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Biodegradation of Pesticide Pollutants by Two Kinds of Enzymes Coexpressed in Genetically Engineered Bacteria

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Pesticides are widely used in insect pest management for crop production, municipal hygiene and disease vector control. In recent years, the land areas affected by the expanding application of pesticides have exceeded 13,000 hektare (ha) in China. This increased the risk of environmental contamination of ground water, food products and water resources. From a survey of 26 provinces in China from 1992 to 1996, there were about 240,000 people poisoned by pesticides and nearly 24,000 of them died (Jiang 2000). There is also an increased threat to the survival of beneficial insects and various wildlife species (Smith 1987). It is becoming important to develop pathways to detoxify and degrade these waste pesticide residues.

Organophosphorus hydrolase (OPH) isolated from the natural soil microorganisms *Pseudomonas diminuta* MG and *Flavobacterium* sp. has been shown to hydrolyze a wide range of organophosphorus pesticides (Mulbry and Karns 1989; Serdar et al. 1989), but it can not hydrolyze chemicals containing carboxylester bonds. The chemical structures of carbamates and pyrethroids correspond to carboxylic acids, which can potentially be hydrolyzed by carboxylesterases (CbEs) (Sogorb and Vilanova 2002). Recent advances in molecular biology have opened up new avenues to move toward the goal of engineering microbes or enzymes to function as biocatalysts, in which certain desirable traits from different organisms are brought together with the aim of performing specific bioremediation. In this study, we transferred the *opd* and *b1* genes into the same bacterium to produce two kinds of enzymes that can simultaneously degrade organophosphates and carboxylesters. Bioassays were used to monitor pesticide residue reduction in polluted water. The method of genetically engineering bacteria has laid the foundation of extending the substrate spectrum of pesticides in future practical use.

MATERIALS AND METHODS

One mosquito strain (*Culex pipiens*), collected from Beijing in 2003, was reared under laboratory conditions (photoperiod L: D = 14 hr: 10 hr; 25 ℃).

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Analytical-grade parathion (93% purity), pirimicarb (99.8% purity) and deltamethrin (98% purity) were obtained from Institute for the Control of Agrochemicals, Ministry of Agriculture, the People's Republic of China.

E.coli strains BL21 (DE3) were used for the expression of the recombinant proteins. Plasmid pETDuet, purchased from Novagen, was used for coexpressing the *opd* gene and the *b1* gene. Plasmid pPNCO33 (granted by Ashok Mulchandani, University of California, Riverside, California.) was used as the template for the *opd* gene, and vector pET28a-*b1* was used as the source of the carboxylesterase B1 gene (Huang et al. 2001). Other reagents, such as T4 DNA ligase, alkaline phosphatase, IPTG, ampicillin and restriction enzymes *Bam*HI, *Bgl*II, *Xho*I and *Hin*dIII, were purchased from TaKaRa (Japan).

To construct an OPH and B1 coexpression plasmid, *opd* and *b1* genes were amplified using PCR. The forward and reverse oligonucleotide primers for the *opd* gene, *opd*-up (5′-ACGGATCCCATGCAAACGAGAAGGG-3′) and *opd*-down (5′-GTAAGCTTCATGACGCCCGCAAGG-3′) contained, respectively, a *Bam*HI restriction site before the *opd* start codon and a *Hin*dIII restriction site at the stop codon (underlined bases). The template for PCR was the pPNCO33 vector. The PCR fragment was subsequently cloned into the *Bam*HI-*Hin*dIII restriction sites of pETDuet to generate the recombinant plasmid pETDuet-*opd*. The His-Tag sequence in pETDuet was fused with the *opd* gene on the 5′-terminus. The forward and reverse oligonucleotide primers of the *b1* gene, *b1*-up (5′-ACAGATCTCATGAGTTTGGAAAGCTTAACCGTTC-3′) and *b1*-down (5′-TACTCGAGAAACAGCTCATCATTCACGTACATTG-3′) contained, respectively, a *Bgl*II restriction site before the *b1* start codon and a *Xho*I restriction site at the 3′-terminus of *b1* gene (underlined bases). The stop codon of the *b1* gene was removed and the *b1* gene was extended inframe with fusion S-Tag sequence in pETDuet. The template for PCR was the pET28a-*b1* vector. The PCR fragment was subsequently cloned into the *Bgl*II-*Xho*I restriction sites of pETDuet-*opd* (previously obtained) to generate the recombinant plasmid pETDuet-*opd-b1*. At the same time the PCR fragment was also cloned into the *Bgl*II-*Xho*I restriction sites of pETDuet to generate the recombinant plasmid pETDuet-*b1*, as a control. The correct sequence of the insert was confirmed by sequencing.

Desired plasmids were transformed into *E.coli* BL21 (DE3) and bacteria were plated on LB agar plates supplemented with ampicillin (50 μg mL^{-1}). Freshly transformed colonies were inoculated into LB medium containing ampicillin (50 μg mL^{-1}) and grown at 37 °C until OD_{600} reached 0.6, then, temperature was lowered to 25 °C and the recombinant expression was induced by addition of isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 1 m*M*, and then culturing was continued for 8 hr. Subsequently, bacteria were centrifuged (5,000 g, 5 min, 4 °C). The pellets were suspended in 50 m*M* phosphate buffer saline (PBS,

pH 7.0) and the cell density was adjusted to OD_{600}=1. Lysozymes were added to a final concentration of 1 μg mL^{-1}. The collected cells were incubated at 30 °C for 15 min. Harvested cells were disrupted by sonication in an ice bath to release recombinant proteins. After centrifuging the resultant cultures at 12,000 g for 15 min, the supernatants or crude enzymes were obtained. pETDuet-*opd-b1*, pETDuet-*opd* and pETDuet-*b1* cell cultures contained 5.5 mg protein mL^{-1}, 4.8 mg protein mL^{-1}, 4.4 mg protein mL^{-1}, respectively, measured using the method described by Bradford (1976).

OPH activity was determined by the method of Shimazu et al. (2001). Standard assay conditions for enzyme activity tests were as follows: 10 μL enzyme solution was added to 880 μL of citrate-phosphate buffer at pH 7.5 and 100 μL of 0.18 m*M* parathion. Methanol was added to a final concentration of 10%. Reaction mixtures were incubated at 30 °C during which time the changes in absorbance (410 nm) were monitored. The same solution with no enzymes was used as a blank. The degradation of parathion was analyzed by measuring the absorbance of the *p*-nitrophenol spectrophotometrically. Enzymatic activities were expressed in units (μmol of parathion hydrolyzed per min), per mg protein.

B1 activity was determined by the method of Van Asperen (1962): 2.5 mL reaction mixture consisted of 20 μL enzyme sample and 0.3 m*M* β-Naphthyl acetate containing 0.5% acetone in 50 m*M* sodium phosphate buffer (pH 7.5). Freshly prepared 0.5 mL Diazoblue SDS reagent (0.3% in fast blue B salt in 3.5% aqueous SDS) was added after 30 min of incubation at 30 °C. The incubation was continued for 15 min. Then colour, developed as a result of β-Naphthol formation, was measured at 555 nm. The enzymatic activities were calculated from a β-Naphthol standard curve and were expressed in units (μmol of β-Naphthyl acetate hydrolyzed per min), per mg protein.

The degradation of the pesticides (pirimicarb and deltamethrin) was done by coexpressed enzymes and detected according to the method described by Leng and Qiao (1986). Pesticides were dissolved in 15 mL of 10 m*M* potassium-phosphate buffer (pH 7.5) and then 5 mL enzyme (55.5 U mL^{-1}) was added. The mixtures were incubated at 37 °C and 1 mL samples were taken from the flask at different lengths of time, to which either 1 mL dichloromethane or petroleum ether was added. The samples were then dried with anhydrous sodium sulfate and the products extracted with redistilled hexane and analyzed by GC. Recoveries of pirimicarb and deltamethrin were 92.2–95.7% and 93.8–97.2%, respectively. The pesticide concentrations were measured with the HP-5890 series- II gas chromatograph, equipped with a nitrogen phosphorus detector on a fused silica capillary column (length 30 m x 0.53 mm id). Pirimicarb were detected using N_2 as the carrier gas at 1 mL min^{-1}. Injector, column and detector temperatures were set at 300 °C, 220 °C and 300 °C, respectively, and the detection limit of pirimicarb was 1 ng μL^{-1}. Deltamethrin was analyzed using the

same GC equipped with an electron capture detector on the same column. Injector, column and detector temperatures were set at 300 °C, 270 °C and 300 °C respectively. The detection limit of deltamethrin was 0.01 ng μL^{-1}. Pesticide concentrations were determined by comparing peak area of the samples to a standard curve.

Bioassays were performed as described by Raymond and Marquine (1994) to evaluate the pesticide degradation. Three pesticides (parathion, pirimicarb and deltamethrin) in ethanol solution were tested. Each bioassay included 5 dosages and triplicates per dosage on sets of 20 early 4th instars (*Culex pipien*) in a total volume of 100 mL of water containing 1 mL of ethanol solution of pesticides. 0.01 U (232 μL OPH, 256 μL OPH+B1), and 100 U (1.669 mL B1) enzymes were used with the parathion solution, respectively. 0.01 U (232 μL OPH), and 100 U (1.802 mL OPH+B1, 1.669 mL B1) enzymes were used with pirimicarb and deltamethrin, respectively. Pesticide solutions without enzymes served as controls. The cups were placed in a dark growth chamber at 28 °C. Mortality was calculated after 24 hr. Complete immobility or paralysis was taken as an indication of neurotoxic activity. Mortality data were analyzed by the log-probit program of Raymond (1993) based on Finney (1971). This program provides LC_{50} and slope for each mortality line, and tests parallelism between two or more mortality lines.

RESULTS AND DISCUSSION

Recombinant proteins were analyzed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie Blue staining. The OPH was purified using His-Bind Purification Kits (Novagen). There was no B1 binding to purified OPH by native-PAGE. This indicated that there is no specific interaction between the two proteins. Proteins were assembled into separate inclusions and their structure formation was independent. However, high-level expression of recombinant proteins in *E. coli* often results in the formation of insoluble and inactive aggregates known as inclusion bodies, but there is still a portion of active proteins in the cell. The expression cells were induced at relatively low temperature for over 8 hr to reduce inclusion body formation. The cells induced for 8 hr at 25 °C were collected, and crude enzymes were obtained to assay the enzymatic activities. The specific yields of enzymes were as in Table 1.

The analytical method for parathion degradation was the same as that for OPH activity determination. Figure 1 shows that parathion was degraded by enzymes produced by cells bearing pETDuet-*opd-b1*. 18 μ*M* parathion rapidly decomposed to 4 μ*M* within 30 min, and was almost completely degraded by coexpressed enzymes within 1 hr. Coexpressed enzymes were very effective in degrading parathion. Figure 2 shows the concentration of pirimicarb was reduced from 100

Table 1. Specific yields of enzyme produced by genetically engineered bacteria.

Enzymes	Parathion as substrate		β-Naphthyl acetate as substrate	
	Sa	Va	Sa	Va
OPH	8.96×10^{-3}	0.043		
OPH+B1	7.09×10^{-3}	0.039	13.6	59.5
B1			10.0	55.5

Sa, Specific activity (Unit: U mg^{-1} protein).
Va, Volumetric activity (Unit: U mL^{-1} medium).

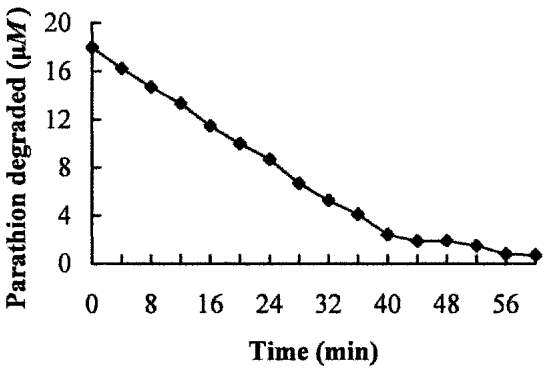

Figure 1. Parathion was degraded by enzymes produced by cells bearing pETDuet-*opd-b1*. The degradation was calculated by release of *p*-nitrophenol.

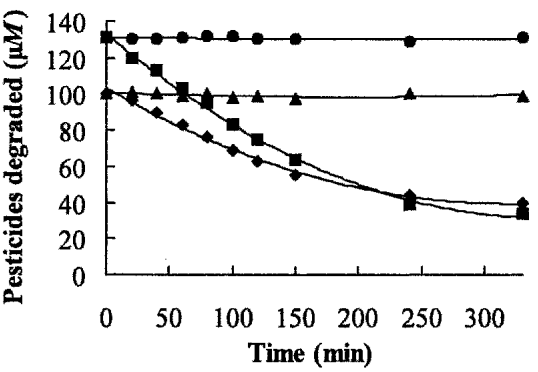

Figure 2. Pirimicarb and deltamethrin were degraded by enzymes produced by cells bearing pETDuet-*opd-b1*. (◆) Pirimicarb degraded, (▲) Pirimicarb control, (■) Deltamethrin degraded, (●)Deltamethrin control.

μM to 60 μM after about 3 hr, and deltamethrin from 130 μM to 40 μM after about 4 hr. In the controls, there was no pesticide lost over the same timeframe. Pirimicarb and deltamethrin were also degraded by coexpressed enzymes, but their degradation effects were poorer than for parathion. Carboxylesterases (CbEs, EC 3.1.1.1) are of great interests for both basic research and industrial purposes. CbEs are a widely distributed family of enzymes in many animals, which hydrolyze carboxylester, amide and thioester bonds in a variety of compounds. Carbamate and pyrethroid pesticides are esters of carboxylesters and can be hydrolysed by carboxylesterases potentially. The differences in the metabolism of carbamates and pyrethroids are dependent on the chemical structures and on the animal species (Sogorb 2002). The enzymatic reaction mechanism reveals that B1 has effectiveness with carbamate and pyrethroid degradation. The results suggest that coexpressed enzymes have good hydrolytic rate for parathion and can hydrolyze carbamate and pyrethroid.

The bioassay originally used to describe the resistant characteristics of insects to toxins. In this study, it proved to be a simple, rapid, economical and sensitive way of assessing whether coexpressed enzymes could efficiently degrade some organophosphorous, carbamate and pyrethroid pesticides. Detailedly, the RR value was derived from the comparison between the LC_{50} values observed in presence and in absence of enzymes, indicating the resistant ratio in pesticide resistant bioassay. In this study, the RR value was considered as an efficient indicator of pesticide degradation by enzymes. When the RR is greater than 1 and the 95 % CI does not include 1, it means that the difference between these two LC_{50} values is significant ($P < 0.05$), so part of pesticide molecules were degraded by the enzymes. The higher the RR value was, greater the pesticide degradation, thereby indicating greater enzyme efficiency. When the 95 % CI included 1, no significant ($P > 0.05$) quantity of pesticide molecules was degraded. None of these enzymes was toxic to the mosquitoes, since no mortality was observed when only crude enzymes were added (data not shown).

Parathion samples treated with OPH or OPH+B1 exhibited a low level of toxicity against 4th instar larvae of mosquito, yielding a 50 % lethal concentration (LC_{50}) of 16.459 mg L^{-1} and 17.840 mg L^{-1} (Table 2). In contrast, Parathion samples treated with B1 and untreated control samples were much more toxic with a LC_{50} of 0.0189 mg L^{-1} and 0.0179 mg L^{-1}, respectively. The reason is that the quantities of pesticide were greatly reduced in OPH or OPH+B1 treated water. The LC_{50} values between samples treated with B1 and the control were not significantly different. B1 could not degrade parathion. Similar results were obtained for pirimicarb and deltamethrin samples. The coexpressed enzymes (OPH+B1) were able to degrade the three pesticides efficiently. With parathion as the substrate, it was mainly OPH that acted in degradation. With pirimicarb and deltamethrin as the substrates respectively, B1 was most efficient. These results are consistent with those derived from the pesticide degradation test.

Table 2. The bioassays of mosquito larvae living in pesticide solutions.

Pesticide samples	LC_{50} (95% CI)	Slope (SE)	RR (95% CI)
Parathion	0.0179 (0.016-0.020)	3.790 (0.357)	
OPH treatment	16.459 (14.813-18.127)	3.778 (0.437)	918.2 (725.4-1162.1)
OPH+B1 treatment	17.840 (16.031-19.604)	4.115 (0.421)	995.2 (778.2-1272.6)
B1 treatment	0.0189 (0.017-0.021)	3.738 (0.388)	1.1 (0.8-1.3)
Pirimicarb	0.298 (0.255-0.351)	2.501 (0.249)	
OPH treatment	0.300 (0.258-0.354)	2.487 (0.250)	1.0 (0.8-1.3)
OPH+B1 treatment	8.410 (7.361-9.802)	2.628 (0.354)	28.2 (22.4-35.6)
B1 treatment	8.284 (7.233-9.621)	2.726 (0.295)	27.8 (21.9-35.2)
Deltamethrin	0.010 (0.008-0.012)	2.310 (0.228)	
OPH treatment	0.010 (0.009-0.012)	2.204 (0.308)	1.0 (0.8-1.3)
OPH+B1 treatment	1.099 (0.911-1.354)	2.187 (0.217)	110.3 (85.8-142.0)
B1 treatment	1.091 (0.907-1.328)	2.162 (0.204)	109.4 (85.6-139.9)

LC_{50} in mg L^{-1}; CI, confidence interval; SE, standard deviation; RR, ratio (LC50 observed in presence of enzyme/LC50 observed in absence of enzyme.)

In summary, we applied the coexpression method for efficient production of the organophosphorus hydrolase together with CbEs in *E.coli*. Therefore, a single bacterium is endowed with the ability to produce two kinds of enzymes simultaneously, which can potentially degrade some organophosphorus, carbamate and pyrethroid pesticides. Thus the spectrum of pesticide degradation was expanded.

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