Expression, Immobilization, and Enzymatic Characterization of Cellulose-Binding Domain-Organophosphorus Hydrolase Fusion Enzymes

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Abstract: Bifunctional fusion proteins consisting of organophosphate hydrolase (OPH) moieties linked to a Clostridium-derived cellulose-binding domain (CBD) were shown to be highly effective in degrading organophosphorus nerve agents, enabling purification and immobilization onto different cellulose materials in essentially a single step. Enzyme kinetics studies were performed for the CBD–OPH fusions using paraoxon as the substrate. The kinetics values of the unbound fusion enzymes were similar to OPH with a modest increase in \( K_m \). Immobilization of the enzymes onto microcrystalline cellulose resulted in a further increase in the \( K_m \) values of approximately twofold. The pH profile of the cellulose-immobilized enzymes was also only minimally affected. The CBD–OPH fusion proteins could be immobilized onto a variety of cellulose matrixes, and retained up to 85% of their original activity for 30 days. The durability of the bound fusions increased with the amount of Avicel used, suggesting that protein/cellulose interactions may have a dramatic stabilizing effect. Repeated hydrolysis of paraoxon was achieved in an immobilized enzyme reactor with 100% degradation efficiency over 45 days. These fusion proteins should prove to be invaluable tools for the development of low cost, OPH-based cellulose materials for the simultaneous adsorption and degradation of stored or spilled organophosphates.© 2000 John Wiley & Sons, Inc. Biotechnol Bioeng 69: 591–596, 2000.

Keywords: CBD; OPH; biodegradation; decontamination; organophosphates; nerve agents

INTRODUCTION

Organophosphorus compounds, which are among the most toxic substances known, are widely used as pesticides. These compounds possess the advantage of quick and effective elimination of pests by the inhibition of acetylcholinesterase (AChE), which occurs in the central nervous system synapses of most animals including humans (Donarski et al., 1989). Exposure to organophosphates results in acetylcholine (ACh) accumulation, which interferes with muscular responses and the function of vital organs. High dosages of organophosphates can prove fatal. Chronic or prolonged exposure to sublethal doses of organophosphates may result in delayed cholinergic toxicity and neurotoxicity (Tuovinen et al., 1994).

Enzymatic degradation of organophosphates has been a subject of considerable attention during the past 10 years. Organophosphorus hydrolase (OPH) isolated from soil microorganisms possesses the capability of hydrolyzing organophosphorus pesticides (P-O bond hydrolysis) as well as organophosphorus phosphonates (P-F or P-CN bond cleavage) that are utilized in nerve gases (Karns et al., 1987). Both native and recombinant OPH’s have been immobilized onto nylon (membrane, powder, and tubing), porous glass, and silica beads, and used as enzyme reactors for the detoxification of organophosphates (Caldwell and Raushel, 1991a; 1991b; Munnecke, 1979). OPH has also been immobilized within polyurethane foams that can be applied as sponges or wipes for the clean-up of pesticide spills (Havens and Rase, 1993; LeJuene and Russell, 1996). Unfortunately, the immobilization of OPH by physical adsorption or covalent binding often results in a significant reduction in operational activity and/or stability due to sensitivity to changes in pH or temperature. In addition, the enzyme kinetics parameters for these modified OPH’s are often less desirable due to the inaccessibility of the enzyme’s active site.

Practical applications for large-scale enzymatic degradation have also been limited by the tedious protocol and the high cost associated with purifying OPH. Although secreted OPH from Streptomyces lividans can be purified from the culture medium without cell disruption (Steiert et al., 1989), the level of production is relatively low and ion-exchange chromatography and gel filtration are still required for purification. Purification and immobilization onto basic resins such as DEAE-Sephadex A-50 has been facilitated by the hybrid enzymes of OPH consisting of repetitive polypep-
tides of [(AG)₈EG]₉ at the N-terminus (Wu et al., 1996). These resins, unfortunately, require costly modification and are too expensive for large-scale applications.

Endo- and exo-cellulases (isolated from a variety of organisms) contain a cellulose-binding domain (CBD) which specifically binds to cellulose and increases the rate of hydrolysis (Greenwood et al., 1992). A cellulose-binding domain not associated with cellulases was also recently found from the cellulose-binding protein A of the cellulolytic bacterium Clostridium cellulovorans (Shoseyov et al., 1992). Because of the high affinity toward cellulose, CBD has been exploited as an affinity tag for the purification and immobilization of heterologous fusion proteins onto cellulose supports (Ahn et al., 1997; Ong et al., 1989). Fusion proteins containing a CBD moiety may be constructed so that little or no decrease in the catalytic efficiency of the fusion enzyme is observed. For example, Ong et al. (1989) observed that the specific activities of β-glucosidase (Abg) and Abg-CBD are almost identical.

An obvious extension of the CBD-fusion technology is to enable a single-step purification and immobilization of OPH by generating active CBD–OPH fusions. The CBD-cellulose affinity system is attractive because it does not require a derivatized matrix, and cellulose is available in a variety of inexpensive forms, such as preformed micro-porous beads, highly adsorbent sponge or cloth and micro-crystalline powders. In this manner, OPH-based cellulose materials could be generated for a variety of relatively low-cost applications such as: (1) immobilized enzyme reactor for the detoxification of hazardous organophosphates; (2) protective clothing for personnel coming in contact with organophosphates; and (3) sponges/clothes for the decontamination of spills and objects/surfaces exposed to these highly toxic nerve agents.

In this article, fusion proteins comprised of CBD of the cellulose-binding protein A from Clostridium cellulovorans at the N-terminus and the OPH from Flavobacterium at the C-terminus were constructed. We demonstrate that purification and immobilization of CBD–OPH fusions is easily achieved in a single step onto a variety of cellulose matrices, and that paraoxon is efficiently degraded by immobilized CBD–OPH. The kinetic properties of CBD–OPH when immobilized onto cellulose or in solution were shown to be similar to the wild-type OPH enzyme.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

Escherichia coli strains BL21(DE3)pLysE (F⁻ ompT hsdSB (rB⁻ mB⁻) gal dcm (DE3) pLysS) (Novagen) and JM105 (endA1, thi, rpsL, sbcB15, hsdR4, Δ(lac-proAB), [F', traD36, proAB, lacI²ZΔM15]) were used in this study. Plasmid pET34b(+) (Novagen, Madison, WI), designed to generate protein fusions with CBDclon, under control of the T7 promoter was used. The opd gene (including its native signal peptide) was PCR amplified from pOP131 (Richins et al., 1997) using the forward and reverse primers, dAAATTCGGATCCGGATGC (P1), and dGGGGATCCAGCTTTCAAAAAAAAGGCCCGTCATTAGGGGGGTCGCCTACA-CCCGCAAGGTCCGTCGACAG (P3), respectively. The resulting 1100 bp fragment was digested with BamHI and HindIII and ligated into similarly digested pET34b(+) (Novagen) to yield pCOPH-C1 (Fig. 1). A similar cbd-opd construct, pCOPH-S1 (lacking the native signal peptide), was engineered by ligating a PCR amplified, BamHI + HindIII digested, 1000 bp, opd gene derived from pOP231 (Richins et al., 1997) into pET34b(+). dAAATTCGGATCCGGATGC (P2) and dGGGGATCCAGCTTTCAAAAAAAAGGCCCGTCATTAGGGGGGTCGCAAGG (P3) were used as the forward and reverse primers, respectively). Plasmid-bearing bacterial cultures were grown in buffered 2× Yeast–Tryptone media (DYT) supplemented with kanamycin to a final concentration of 50 μg/mL. Initial screening of plasmids was performed in E. coli strain JM105. Expression of CBD–OPH fusions was carried out in E. coli strain BL21 (DE3) pLysE (Novagen).

Production of CBD–OPH Fusions

Expression of CBD–OPH fusions was achieved by growing 40 mL cultures of BL21(DE3)pLysE carrying either pCOPH-C1 or pCOPH-S1 at 37°C to an OD₆₀₀ of 2.0. Cultures were then transferred to either 30 or 37°C and induced with 1.0 mM IPTG. Cells were collected by centrifugation and resuspended in 1/2 volume of PC buffer [50 mM disodium phosphate titrated to pH 8.0 with citric acid; 100 μM CoCl₂]. Lysozyme was added to the resuspended cells to give a final concentration of 10 μg/mL. Following a 15-min incubation at room temperature, the cells were cooled to 0°C, then passed through a French pressure cell (15,000 to 20,000 psi). The solution was centrifuged twice for 10 min at 20,000g. The resulting supernatant was used for binding and enzymatic studies. For the production of larger quantities of fusion protein, cultures were grown in 4 L of DYT

For comparative purposes, OPH was expressed as free enzyme from JM105 cells containing the plasmid pWM513. This strain was cultured and processed identically to the pCOPH strains.

**Binding of Fusion Proteins to Cellulose Matrices**

Binding and washing was performed with PCST (PC buffer + 50 mM NaCl + 0.1% v/v Triton-X100) buffer. Sodium chloride and Triton-X100 were added to the crude extract to give a concentration of 50 mM and 0.1% (v/v), respectively. Cellulose matrices (either Avicels, fabrics or sponges) were washed twice with PCST prior to exposure to the crude extract. Binding was performed for 30–60 min at room temperature on an orbital shaker at 60 rpm. Samples were then rinsed extensively with PCST, then finally with two to four washes of PC buffer. For longterm studies, sodium azide was added to the immobilized enzyme to give a final concentration of 0.02% (w/v).

**Enzymatic Analyses and Storage Stability**

Enzyme activities were determined in 4 mL of PC buffer at 37°C. Rates of p-nitrophenol production were determined at 412 nm using a Beckman Model DU 640 spectrophotometer. Paraoxon (diethy p-nitrophenyl phosphate, Sigma Chemical Co., St. Louis, MO) was added to the samples immediately prior to the 2-min kinetic analyses. Enzyme assays were carried out in a well-stirred microreactor with a total volume of 30 mL. The agitation rate was selected to eliminate any external mass-transfer limitation (Banexy et al., 1989). Enzyme kinetic analyses utilized paraoxon concentrations ranging from 2 μM to 2.5 mM from three independent experiments. For most binding and stability studies, a paraoxon concentration of 0.5 mM was employed. For pH profiling, the 50 mM phosphate-citrate buffer was adjusted to the desired pH’s using either concentrated HCl or saturated NaOH. The performance of the C1 and S1 fusions was compared to purified OPH prepared via the procedure described by Mulchandani et al. (1999). For longterm storage stability, a small quantity of Avicel with bound OPH was removed for activity assay each day.

**Protein Quantification**

Protein concentration was estimated using the BioRad’s (Bradford; Hercules, CA) protein assay kit. For quantification of cellulose-bound proteins, an aliquot of CBD–OPH-cellulose was first extracted twice with 0.2M NaOH (5 min each at 80°C). The NaOH-extracted samples were then neutralized with an equal volume of 0.2M HCl, then buffered with 1/10 volume of 10× phosphate-citrate buffer prior to the protein assay. For all assays, the microassay method was employed. Absorbance values of test samples (595 nm) were compared to values obtained using a bovine plasma gamma-globulin standard.

**SDS and Western Blot Analysis**

Crude extracts and “purified” fusion proteins (released from Avicel by treatment with boiling SDS) were electrophoresed through 12.5% SDS-PAGE gels (Laemmli, 1977). Samples were electroblotted onto nitrocellulose membranes, then subjected to Western analyses using a BioRad Immun-Blot GAR-AP kit (BioRad, Hercules, CA). Antiserum to purified OPH was prepared by Robert Sergeant Polyclonal Antibody Production Services (San Diego, CA). Antiserum to CBD was obtained from Novagen.

**Repeated Degradation of Paraoxon by Immobilized CBD–OPH Columns**

Cell extracts were incubated with CF11 cellulose (Whatman) in the presence of 0.1% triton. After centrifugation and washing twice with phosphate-citrate buffer, the CBD–OPH-cellulose complex was then packed into a 5 x 1 cm diameter column. Paraoxon solutions of 2 mM concentration were passed through the column at different flow rates, and the hydrolyzed products were collected and p-nitrophenol was measured spectrophotometrically at 405 nm. Repeated degradation of paraoxon with the immobilized column was tested on a daily basis for 45 days. Column was stored in the same buffer at 25°C between experiments.

**RESULTS AND DISCUSSION**

**Production of CBD–OPH Fusions**

Because OPH activity can be successfully retained as a C-terminus fusion with Lpp-OmpA (Richins et al., 1997), we hypothesized that CBD–OPH fusions could be similarly constructed while retaining OPH activity. In fact, attempts to generate active OPH–CBD fusions were not successful. OPH is normally a membrane-associated protein with an N-terminal signal sequence responsible for controlling cellular localization (Serdar et al., 1989) and the intracellular expression of OPH in E. coli has been reported to be greatly enhanced by deletion of the signal peptide (Mulbry and Karns, 1988). To explore whether inclusion of the OPH signal sequence (29 amino acids) would affect the stability and folding of the fusion proteins, two recombinant plasmids were created with one encoding an CBD–OPH fusion enzyme containing the native OPH signal sequence (C1) and one without the signal sequence (S1).

Maximum recovery of the fusion proteins in the soluble fraction (∼ 10 ng per liter of culture) was achieved 2 to 3 h after induction at 30°C. During that time interval, over 60% of the total OPH activity could be recovered in the soluble fraction. Both S1 and C1 fusions retained OPH activity as
demonstrated by the hydrolysis of paraoxon by the whole cells. Expression of the fusion protein at 37°C or prolonged induction (> 6 hours) at 30°C resulted in a marked decrease in soluble enzymes. It is likely that most of the fusions were produced as inclusion bodies as reported for other CBD fusions (Shpigel et al., 1999). Production of CBD–OPH fusions in the soluble fractions was confirmed by Western blot analysis. Protein bands with apparent MWs of 60 kDa were detected with both CBD and OPH antisera (Fig. 2). Neither fusion proteins appeared to undergo significant proteolysis as only limited amounts of degraded products were detected. The presence of the CBD moiety may have protected the signal sequence from proteolysis (Dave et al., 1993; Mulbry and Karns, 1988); the reduction in proteolysis by expressing proteins as fusions has been previously observed (Yang et al., 1995). Moreover, presence of the signal sequence did not play a significant role in affecting the folding or stability of the fusions. Similar whole-cell activities and protein yields were obtained from the C1 and S1 strains indicating that the presence of the signal peptide does not significantly affect the expression or activities of the fusion proteins.

**Binding of CBD–OPH to Celluloses**

Purification and immobilization of the OPH fusions was achieved by incubating cell extracts with Avicel A1 (Sigma) in the presence of 0.1% Triton-X100. After incubation, 80% of the OPH activity was removed from the supernatants of both the C1 and S1 fusion. The immobilized enzymes were as active as their free-solution counterparts as all OPH activity removed from the solution was recovered on the cellulose. The unbound fraction in the supernatant was subjected to Western blot analysis with both OPH and CBD antisera (Fig. 3). The unbound material consisted primarily of truncated or degraded products. As a control, cell extracts from pWM513, which expresses OPH intracellularly, was subjected to the same incubation. In contrast to the CBD–OPH fusions, no significant adsorption of OPH onto cellulose was observed.

SDS-PAGE analysis of the bound proteins revealed that only CBD–OPH fusions were immobilized tightly to the cellulose. Essentially, all nonspecific binding was removed in the presence of 0.1% Triton-X100. In addition to Avicel, both S1 and C1 fusions also bind to other cellulose materials including CF11 cellulose (Whatman), nonwoven cellulose fabric (Asahi Chemical Industry, Japan), and different cellulose sponges/clothes. The binding efficiency to CF11 cellulose was similar to Avicel; however, binding to cellulose fabrics and sponges was noticeably less, an observation in line with the binding characteristics of the purified CBDcloset (Goldstein et al., 1993).

**Catalytic Activity of CBD–OPH Fusions**

The kinetic characteristics of the free- and bound-C1 fusions were determined. Table I shows the $K_m$ and $k_{cat}$ values for the hydrolysis of paraoxon. The specific activity of the CBD–OPH fusions was 3196 U/mg, which is substantially

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<th>$K_m$ (mM)</th>
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<tr>
<td>OPH</td>
<td>0.058</td>
<td>3170</td>
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<tr>
<td>C1</td>
<td>0.126</td>
<td>3480</td>
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<td>Avicel-bound C1</td>
<td>0.220</td>
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higher than the 1976 U/mg reported for the [(AG)3PEG]16-OPH hybrids (Dong et al., 1994). The $k_{cat}$ values for the unbound CBD–OPH fusion was 10% higher than soluble OPH and the $K_m$ values increased by 100%, indicating that presence of the CBD moiety has a moderate impact on the catalytic performance of OPH. Immobilized CBD–OPH fusions on Avicel A1, on the other hand, showed a further twofold increase in the $K_m$ and a 20% drop in the $k_{cat}$ value compared to the free-fusion enzymes. The presence of Avicel alone appears not to exert any noticeable effect on enzymatic activity, because no change in the $K_m$ for the free OPH was observed in the presence of Avicel (data not shown). Rather, the differences in kinetic parameters may be attributed to the conformational or steric effects as a result of binding to Avicel.

The pH dependence of the immobilized CBD–OPH fusions is shown in Figure 4. The pH profile was identical for both fusions with maximum activity detected at pH 9.5. This profile is very similar to that of OPH with the fusions showing a small acidic shift. This suggests that the CBD portion does not affect in a significant way the behavior of the key ionizable groups of OPH.

Stability of the Immobilized CBD–OPH Fusions

The stability of the immobilized CBD–OPH fusions was studied. Immobilized fusions maintained close to 85% (Fig. 5) of the initial activity after 30-d incubation at 25°C. Because no detectable desorption of the fusion proteins from the cellulose matrix was observed under these conditions, this result reflects the intrinsic stability of the fusion enzyme. This is a dramatic improvement compared to soluble OPH which has been shown to lose more than 80% activity at 25°C even after 3-d incubation (Lejune and Russell, 1996). Although similar stability has been reported with immobilized polyurethane foam-entrapped OPH, the immobilized CBD–OPH is easier to prepare and retains better activity.

The presence of microcrystalline cellulose (e.g., Avicel A1) had a significant stabilizing effect on OPH activities of the fusion proteins. As the amount of Avicel used for the binding of a fixed amount of the fusion proteins was increased, the durability of the bound CBD–OPH increased (Fig. 5). The presence of a large quantity of Avicel significantly reduced the initial drop in OPH activity for the fusion enzymes. Surprisingly, Avicel also had a stabilizing effect on purified OPH. Highly diluted OPH (ca. 0.2 ng/mL) was observed to lose activity very quickly (data not shown); the addition of Avicel helped to stabilize the OPH (probably by nonspecific hydrophobic binding of the enzyme). The stabilizing effect of the Avicel on the purified OPH was not nearly as strong as for the CBD–OPH fusion protein. Nevertheless, the observation suggests that protein/cellulose interactions appear to have a stabilizing effect on OPH in much the same manner as certain adjuncts such as BSA and glycerol stabilize many enzymes.

Immobilization onto cellulose also improves the stability of the enzyme in repeated operation. The CBD–OPH-cellulose complex was packed into a 5 × 1 cm diameter column. A 2 mM solution of paraoxon was passed through the column at 100 mL/h, and the hydrolyzed products ($p$-nitrophenol) were collected and analyzed spectrophotometrically at 412 nm. Essentially 100% of the paraoxon was degraded (Fig. 6). In addition, the 100% hydrolysis efficiency was maintained over a period of 45 days.

CONCLUSIONS

Bifunctional enzymes composed of CBD and OPH were generated, enabling single-step purification and immobilization onto different cellulose materials. The enzymatic
characteristics of the immobilized CBD–OPH fusions were similar to OPH with only modest increase in $K_m$. The storage stability of the immobilized fusions is significant, retaining more than 85% activity over 45 days. An immobilized CBD–OPH reactor was used to achieve repeated degradation of paraoxon for over 45 days. Other cellulose materials such as fabrics and sponges can also be used to immobilize CBD–OPH without any loss of activity. In addition to bioreactor applications, CBD–OPH fusions could also be useful as protective clothing or decontamination wipes for pesticide spills when immobilized onto highly absorbent/water-retaining cellulose cloths/sponges.

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References


