Coexpression of two detoxifying pesticide-degrading enzymes in a genetically engineered bacterium

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Abstract

Degradation of pesticides is usually beneficial, since the reactions that destroy pesticides convert most pesticide residues in the environment to inactive, less toxic, harmless compounds. This paper describes a novel strategy using a coexpression vector for the purpose of developing bacteria that can detoxify different pesticides. The vector pETDuet was designed for coexpression of two target genes simultaneously. The organophosphate hydrolase gene (*opd*) from *Flavobacterium* sp. and carboxylesterase B1 gene (*b1*) from *Culex pipiens* were cloned in the coexpression vector. The expressed enzymes of OPH and B1 had a molecular mass of approximately 35 and 65 kDa, respectively. A single microorganism was capable of producing both enzymes for degradation of organophosphorus, carbamate and pyrethroid pesticides. The technical capability of genetically engineering bacteria with more enzymes should open up new opportunities for extending the wide range of pesticides that can be biodegraded in the future.
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1. Introduction

Pesticides are used in controlling crop pests to minimize losses of agricultural products and control insect vectors to prevent the outbreak of human and animal epidemics. Food shortages have resulted in increasing use of insecticides in agriculture. For example, in India, almost 30% of agricultural output is lost because of pest infestation, and pesticide consumption for protecting crops accounts for about 3% of the total world consumption (Bhadbhade et al., 2002). However, pesticide residues can adversely affect human health and also cause environmental pollution. Worldwide unintentional overexposure results in $> 3 \times 10^6$ cases of pesticide poisoning annually. Excessive pesticide use has also created global problems of pest resistance, resurgence and pesticide residues in crops and soil (Qiao et al., 2003). However, pesticides continue to play an important role in controlling economically harmful populations of insects. Many believe that this conflict is one of the most critical current problems requiring to be tackled nowadays.

The most popular pesticides for agriculture purposes are organophosphates, carbamates and pyrethroids. These groups cause neurotoxicity in mammals. Compounds of these three families are spontaneously hydrolyzed and undergo enzymic degradation by hydrolases (Sogorb and Vilanova, 2002). Synthetic organophosphates (OPs) are widely used as pesticides. OPs contain three phosphoester linkages and hydrolysis of one of the phosphoester bonds dramatically reduces the toxicity of the pesticides by eliminating their acetylcholinesterase inactivating properties (Horne et al., 2002). Several enzymes capable of detoxifying OPs have been isolated from microorganisms able to use OPs as a carbon source. The most widely characterized phosphotriesterase is the bacterial organophosphate hydrolase (OPH, E.C.8.1.3.1), which has been isolated from both *Flavobacterium* sp. ATCC 27551

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(Mulbry and Karns, 1989) and *Pseudomonas diminuta* MG (Serdar et al., 1989). It is one of the most crucial enzymes in the detoxification of organophosphorus compounds, such as paraoxon, parathion, coumphos and diazion (Wu et al., 2002). However, the enzyme does not catalyze the cleavage of carbonyl groups such as those found in *p*-nitrophenyl acetate (Raushel, 2002). Since the chemical structures of carbamates and pyrethroids correspond to carboxylic acids, carboxylesterase (CbE E.C.8.1.1.1) can potentially hydrolyze all these compounds. Many CbEs have been discovered in insects. B1 esterase is a CbE and plays a key role in mosquito resistance to OPs (Raymond and Pasteur, 1989). The enzymatic hydrolysis of carboxyl esters by CbEs is based on the reversible acylation of a serine residue with the active centre of the alcohol moiety of the carboxyl ester and of the corresponding covalently acylated enzyme. This acylated intermediate is hydrolyzed by nucleophilic attack of water that releases the corresponding carboxylic acid moiety, plus the free active enzyme again ready to initiate a new catalytic cycle (Miguel and Vilanova, 2002). Accordingly, B1 has been the subject of intense study in an attempt to develop technologies for the biodegradation of pesticides (Yan et al., 2000).

A number of choices for coexpression of multiple target proteins in *Escherichia coli* are currently available, of which the plasmid pETDuet has been designated for coexpression of two target genes. This vector contains two multiple cloning sites (MCS), each of which is preceded by a T7 promoter/*lac* operator and a ribosome binding site (RBS). The vector also carries the pBR322-derived ColE1 replicon, *lacI* gene and ampicillin resistance gene. The pETDuet vector is designed with the option of producing native unfused proteins or fusions to His-Tag and S-Tag sequences for detection and purification of protein complexes. Coexpression in *E. coli* facilitates the identification and characterization of the interaction of different proteins.

In this study we investigated the expression of two different enzymes and this relative enzymatic activity. The target proteins were produced and the bioactivities of products were measured.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli strain BL-21 (DE3) was used as the expression host throughout the experiments. Plasmid pETDuet (Novagen) was used for coexpressing the OPH gene and the B1 gene carboxylesterase. Plasmids were maintained and propagated in *E. coli* DH5α according to Sambrook et al. (1989). Plasmid pPNCO33 (kindly donated by A. Mulchandani, University of California, Riverside, CA) carrying an *opd* gene, was used as the template of the opd gene. Expression vector pET28a-*b1* (Huang et al., 2001), carrying a *b1* gene, was used as the source of that gene. Other reagents, viz. T4 DNA ligase, alkaline phosphatase, isopropyl thiogalactoside, ampicillin and restriction enzymes *Bam*HI, *Bgl*II, *Xho*I and *Hind*III, were purchased from TaKaRa (Japan).

2.2. Media and culture conditions

Strains bearing plasmids were grown in Luria–Bertani (LB) medium supplemented with ampicillin to a final concentration of $50 \mu g \, ml^{-1}$. Cells were grown at various temperatures in 250 ml flasks in an Innova 4000 shaker (New Brunswick Scientific) with vigorous agitation (260 rpm). Bacteria harbouring expression vectors were grown to an $OD_{600} = 0.6$ before induction with 1 mM IPTG. At 1 h after induction, $CoCl_2$ was added to a final concentration of 1 mM. Cells continued to grow under the different conditions.

2.3. Construction of the expression plasmids

To construct an OPH and B1 coexpression plasmid, *opd* and *b1* genes were amplified using PCR. The upstream and downstream oligonucleotide primers of *opd* gene, *opd*-up (5′-ACGGATCCCATGCAAACGA-GAAGGG-3′) and *opd*-down (5′-GTAAGCTTCATG ACGCCCGCAA GG-3′) contained, respectively, a *Bam*HI restriction site before the *opd* start codon and a *Hind*III restriction site at the stop codon (underlined bases). The template for this PCR was the *p*PNCO33 vector obtained from Mulchandani. The PCR fragment was subsequently cloned into the *Bam*HI-*Hind*III restriction sites of pETDuet (Novagen) to generate the recombinant plasmid pETDuet-*opd*. The His-Tag sequence in pETDuet was fused with *opd* gene on the 5′-terminus.

The upstream and downstream oligonucleotide primers of *b1* gene, *b1*-up (5′-ACAGATCTCATGAGTTTGGAAAGCTTAACCGTTC-3′) and *b1*-down (5′-TACTCGAGAAACAGCTCATCAT TCACGTACATTG-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 *b1* gene was removed and the *b1* gene extended inframe with fusion S-Tag sequence in pETDuet. The template for this PCR was the pET28a-*b1* vector. The PCR fragment was subsequently cloned into the *Bgl*II-*Xho*I restriction sites of pETDuet-*opd* 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 control. The construction scheme is shown in Fig. 1. Three plasmids were designed to produce fusions of His-Tag and S-Tag sequences for detection and purification of target protein. The correct sequence of the insert was confirmed by sequencing.

2.4. Transformation of E. coli strain BL-21 (DE3) and expression

Expression studies were carried out using *E. coli* BL-21 (DE3). Transformation was performed according to the pET System Manual (10th edn. TB055, Novagen 2002). The vectors of pETDuet-*opd*-*b1*, pETDuet-*opd* and pETDuet-*b1* were transformed. One colony from freshly transformed cells was used to inoculate 2 ml medium containing $50 \mu g \, ml^{-1}$ ampicillin and incubated for 8–10 h at 37 °C. Then, 2 ml cell suspension was used to inoculate 50 ml LB medium supplemented with $50 \mu g \, ml^{-1}$ ampicillin. Optimal production of recombinant protein was obtained when mid-exponential-phase cells ($OD_{600} = 0.6$) were induced with 1 mM IPTG for 5 h at 37 °C. After collecting induced cells by centrifugation at 5000*g* for 5 min, the cell density was adjusted to $OD_{600} = 1$ with PBS. Lysozymes were added to a final concentration of $1 \mu g \, ml^{-1}$. Then, 1% Triton X-100 was added to 1/10 by volume. The cells were incubated at 30 °C for 15 min, harvested and disrupted by sonication in an ice bath, and the expression of target genes was assessed by analysis of total cell protein by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie blue staining.

2.5. Purification and identification recombinant OPH and B1

The expressed OPH contained a sequence of histidine residues (His-Tag sequence) at the N-terminus and was purified using His Bind Kits (Novagen). Disruption of the cells and precipitation steps were mainly
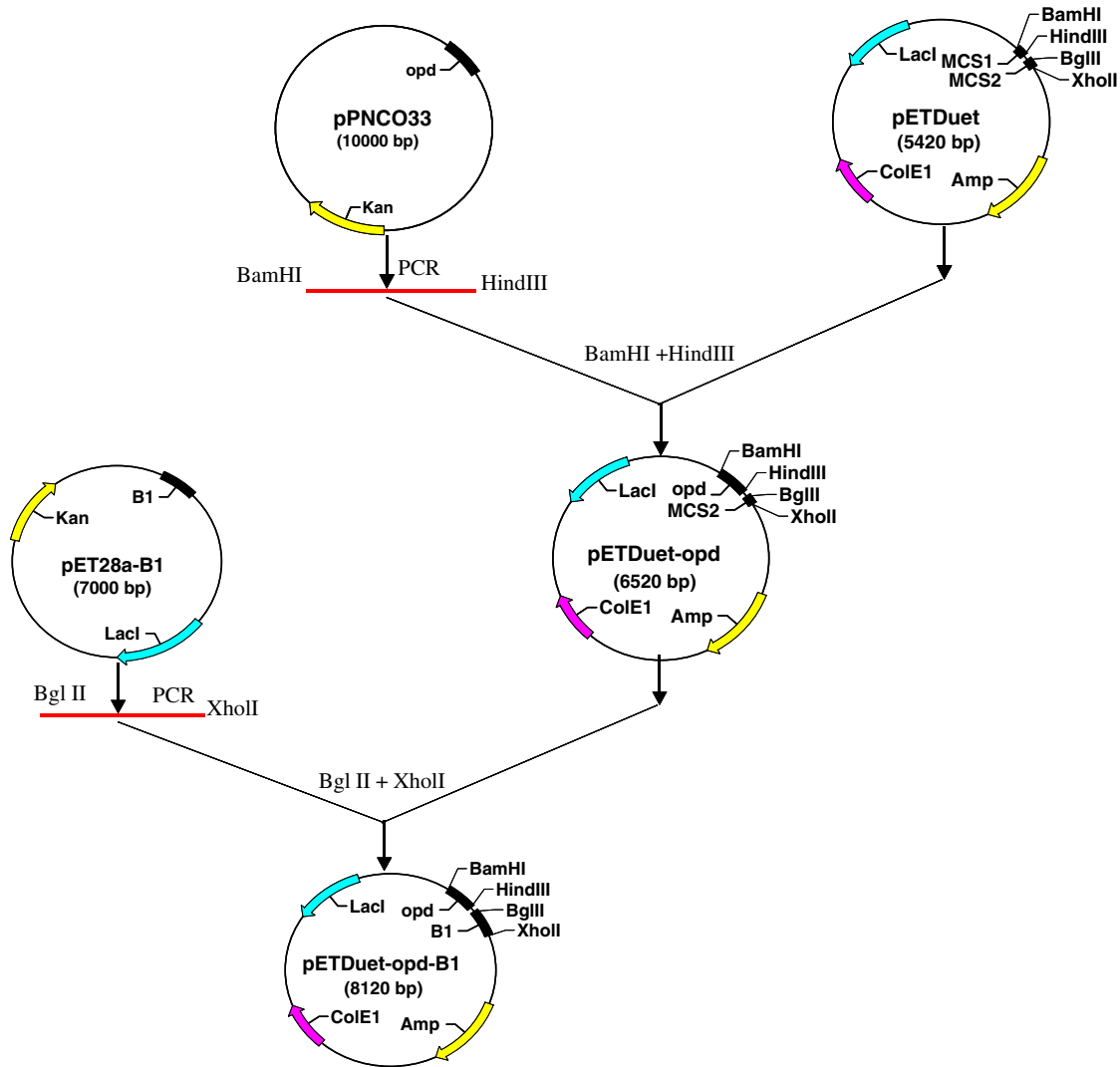
Fig. 1. Construction of recombinant plasmids.

carried out as described in the Novagen user's manual. The process was carried out using a cell extract from 100 ml culture broth. Before the crude cell extract was loaded onto an ion exchange chromatography column, the binding buffer was allowed to drain to the top of the column bed. A flow rate of about 10 column volumes h^{-1} was optimal for efficient purification. The column was washed with 10 volumes of $1 \times$ binding buffer. Then washed with 6 volumes of $1 \times$ wash buffer. The bound protein was eluted with 6 volumes of $1 \times$ elute buffer and the eluate collected in a fraction collector.

The expressed B1 esterase, containing a sequence of S-Tag residues at the C-terminus, was purified using an S-Tag rEK purification kit according to the kit protocol. The induced cells were harvested by centrifugation and resuspended in 10 ml ice-cold $1 \times$ bind/wash buffer. The cells were sonicated to shear the DNA, and the lysate was then centrifuged at 39,000g for 20 min to remove debris. The supernatant was filtered through a 0.45-μm membrane to prevent clogging of the resin. Then, 2 ml slurry of settled resin were added to the sample and mixed thoroughly. It was incubated at room temperature on an orbital shaker for 30 min. Unbound protein was washed away by repeating centrifugation and resuspension of the S-protein agarose in 5 ml $1 \times$ bind/wash buffer. The washed resins were resuspended in 1.5 ml 3 M MgCl$_2$ elution buffer. The entire reactions were transferred to a spin filter in a collection tube and centrifuged at 500g for 5 min. The clear filtrate containing the purified target protein was collected on recombinant protein detected on 12% SDS–PAGE gels run on the Bio-Rad Mini-Protein II apparatus according to Laemmli (1970). Proteins were visualized by staining with Coomassie Blue.

2.6. Assay of enzymatic activity

OPH activity was determined by the method of Shimazu et al. (2001). The standard assay for enzyme activity tests involved addition of 20 μl enzyme solution (OD$_{600}$ = 1.0 whole cell protein in 100 μl PBS) to 880 μl citrate-phosphate buffer at pH 7.5 and 100 μl of 20 mM parathion. Methanol was added to a final concentration of 10%. Reaction mixtures were incubated at room temperature or 30 °C, and during incubation the changes in absorbance (410 nm) were monitored. Enzymatic activities are expressed as μmoles parathion hydrolyzed min^{-1}, OD$_{600}$ whole cells^{-1}.

B1 activity was determined by the method of van Asperen (1962); in which 2.5 ml reaction mixture comprised 20 μl enzyme sample (OD$_{600}$ = 1.0 whole cell protein in 100 μl PBS) and 0.3 mM β-naphthyl acetate containing 0.5% acetone in 50 mM sodium phosphate buffer (pH 7.5). After incubation for 30 min at 30 °C, 0.5 ml freshly prepared diazoblue SDS reagent (0.3% in fast blue B salt in 3.5% aqueous SDS) was added and the incubation was continued for 15 min. Thereafter, the solution was centrifuged at about 1000g After centrifugation, the colour developed as a result of β-naphthol formation was measured at 590 nm spectrophotometrically. The enzyme activity was calculated from a

β-naphthol standard curve and enzymatic activities are expressed as μmoles β-naphthyl acetate hydrolyzed^{-1} per OD_{600} whole cells.

2.7. Insect bioassay

A laboratory colony of the mosquito *Culex pipiens* was cultured in the laboratory and maintained by standard procedures. In the bioassays, groups of 80 early fourth instar larvae were exposed to a range of concentrations of different pesticides in 100 ml deionized water contained in 237 ml plastic cups. Stock concentrations of pesticides were prepared in ethanol. The final concentrations of parathion, pirimicarb and decamethrin were, respectively, 0.02, 2.2 and 0.005 ppm, which were 90% lethal concentrations (LC_{90}) for the larvae. The parathion solutions were then treated with 5 U OPH or OPH + B1 enzyme, and the pirimicarb and decamethrin solutions with 500 U OPH + B1 or B1 enzyme. Groups with only pesticide solution in the cups served as negative controls, and those with only enzymes served as positive control. All treatments were in triplicate and all experiments were repeated three times, with the cups being in a growth chamber at 28 °C and mortality being evaluated at 24 °C. Complete immobility or paralysis was taken as indicating for neurotoxic activity towards the insect larvae.

3. Results and discussion

3.1. Expression of OPH and carboxylesterase (CbEs) B1

For coexpression, two strategies can be used. In the first, target genes are cloned on two plasmids with two different antibiotic resistances, i.e. independent expression strategy (Johnston et al., 2000; Fribourg et al., 2001). Although the use of two plasmids with the same origin of replication was reported by Yang et al. (2001), most studies are performed with ColEl derivatives combined with compatible plasmids bearing a p15A replicon. In the second strategy, the target genes are cloned in tandem in a transcription unit on a unique plasmid, i.e. single operon strategy (Henricksen et al., 1994; Li et al., 1997). The former is a relatively simple process of DNA manipulation, but the copy numbers of two plasmids differ. The high copy number of the ColE1-based vectors would be likely to strongly bias expression in favour of the genes carried by them and create an imbalanced protein expression in cell. The latter strategy seems to be complicated for constructing the coexpression strain, but the relative amount of the protein produced is similar. Here the Duet vector was used to coexpress OPH and B1 simultaneously in *E. coli* BL-21 (DE3), and OPH and B1 were expressed alone as a control to study whether the two enzymes from different species interacted with each other in a single cell.

E. coli BL-21 (DE3) is lysogenic for bacteriophage λ DE3, which contains the T7 bacteriophage gene (encoding T7 RNA polymerase) under the control of lac UV5 promoter. Two target genes of *opd* and *b1* cloned downstream of T7 promoters of pETDuet plasmid. Coding regions of *opd* and *b1* are both controlled by T7 promoter in recombinant pETDuet-*opd-b1*. Addition of IPTG to the cell-growing culture will induce expression of both OPH and B1. The molecular weights of OPH and B1 are about 35 and 65 kDa, respectively, and the desired products
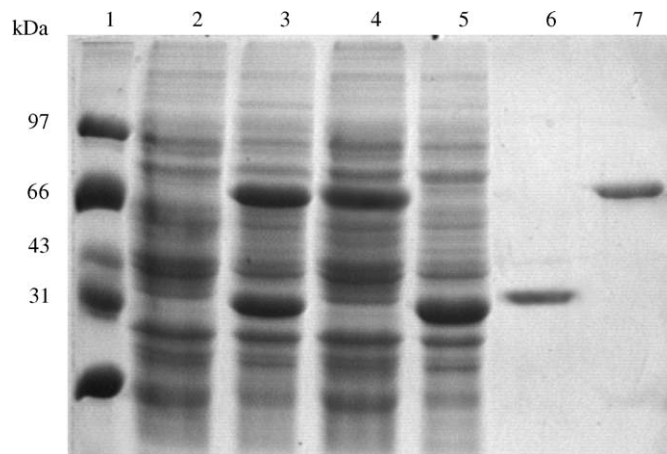

Fig. 2. SDS–PAGE analysis of recombinant proteins coexpressed in *E. coli* BL21(DE3). (Lane 1, protein molecular weight standards; lane 2, whole cell lysates of induced *E. coli* harbouring plasmid pETDuet; lane 3, expression of OPH and B1 in *E. coli* carrying pETDuet-opd-b1; lane 4, expression of B1 in *E. coli* carrying pETDuet-b1; lane 5, expression of OPH in *E. coli* carrying pETDuet-opd; lane 6, purified OPH; lane 7, purified B1. The molecular masses of standards are shown in the left margin.)

comprised about 32% and 35% of total cell protein by electrophoresis gel scanning (Fig. 2).

The His-Bind family of products offers a wide selection of supports designed for rapid one-step purification of proteins containing the His-Tag sequence by immobilized metal affinity chromatography (IMAC). The His-Tag sequence (6, 8 or 10 consecutive histidine residues) binds to divalent cations (Ni^{2+}) immobilized on NTA and IDA-based His-Bind resins. After the unbound proteins are washed away, the target protein is recovered by elution with either imidazole or slight decrease in pH. This versatile system enables proteins to be purified under gentle, non-denaturing conditions. If a complex contains the two proteins, they will both be obtained by affinity chromatography. The complexes obtained through coexpression are highly specific and coexpression can be used as a tool to study protein–protein interactions (Fribourg et al., 2001).

To find out whether the two proteins interact with each other, the crude extract of coexpression cells and proteins retained on Ni-IDA affinity resin were analysed using 12% SDS–PAGE after Coomassie blue staining. No B1 binding to OPH was observed through purification under non-denaturing conditions. No specific interaction between the two proteins was detected (Fig. 2). The proteins were assembled into separate inclusions and their structure formation was independent.

3.2. Enzymatic activity of recombinant OPH and B1

High-level recombinant proteins were coexpressed in *E. coli* BL-21. However, high-level expression of recombinant proteins in *E. coli* often results in the formation of

insoluble and inactive aggregates known as inclusion bodies. However, there was still a proportion of active protein in the cell. Time-course studies of B1 (Figs. 3 and 4) and OPH expression (Figs. 5 and 6) were also carried out at different temperatures, with the same amount of cells ($OD_{600} = 1$) being collected at 2-h intervals, and the relative enzyme activities determined. In recombinant cells, the active protein was produced earlier, but in lesser amount at high temperature than at low temperature. Overall, it was considered that 25 °C was optimum for active protein production.

In general, cells induced for 8 h at 25 °C were collected for assaying the enzymatic activity of OPH and B1. The V_{max} value for parathion was 0.01 µmol min^{-1} mg^{-1} of OPH protein in cells containing pETDuet-opd. V_{max} value for β-naphthyl acetate was 0.2 µmol/min/mg of protein in cells contain pETDuet-b1. In cells containing pETDuet-opd-b1, the V_{max} for parathion was 0.003 and for β-naphthyl acetate 0.2 µmol min^{-1} mg^{-1}.

To explore whether the differences between coexpression and expression alone would lead to a change in the activity of B1 or OPH in the coexpression cells, the activity of OPH in coexpression cells were compared with that expressed alone and at the same time, the same comparison was made for B1. To demonstrate the functional expression of B1, both recombinant cells were grown at 30 °C. After induction, CoCl$_2$ was added to give a final concentration of 1 mM in the coexpression cell culture. Whole cell activity

was measured as described above. As shown in Figs. 3 and 4, cells carrying pETDuet-b1 had similar activity against β-naphthyl acetate to cells bearing pETDuet-opd-b1. The temperature of incubation resulted in the active protein being produced at different times. The divalent cation Co^{2+} did not affect the activity of the enzyme of B1.

However, OPH assays revealed the decrease of activity in coexpression cells (Fig. 6). It was postulated that the low OPH activity in coexpression cells bearing pETDuet-opd-b1 was due to the competition for substrate between OPH and B1, but not a lower level of gene expression. In this study, the same amount of cell protein (harvested cell $OD_{600} = 1$) was used and the expression level was observed on SDS–PAGE gel (Fig. 2). This indicated a similar expression level in different expressing cells. To justify this hypothesis, purified OPH and B1 were used. The mixtures of OPH and B1 showed lower OPH activity than the same amount of OPH, indicating that B1 competed for the substrate with OPH and affected the degradation efficiency of parathion. This agrees with the detoxification of OPs by CbEs in insects. In several Culex spp. and peach-potato aphids, resistance to OPs is associated with increased esterase activity resulting from amplification of the corresponding structural gene (Mouches et al., 1987). The CbE is thought to combine almost irreversibly with OP insecticides (so-called sequestration) and prevent their access to the target site, acetylcholinesterase. In vivo, CbEs cannot degrade the Ops, just as OPH breaks the bond
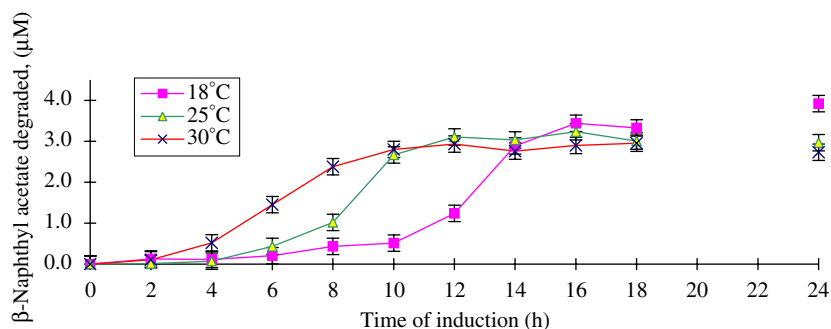

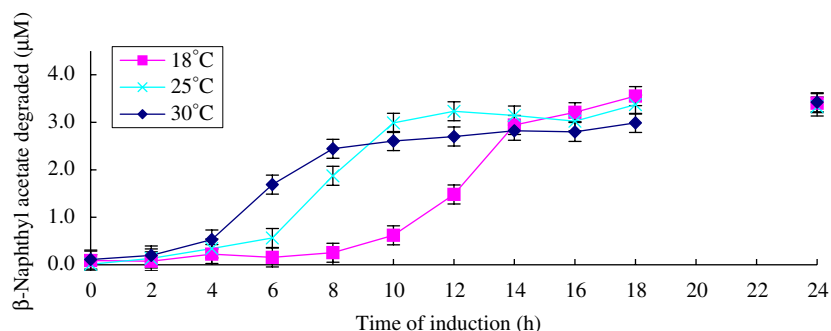Fig. 3. Induction of active protein B1 in pETDuet-b1.

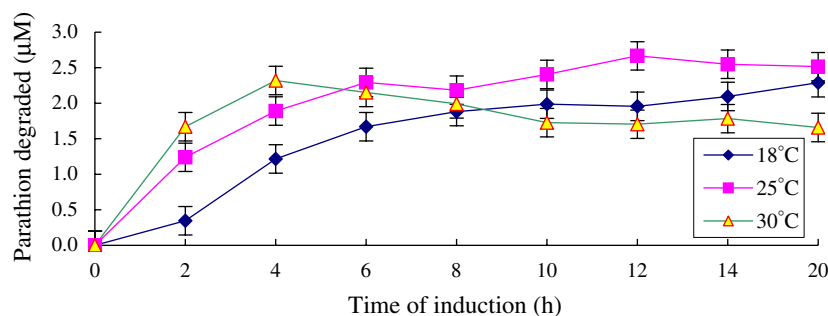Fig. 4. Induction of active protein B1 in pETDuet-opd-b1.

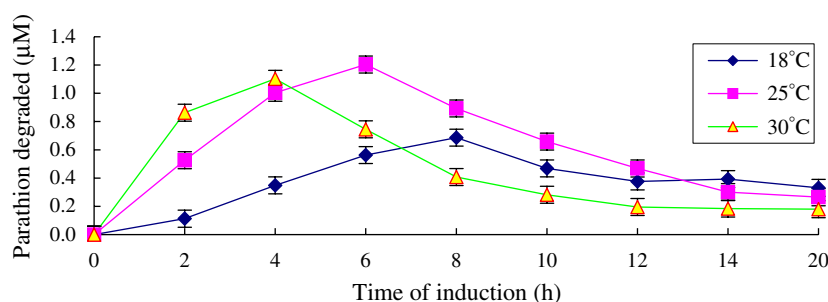Fig. 5. Induction of active protein OPH in pETDuet-opd.

Fig. 6. Induction of active protein OPH in pETDuet-opd-b1.

Table 1
Approximate survival rates of mosquito larvae exposed to pesticide solutions

Pesticide	Percentage survival of larvae						
	Control	Exposed to pesticide for 5 h			Exposed to pesticide for 10 h		
		OPH	OPH + B1	B1	OPH	OPH + B1	B1
Parathion	10	100	100	0	100	100	0
Pirimicarb	10	0	0	0	0	50	50
Deltamethrin	10	0	25	25	0	75	75
Water	100	100	100	100	100	100	100

between the phosphorus atom and the releasing group, otherwise each molecule of CbEs is able to "scavenge" a minimum of one molecule of OPs before they reach targets in the nervous system (Bisset et al., 1999).

3.3. Insect bioassay

The bioassay was performed three times in triplicate. The results shown in Table 1 are representative of one experiment. OPH was highly efficient in degrading parathion-allowing survival of larvae at low concentration (0.1 mg cell protein, dry weight). However, B1 showed low percentage degradation of pirimicarb and decamethrin (excess enzyme was tested against 100 ml pesticide solution). Elsewhere it has been shown that OPH can hydrolyze a broad variety of OP neurotoxins, including chemical warfare agents and many widely used pesticides (Di Sioudi et al., 1999; Mulchandani, 1999). However, in vitro there is

no evidence that B1 could degrade pesticide as efficiently as OPH. The present research suggests that OPH has good catalytic rate for neurotoxic OP compounds, but B1 has poor substrate affinity. In mosquitoes, CaE is the primary mechanism conferring resistance to OP insecticide as well as a secondary mechanism for carbamate resistance (Peiris and Hemingway, 1993). In vivo, esterase acts as a resistance mechanism by sequestering the insecticide, i.e. rapid binding and slow release of the insecticide metabolites. This type of resistance requires the presence of increased quantities of esterase, due to the 1:1 stoichiometry of the reaction and the slow metabolic rate. Such a mechanism is not easily detected by classical metabolic studies. The enzymatic reaction mechanism reveals that B1 is only weakly effective in degrading carbamate or pyrethroid. To harness the catalytic potential of CaE, the active site must be reshaped in order to optimize the mutant enzyme for a specific class of substrate targets. This

can be realized through rational modifications of the substrate-binding cavities and the catalytic centre. Modified B1 might potentially be used to biodegrade carbamate and pyrethroid.

Acknowledgements

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