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Detoxication of Pyrethroid Insecticides by Recombinant Carboxylesterase from Peach-Potato Aphid (*Myzus persicae*)

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Pyrethroids are among the most effective group of insecticides, which account for more than 30% of global use (Leahay 1985). Besides the manifold discussions about neurotoxic effects of pyrethroids on humans, the cytotoxic and genotoxic potential of pyrethroids should also be taken into consideration (Wolfgang et al. 1999). Current methods for their disposal, including chemical treatment, incineration, and landfills, are problematic due to the secondary risk of exposure and, in some cases, economically restrictive (Chen and Mulchandani 1998). Therefore, economically feasible methods for detoxification of pyrethroids accumulated in food products and water, which is now a growing public concern, need to be developed.

Generally, microorganisms seem to be the most efficient bioremediation agents (Picord et al. 1992; Tsai and Olson 1992). Numerous novel approaches for bioremediation are possible and should be investigated with the development of biotechnology. The search for appropriate enzymes for pesticides degradation extends to pesticide-resistant insects. Some of these insects become resistant to a wide range of pesticides by the increased synthesis of carboxylesterases (CarBs) that sequester or hydrolyze pesticidal esters (Devonshire 1977). CarBs are a class of lipolytic enzymes that can hydrolyze water-soluble, ester-containing chemical molecules. Since the chemical structures of pyrethroids correspond to carboxylesters, CarBs could potentially hydrolyze these compounds.

In peach-potato aphid, carboxylesterase E4 (CarB E4) may result in resistance to organophosphorus, carbamate and pyrethroid insecticides. Purified CarB E4 from insect homogenate could hydrolyze organophosphorus and carbamate esters, but the turnover of these substrates was very slow (Devonshire and Moores 1982). However, there is no report on the heterologously-expressed CarB E4 for degradation of pyrethroid insecticides. The present study describes expressing the CarB E4 gene from *Myzus persicae* to evaluate the potential of a genetically-engineered enzyme to detoxify aqueous pyrethroids, specifically, tetramethrin, bifenthrin, permethrin, cypermethrin, cyfluthrin, deltamethrin and fenvalerate (Figure 1).

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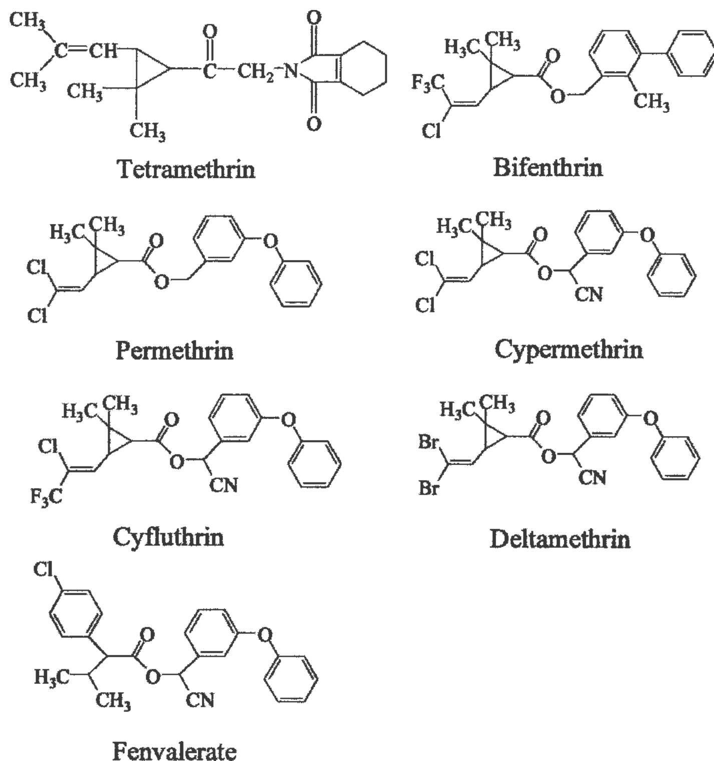

Figure 1. Chemical structures of tetramethrin, bifenthrin, permethrin, cypermethrin, cyfluthrin, deltamethrin and fenvalerate.

MATERIALS AND METHODS

Analytical-grade tetramethrin (96% purity), bifenthrin (95% purity), permethrin (95.4% purity), cypermethrin (97% purity), cyfluthrin (93% purity), deltamethrin (98% purity), and fenvalerate (97.0% purity) were obtained from the Institute for the Control of Agrochemicals, Ministry of Agriculture, P. R. China.

The CarB E4 gene was cloned into plasmid pET-28 (Lan et al. 2005). The transformed *E. coli* BL21 with plasmid pET-28E4 was stored at -70 °C. Inoculums were prepared by transferring the cells into the culture tube containing 2 mL Luria-Bertani (LB) medium (tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; and kanamycin, 50 mg; per liter distilled water, pH 7.0). After incubating at 37 °C for 18 hrs, cultures were inoculated into 100 mL of the same medium in an Erlenmeyer flask, and then were shaken at 200 rpm and 37 °C until the OD_{600} was approximately 0.6. Then isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to give 1 m*M* and the cultures were incubated at 30 °C for 12 hrs to induce the target protein. Cells were harvested by centrifuging the cultures at 6,000 × *g*

for 10 min. 15 mL of 50 m*M* Tris/HCl buffer (pH 8.0) was added and well mixed, and then 30 μL of 0.5 mg/mL lysozyme and 1.5 mL of 1% Triton X-100 were added to the suspension. After incubation at 30 °C for 15 min, cells were disrupted by sonication in an ice bath. After centrifuging at 12,000 × *g* for 15 min, the supernatant was collected. Crude enzyme was obtained by mixing the resulting supernatants from 10 batches of cell cultures, containing 6mg/mL of total protein, measured using the method described by Bradford (1976).

Assay of optimal temperature and pH of CarB E4 activity was carried out using β-naphthyl acetate (β-NA) as the substrate at different temperatures and pH. Relative enzyme activity was assayed according to the method described by Asperen (1962). For each assay, 6 mL phosphate buffer containing 5×10^{-4} *M* β-NA and 3 mL crude enzyme was incubated in a flask. Each reaction was repeated three times, and 3 mL buffer alone was used for the control. After reacting for 5 min, 3 mL of aliquot sample was taken from each flask and mixed with 0.5 mL diazo blue/lauryl sulphate reagent, and variation in absorbance was immediately estimated at 550 nm with a Beckman spectrophotometer.

Degradation of the pyrethroids was carried out by incubating them with or without expressed enzymes respectively, and estimated according to the method described elsewhere (Leng and Qiao 1986). For each assay, the pyrethroid was dissolved in 15 mL of 10 m*M* potassium-phosphate buffer (pH 7.5) and then 5 mL enzyme (55.5 U mL^{-1}) (or buffer without enzyme) was added. The mixture was incubated at 37 °C. At regular intervals, 1 mL of sample was taken from the flask and mixed with 1 mL petroleum ether, and then was dried with anhydrous sodium sulfate. The product was extracted with redistilled hexane and analyzed by gas chromatography (GC). GC analysis was performed on a Hewlett-Packard 5890 series II GC equipped with an HP 7673 autosampler and electron-capture detector (ECD), using N_2 as the carrier gas at 1 mL/min. A fused silica capillary column (0.53 mm id × 30 m × 0.5 μm film thickness, Supelco Corp. USA) was used in this assay. Injector, column and detector temperatures were set at 300°C, 220°C and 300°C, respectively. 1 μL sample was splitless injected each time. Concentration of each pyrethroid was determined by comparing the peak area to a standard curve. The recoveries and detection limits of these pyrethroids were shown in Table 1.

RESULTS AND DISCUSSION

All of the enzymes used in this study from different batches were obtained directly from the transformed *E. coli* BL21 with plasmid pET-28E4 and were not purified prior to use. They exhibited good batch-to-batch reproducibility on the enzyme activities of degradation of β-NA and pyrethroids (data not shown). Therefore, 10 batches of the crude enzymes were mixed and used for subsequent studies.

Table 1. Recoveries and detection limits of pyrethroids of tetramethrin, bifenthrin, permethrin, cypermethrin, cyfluthrin, deltamethrin and fenvalerate.

Pyrethroid	Recovery (%)	Detection limit (μL^{-1})
Tetramethrin	85.9-89.3	
Bifenthrin	87.4-91.5	
Permethrin	92.3-97.1	
Cypermethrin	92.7-96.6	0.01 ng
Cyfluthrin	90.4-93.8	
Deltamethrin	93.8-97.2	
Fenvalerate	90.8-94.1	

The reacting temperature affected the activity of the crude enzyme at the same pH (in this case was 7.0). The activity increased with temperature, reached its maximum at 37 °C and then decreased (Figure 2). As shown in Figure 3, the operating pH was also an important factor for enzyme activity at the optimal temperature of 37 °C, and at pH 7.0 the enzyme showed the maximum activity. Therefore, the CarB E4 activity was maximal for subsequent studies at a temperature of 37 °C and pH of 7.0.

Figures 4, 5 and 6 show the degradation of tetramethrin, bifenthrin and permethrin at different time intervals. The recombinant CarB E4 hydrolyzed 66% of tetramethrin within 1.5 hrs, 69% of bifenthrin and 71% of permethrin within 3 hrs. The degradation rate of tetramethrin by CarB E4 was about 2 times that of either bifenthrin or permethrin. However, hydrolase activity of CarB E4 was undetectable for cypermethrin, cyfluthrin, deltamethrin and fenvalerate.

Substrate specificity was measured with tetramethrin, bifenthrin and permethrin at different concentrations. As the activity apparently obeyed Michaelis-Menten kinetics for all active substrates, the Michaelis constant (*Km*) and the maximal velocity (*Vmax*) values were calculated for each substrate (Table 2). These data showed that the best substrate of the three pyrethroids was tetramethrin, with *Vmax* value of 25 μmol mg^{-1} min^{-1} and *Km* value of 1.68 μM. Figure 7 showed the degradation of the mixture of 10 μM tetramethrin, bifenthrin and permethrin by CarB E4. It indicated that the degradation rate of tetramethrin was much faster than those of bifenthrin and permethrin.

In our study, 7 pyrethroid insecticides, that all have carboxyl ester moieties in the chemical structures, were tested as substrates of CarB E4. However, only tetramethrin, bifenthrin and permethrin could be obviously hydrolyzed by CarB E4. It suggests that the hydrolase activity of CarB E4 has specificity. Interestingly enough, all 4 pyrethroid pesticides that could not be hydrolyzed by CarB E4 have the same functional group: a cyano moiety near the carboxyl ester moiety, where the other three have no such molecular structure. This suggests that the cyano group may influence the enzyme activity of CarB E4.

Table 2. *Vmax* and *Km* values of CarB E4 to tetramethrin, bifenthrin and permethrin.

Pyrethroid	*Vmax* (μmol mg^{-1} min^{-1})	*Km* (μ*M*)
Tetramethrin	25.03	1.68
Bifenthrin	18.83	1.47
Permethrin	17.06	1.40

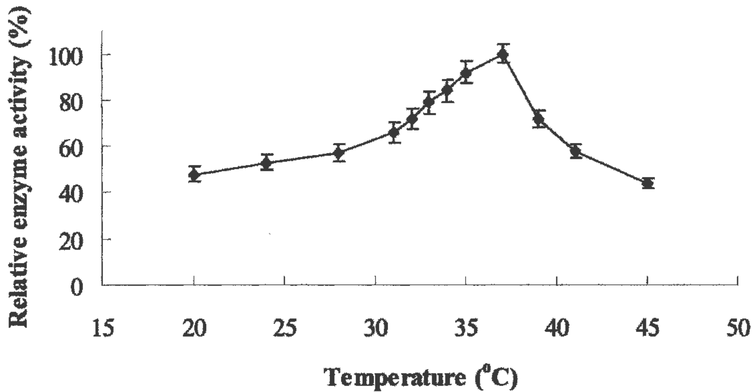

Figure 2. Effect of temperature on the degradation of β-NA.

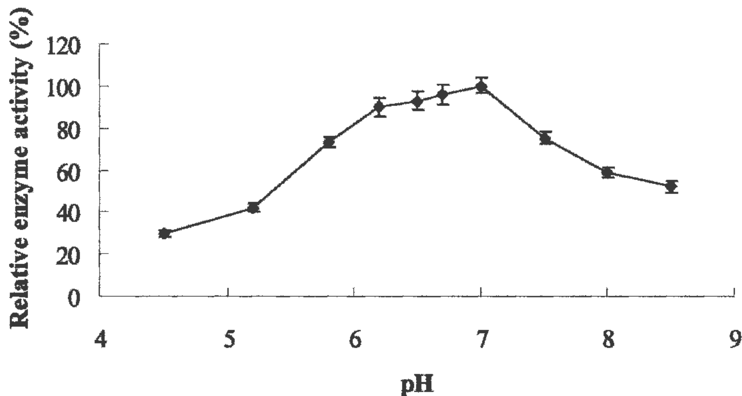

Figure 3. Optimum of pH on β-NA with crude enzyme.

The hydrolysis mechanism of carboxyl esters by carboxylesterase is demonstrated in Figure 8. The substrate binds to a specific recognition site in the carboxylesterase. The size and shape of this binding site are complementary to those of the substrate. Binding is assisted by the negatively-charged carboxylate group of a glutamate residue in the enzyme. The next step is the transfer of the carboxylic acid moiety to the alcohol group of a serine residue in the enzyme.

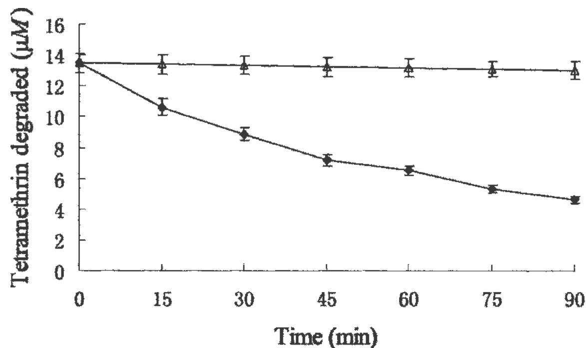

Figure 4. Degradation of tetramethrin by CarB E4 in aqueous medium with (♦) or without (△) enzyme.

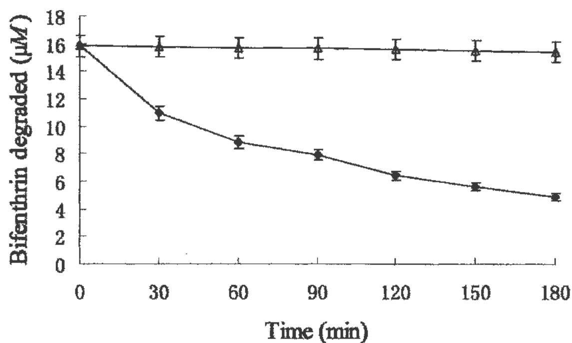

Figure 5. Degradation of bifenthrin by CarB E4 in aqueous medium with (♦) or without (△) enzyme.

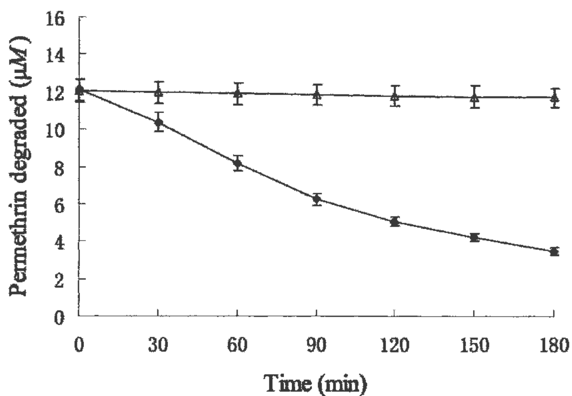

Figure 6. Degradation of permethrin by CarB E4 in aqueous medium with (♦) or without (△) enzyme.

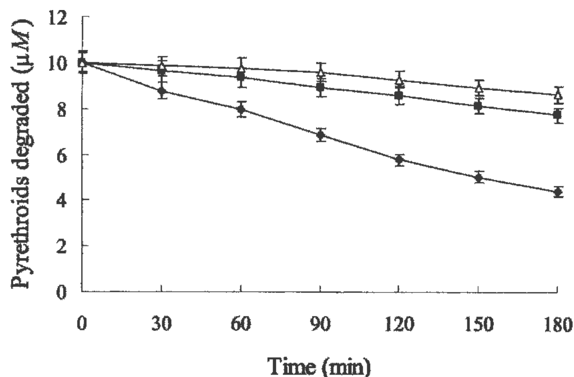

Figure 7. Degradation of tetramethrin (♦), bifenthrin (■) and permethrin(△) mixtures by CarB E4 in aqueous medium.

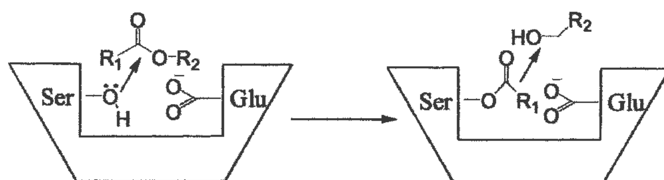

Figure 8. Theoretical substrate degradation by carboxylesterase.

This transacetylation (a form of transesterification) affects the hydrolysis of the substrate and the release of the corresponding alcohol moiety, involving nucleophilic attack of a serine on a carbonyl carbon of the ester. A catalytic triad enabling a charge transfer through histidine from glutamate or aspartate enhances the reactivity of the serine. The glutamate and serine residues are oriented in three dimensions to geometrically favour this transesterification when the substrate is bound in the active site.

Pyrethroids containing a cyano group may exhibit lower reactivity and binding ability to the specific recognition site in the CarB E4 than the pyrethroid without a cyano group. The cyano group of pyrethroids repulse the binding assisted by the negatively-charged carboxylate group of a glutamate residue in the enzyme. Therefore, the pyrethroid containing a cyano group is difficult to bind to the specific recognition site in the carboxylesterase.

To our knowledge, enzyme preparations from insects for degrading pyrethroids have not been reported to date, although some bacterial isolates that degrade pyrethroids have been investigated previously (Lee et al. 2004). Our results show that the recombinant CarB E4 has been expressed in *E. coli* successfully that can degrade tetramethrin, bifenthrin and permethrin. This result makes it a promising

candidate for the decontamination of wastes containing pyrethroid insecticides.

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