1 activity. This makes our system easier and safer to use CaE B1 to degrade organophosphorus compounds in practice.

Pseudomonas syringae produces an INP that normally resides on the surface of cells. It is membrane-anchored via the glycosylphosphatidylinositol (GPI)-anchor sequence, and this is quite unique for a prokaryote since this motif is normally found only in eukaryotic cells (*23*). By fusing the gene of interest to the C-terminus of INP and expressing this structure in *E. coli*, various active

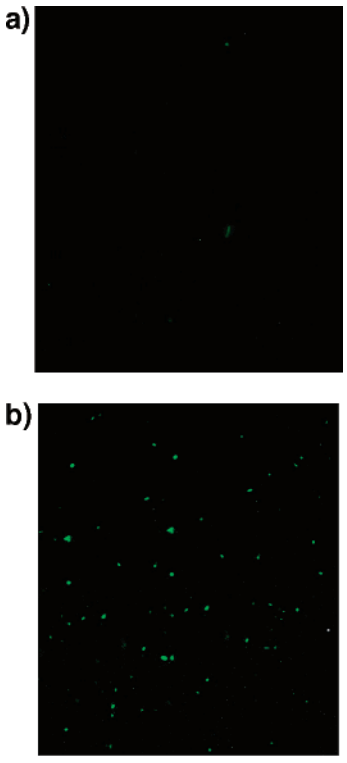

Figure 3. Immunofluorescence micrographs of E. coli BL21cells harboring pUC 18 (a) and XL1-Blue containing pUC-NC-B1 (b).

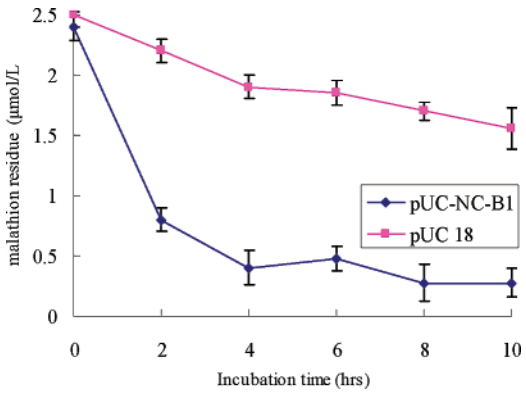

Figure 4. Biodegradation of malathion by whole cells containing plasmid pUC-NC-B1.

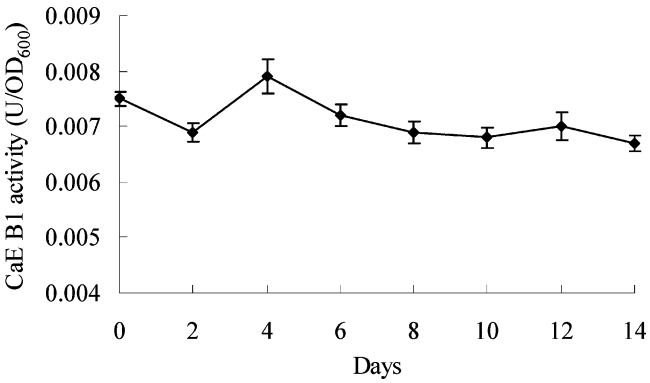

Figure 5. CaE B1 activity in suspended *E.coli* XL1-Blue cultures expressing CaE B1 constitutively on the surface. Every 2 days, 0.5 mL samples were removed and assayed for CaE B1 activity.

enzymes (*18*, *19*, *24*, *25*) and single-chain antibodies (*26*) have been successfully anchored to the cell surface. Our results in this paper demonstrate that CaE B1 can be

similarly expressed on the cell surface with good stability using the truncated version of the INP protein (INPNC). as confirmed by Western blotting analysis and immunofluorescence microscopy. Furthermore, proteinase accessibility and cell fractionation studies suggested that more than 50% of the enzymatically active CaE B1 was exported across the membrane and anchored onto the cell surface. Degradation of malathion was significantly improved by whole cells with surface-expressed CaE B1 compared to cells expressing CaE B1 intracellularly. The high biodegradation activity for malathion and stability of CaE B1 may provide useful tools for the successful decontamination of malathion and other organophosphorus pesticides.

In conclusion, surface expression of CaE B1 offers improved biodegradation of organophosphorus neurotoxins. From a practical standpoint, the utility of live biocatalysts with CaE B1 expressed on the cell surface could replace more conventional cleanup strategies for organophosphorus compounds. This system may be ideal for the development of whole-cell degradation systems for these organophosphorus neurotoxins.

Acknowledgment

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