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

Richard D. Richins, Ashok Mulchandani, Wilfred Chen

Department of Chemical and Environmental Engineering, University of California, Riverside, California 92521; telephone: 909-787-2473; fax: 909-787-2425; e-mail: Wilfred@engr.ucr.edu

Received 2 November 1999; accepted 20 February 2000

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 organophosphate 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 organophosphate wastes. © 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-

Correspondence to: W. Chen and A. Mulchandani
Contract grant sponsor: National Science Foundation
Contract grant number: BES9731513

tides of $[(AG)_xEG]_n$ 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 microporous beads, highly adsorbent sponge or cloth and microcrystalline 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 matrixes, 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* $hsdS_B(r_B^- m_B^-)$ *gal dcm (DE3) pLysS*) (Novagen) and JM105 (*end*A1, *thi*, *rps*L, *sbc*B15, *hsd*R4, Δ(*lac-pro*AB), [F′, *tra*Δ36, *pro*AB, *lac*I^qZΔM15]) were used in this study. Plasmid pET34b(+) (Novagen, Madison, WI), designed to generate protein fusions with CBD_{clos}, 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, dAATTTCGGATCCCGGGATGC (P1), and dGGGGAATTCAAGCTTCCAAAAAAAAGCCCGCTCATTAGGCGGGCTGCGTCATA-CGCCCAAGGTCGGTGACAG (P3), respectively. The resulting 1100 bp fragment was digested with *BamH*1 and *Hind*III 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, *BamH*I + *Hind*III digested, 1000 bp, *opd* gene derived from pOP231 (Richins et al., 1997) into pET34b(+). dAATTTCGGATCCCGGGTCGA (P2) and dGGGGAATTCAAGCTTCCAAAAAAAA-GCCCGCTCATTAGGCGGGCTGCGTCATGACGCCCGCAAGGTCGGTGACAAG 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 OD_{600} of 2.0. Cultures were then transferred to either 30 or 37°C and induced with 1.0 m*M* IPTG. Cells were collected by centrifugation and resuspended in 1/2 volume of PC buffer [50 m*M* disodium phosphate titrated to pH 8.0 with citric acid; 100 μ*M* $CoCl_2$]. 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 20,000*g*. 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

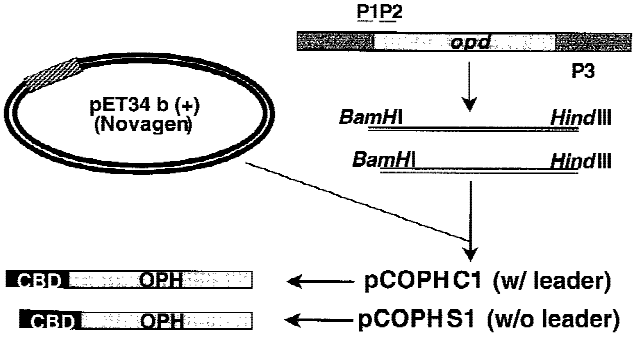

Figure 1. Construction of pCOPHC1 and pCOPHS1 encoding the CBD–OPH fusions. Details are given in Materials and Methods.

using a BioFlow 3000 fermenter (New Brunswick Scientific, Edison, NJ).

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 m*M* 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 m*M* 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 (Baneyx et al., 1989). Enzyme kinetic analyses utilized paraoxon concentrations ranging from 2 μM to 2.5 m*M* from three independent experiments. For most binding and stability studies, a paraoxon concentration of 0.5 m*M* was employed. For pH profiling, the 50 m*M* 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 0.2*M* NaOH (5 min each at 80°C). The NaOH-extracted samples were then neutralized with an equal volume of 0.2*M* 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_{CLOS} 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 × 1 cm diameter column. Paraoxon solutions of 2 m*M* 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 controling 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 mg 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.

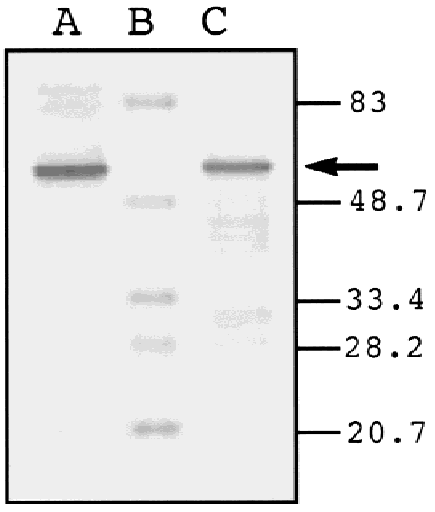

Figure 2. Production of the CBD–OPH fusions. Western blots of CBD–OPH fusion protein (C1) probed with anti-CBD antiserum (A) or with anti-OPH antiserum (C). The molecular weights of the protein standards (B) are shown to the right of lane C.

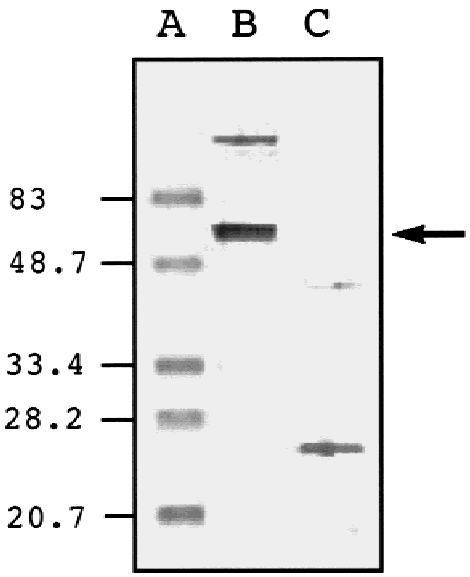

Figure 3. Binding efficiency of CBD–OPH. Western blot (probed with anti-OPH antiserum) of: material bound to Avicel (B) and unbound proteins remaining in the supernatant following Avicel binding (C). Protein standards appear in land A. The molecular weights of the standard proteins are shown on the left.

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 CBD_{clos} (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

Table I. Kinetic characteristics of the CBD–OPH fusion enzymes.

	K_m (m*M*)	k_{cat} (s^{-1})
OPH	0.058	3170
C1	0.126	3480
Avicel-bound C1	0.220	2840

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.

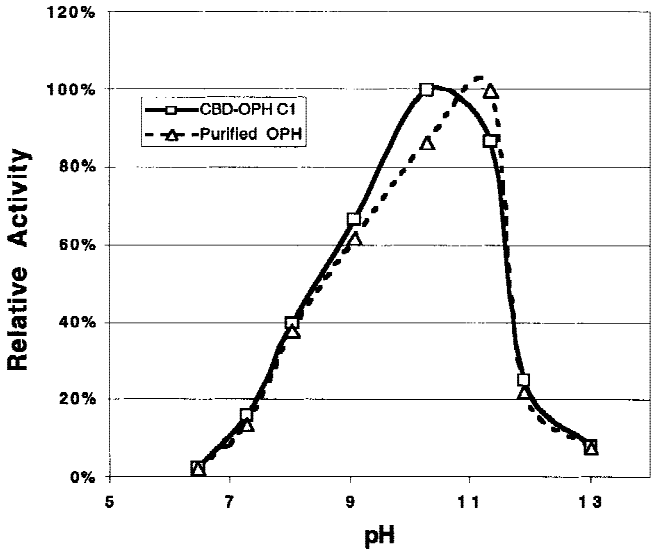

Figure 4. Effect of pH on the activity of OPH and CBD–OPH enzymes. Normalized activities (maximum activity = 100%) are shown for the degradation of paraoxon for purified OPH and the Avicel-immobilized fusion proteins. Reactions were carried out in PC buffer adjusted to various pH's (as indicated). Degradation due to base hydrolysis of paraoxon has been subtracted.

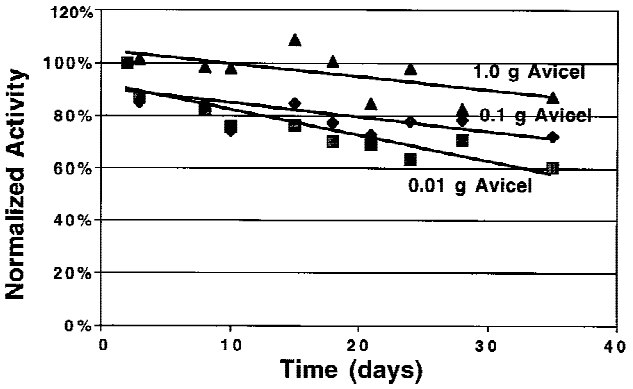

Figure 5. Stability of CBD–OPH adsorbed to Avicel at 25°C. Equal amounts of CBD–OPH were bound to 0.01, 0.1, and 1.0 grams of Avicel A1. Normalized activities (initial activity equals to 100%) are shown for the degradation of paraoxon over time.

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 m*M* 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

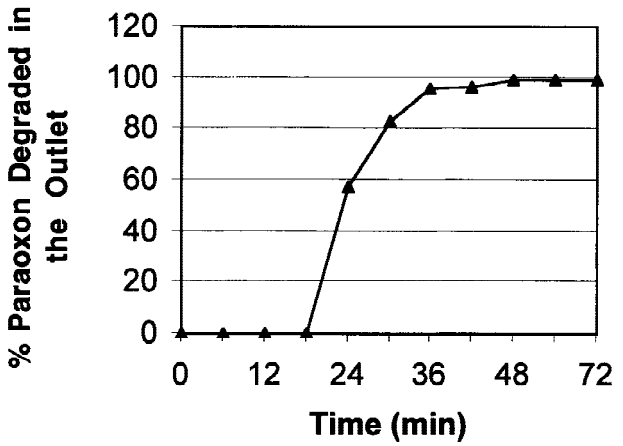

Figure 6. Continuous degradation of paraoxon by an immobilized CBD–OPH column at 25°C. 2 m*M* paraoxon solution was passed through the column at a flow rate of 100 mL/h.

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.

We thank Dr. Ayman Mansee and Cathy Cho for their help with some of the experiments.

References

Ahn DH, Kim H, Pack, MY. 1997. Immobilization of β-glucosidase using the cellulose-binding domain of *Bacillus subtilis* endo-β-1,4-glucanase. Biotechnol Lett 19:483–486.

Baneyx F, Schmidt C, Georgiou G. 1989. Affinity immobilization of a genetically engineered bifunctional hybrid protein. Enzyme Microb Technol 12:337–342.

Caldwell SR, Raushel FM. 1991a. Detoxification of organophosphate pesticides using a nylon based immobilized phosphotriesterase from *Pseudomonas diminuta*. Appl Biochem Biotechnol 31:59–73.

Caldwell SR, Raushel FM. 1991b. Detoxification of organophosphate pesticides using an immobilized phosphotriesterase from *Pseudomonas diminuta*. Biotechnol Bioeng 37:103–109.

Dave KI, Miller CE, Wild JR. 1993. Characterization of organophosphorus hydrolases and the genetic manipulation of the phosphotriesterase from *Pseudomonas diminuta*. Chem Biol Interactions 87:55–68.

Donarski WJ, Dumas DP, Heitmeyer DP, Lewis VE, Raushel FM. 1989. Structure-activity relationships in the hydrolysis of substrates by the phosphotriesterase from *Pseudomonas diminuta*. Biochem 28: 4650–4655.

Dong W, Fournier MJ, Mason TL, Tirrell DA. 1994. Bifunctional hybrid artificial proteins. Polymer Preprints 74:419–420.

Goldstein MA, Takagi M, Hashida S, Shoseyov O, Do RH, Segel IH. 1993. Characterization of the cellulose-binding domain of the *Clostridium cellulovorans* cellulose-binding protein A. J Bacteriol 175:5762–5768.

Greenwood JM, Ong E, Gilkes NR, Warren RAJ, Miller RC, Kilburn DG. 1992. Cellulose-binding domains: Potential for purification of complex proteins. Prot Eng 5:361–365.

Havens PL, Rase HF. 1993. Reusable immobilized enzyme/polyurethane sponge for removal and detoxification of localized organophosphate pesticide spills. Ind Eng Chem Res 32:2254–2258.

Karns JS, Muldoon MT, Mulbury WW, Derbyshire MK, Kearney PC. 1987. Use of microorganisms and microbial systems in the degradation of pesticides. In: LeBaron HM, Mumma RO, Honeycutt RC, Duesing JH, editors. Biotechnology in agricultural chemistry. ACS Symposium Series. No. 334. Washington, DC: American Chemical Society. p 156–170.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

LeJuene KE, Russell AJ. 1996. Covalent binding of a nerve agent hydrolyzing enzyme within polyurethane foams. Biotechnol Bioeng 51: 450–457.

Mulbry WW, Karns JS. 1988. Parathion hydrolase specified by the *Flavobacterium opd* gene: Relationship between the gene and protein. J Bacteriol 171:6740–6746.

Mulchandani P, Mulchandani A, Kaneva I, Chen W. 1999. Biosensor for direct determination of organophosphate nerve agents. 1. Potentiometric enzyme electrode. Biosens Bioelectron 14:77–85.

Munnecke DM. 1979. Hydrolysis of organophosphate insecticides by an immobilized-enzyme system. Biotechnol Bioeng 21:2247–2261.

Ong E, Gilkes NR, Antony R, Warren J, Miller RC, Kilburn DG. 1989. Enzyme immobilization using the cellulose-binding domain of a *cellulomonas fimi* exoglucanase. Bio/Technol 7:604–607.

Richins R, Kaneva I, Mulchandani A, Chen W. 1997. Biodegradation of organophosphorus pesticides using surface-expressed organophosphorus hydrolase. Nature Biotechnol 15:984–987.

Serdar CM, Murdock DC, Rohde MF. 1989. Parathion hydrolase gene from *Pseudomonas diminuta* MG: subcloning, complete nucelotide sequence, and expression of the mature portion of the enzyme in *Escherichia coli*. Bio/Technol 7:1151–1155.

Shpigel E, Goldlust A, Efroni G, Avraham A, Eshel A, Dekel M, Shoseyv O. 1999. Immobilization of recombinant Heparinase I fused to cellulose-binding domain. Biotechnol Bioeng 65:17–23.

Shoseyov O, Takagi M, Goldstein MA, Doi RH. 1992. Primary sequence analysis of *Clostridium cellulovorans* cellulose binding protein A. Proc Natl Acad Sci USA 89:3483–3487.

Steiert JG, Pogell BM, Speedie MK, Laredo J. 1989. A gene coding for a membrane-bound hydrolase is expressed as a secreted soluble enzyme in *Streptomyces lividans*. Bio/Technol 7:65–68.

Tuovinen K, Kalistekorhonen E, Raushel FM, Hanninen O. 1994. Phosphotriesterase—A promising candidate for use in detoxification of organophosphates. Fund Appl Toxicol 23:578–584.

Wu D, Fournier MJ, Mason TL, Tirrell DA. 1996. Hybrid artificial proteins: A new class of reactive polymers. PMSE Preprints 74:71–72.

Yang S, Veide A, Enfors SO. 1995. Proteolysis of fusion proteins: Stabilization and destabilization of staphylococcal protein A and *Escherichia coli* β-galactosidase. Biotechnol Appl Biochem 22:145–159.