Biotechnology Letters (2005) 27: 1141–1146
DOI 10.1007/s10529-005-8464-x

Expression and characterization of carboxylesterase E4 gene from peach–potato aphid (*Myzus persicae*) for degradation of Carbaryl and Malathion

Wen-sheng Lan[1,2], Jian Cong[3], Hong Jiang[1], Shu-ren Jiang[3] & Chuan-ling Qiao[1,*]
[1]*State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology Chinese Academy of Sciences, 100080, Beijing, P.R. China*
[2]*Graduate School of the Chinese Academy of Sciences, 100039, Beijing, P.R. China*
[3]*College of Science, China Agricultural University, 100094, Beijing, P.R. China*
**Author for correspondence (E-mail: Qiaocl@ioz.ac.cn)*

Received 6 January 2005; Revisions requested 26 January 2005 and 23 March 2005; Revisions received 15 March 2005 and 26 May 2005; Accepted 30 May 2005

Key words: carboxylesterase, degradation, pesticide

Abstract

An abridged carboxylesterase E4 (CbE E4) gene was cloned from the peach–potato aphid, *Myzus persicae*, by reverse transcription-PCR and subcloned into the expression vector pET28b. The abridged CbE E4 gene was successfully expressed in *E. coli* BL21 (DE3). The recombinant CbE E4 hydrolyzed β-naphthyl acetate and Carbaryl by 64% within 2.5 h, Malathion by 80% within 1.25 h. However, the hydrolysis of other pesticides (Dichlorovos, Parathion, Pirimicarb and Deltamethrin) was not detected.

Introduction

Pesticide residues can cause adverse effects on human health and environmental pollution (Blain 1990, Brown *et al.* 1996). Thus there is a need to detoxify the large quantities of pesticide residues generated in the general process of pesticide usage. Since, most carbamates and pyrethroids are esters (see Figure 1), they can potentially be hydrolyzed by carboxylesterases (CbEs) (Sogorb & Vilanova 2002). CbEs (EC 3.1.1.1) are widely distributed from microorganisms to plants and animals. The search for appropriate enzymes for pesticide degradation has extended to pesticide-resistant insects and their means of surviving pesticide applications. Some insects become resistant to a wide range of pesticides by an increased synthesis of CbEs (Devonshire 1977, Kao *et al.* 1984). Because these resistant insects can detoxify many kinds of pesticides, the enzymes encoded by resistant genes of the insects may be useful to degrade pesticide pollutants in the environment.

In the peach–potato aphid, CbE E4 activity is a major mechanism responsible for its resistance to organophosphate, carbamate and pyrethroid pesticides. CbE E4 purified from insect homogenates can slowly hydrolyze organophosphates and carbamate esters (Devonshire & Moores 1982). However, there is no report on the heterologous-expressed CbE E4 for degradation of pesticides *in vitro*. In the present study, we cloned and expressed the CbE E4 gene from *Myzus persicae* to obtain insight into the characteristics and pesticide degradation potential of the recombinant CbE E4.

Materials and methods

Experimental animals, bacterial strains, plasmids and reagents

An organophosphate-resistant colony of the peach–potato aphid (*Myzus persicae*) was from

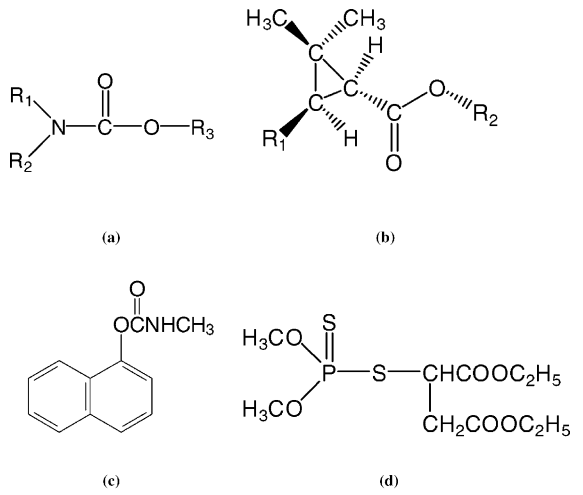

Fig. 1. Chemical structure of pesticides: the carbamate (a) and pyrethroid (b) families, Carbaryl (c) and Malathion (d). In the structures R_1, R_2 and R_3 are usually organic radicals, but R_1 or R_2 may also be hydrogen.

Yan-chuan Tian, Institute of Microbiology, Chinese Academy of Sciences, P. R. China. *M. persicae* aphids were maintained on tobacco (*Nicotiana tabacum* L.) plants at 22 °C with a photoperiod of 16 h per day. This colony has been maintained in our laboratory and further treated with organophosphorus pesticides.

E.coli strain BL21 (DE3) was used as host strain for expression of the recombinant proteins. Plasmid pET28b, purchased from Novagen, was used for expressing the CbE E4 genes. The pGEM-T Easy (TA cloning kit of Invitrogen Corp., USA) is a TA cloning vector with 3′-T overhangs. Plasmids were maintained and propagated in *E. coli* DH5α. TRI*zol* were from GibcoBRL and His-Bind Purification Kits from Novagen. Other reagents including T4 DNA ligase, alkaline phosphatase, ExTaq DNA polymerase, IPTG, ampicillin, kanamycin and restriction enzymes *Bam*HI and *Hin*dIII were products from TaKaRa Biotechnology Co., Ltd. (TaKaRa, Dalian).

Analytical-grade Malathion (99% purity), Carbaryl (99.5% purity) and other pesticides (Dichlorovos, 99.5% purity; Parathion, 93% purity; Pirimicarb, 99.8% purity; Deltamethrin, 99.5% purity) were obtained from Institute for the Control of Agrochemicals, Ministry of Agriculture, P. R. China.

DNA manipulation

The total RNA was extracted from an organophosphate-resistant peach–potato aphid using TRI*zol* reagents. The reverse transcription of poly(A)$^+$ RNA was performed on 1 μg heat-denatured poly(A)$^+$ RNA at 37 °C for 1 h in a reaction including 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 2 μM of oligo dT_{18} and 200 units of M-MLV reverse transcriptase according to the manufacturer's manual. The single strand cDNA obtained from RNA was used as a PCR template. The amplification program used 95 °C for 3 min followed by 33 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 58 °C for 1.5 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. The primers were as follows: P1-a: 5′-GGA TCC AAT GAA AAA TAC GTG TGG AAT-3′. P1-b: 5′-GGA TCC GAT GTC TAA TAC GCC TAA GGT G-3′. P2: 5′-AAG CTT CAT TTA ATG GTA AGC TAC TCC-3′. P1-a and P1-b are the forward primers that contain a *Bam*HI restriction site before the start codon. P2 is a reverse primer that contains a *Hin*dIII restriction site after the stop codon (underlined bases). The primers were designed according to the CbE E4 gene sequence, obtained from GenBank data base (GenBank accession number X74554). The intact CbE E4 gene was amplified using P1-a and P2, which contained the whole open reading frame (ORF) of the gene, and the abridged CbE E4 gene (the 69 forepart nucleotides of signal peptide sequence was abridged) was also amplified using P1-b and P2. PCR products were ligated with pGEM-T Easy vector. Both the intact and the abridged CbE E4 genes were sequenced by TaKaRa Biotechnology Co., Ltd. (TaKaRa, Dalian). The two genes were respectively subcloned into pET28b, resulting in the recombinant plasmids of pET28b-intact E4 and pET28b-abridged E4.

Expression of the CbE E4 gene

Expression studies were carried out using *E. coli* BL21 (DE3). The transformed cells with plasmids of pET28b-intact E4 and pET28b-abridged E4 were established. Inocula were prepared by

transferring a transformant into a 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). Cultures were initially grown overnight at 37 °C, then inoculated into 100 ml LB medium, which were shaken at 200 rpm until the OD_{600} was approx. 0.6. IPTG was added to give 1 mM and the cultures were incubated at 30 °C for a further 12 h to induce the protein. Cells were harvested by centrifugation at $6000 \times g$ for 10 min, washed with 50 mM Tris/HCl buffer (pH 8.0), then dried at 30 °C to a constant weight. Harvested cells were disrupted by sonication in an ice bath, centrifuged at $12\ 000 \times g$ for 15 min, and the supernatant was used as source of crude enzyme. The expression of CbE E4 gene was assessed by SDS-PAGE (10% polyacrylamide) followed by Coomassie Blue staining.

Purification of the recombinant CbE E4

The recombinant CbE E4 contains a sequence of histidine residue (His-Tag sequence) at the *N*-terminus, which can be purified by Ni–NTA affinity column chromatography using His-Binding Purification Kits (Novagen). The above procedure was applied to 2 l cultures for production of target proteins. The crude enzymes obtained from the induced cells were applied onto the nickel-nitriloacetic (Ni–NTA) agarose column, which was pre-equilibriumed with 10 mM phosphate buffer containing 10 mM imidazole (pH 8.0). After washing the column with 20 mM imidazole in 10 mM phosphate buffer (pH 8.0), the His-tagged CbE E4 was eluted by 250 mM imidazole in 10 mM phosphate buffer (pH 8.0). The eluted fraction were pooled and dialyzed to 20 mM phosphate buffer saline (PBS), pH 7.0. The elution of the enzyme from Ni–NTA column was determined by Coomassie Blue staining of SDS-PAGE.

Assay of enzymatic activity

The protein concentration of purified product was determined by Bradford method with bovine serum albumin (BSA) as standard. The activity of CbE E4 was determined by the method of Van Asperen (1962) with some modifications. 2.5 ml reaction mixture consisted of 20 μl enzyme sample and 0.3 mM β-naphthyl acetate in 0.5% acetone, in 20 mM sodium phosphate buffer (pH 7.5). After 10 min at 37 °C, a freshly prepared 0.4 ml Diazoblue SDS reagent (0.3% in Fast Blue B salt in 3.5% aqueous SDS) was added. The reaction continued for 10 min, thereafter, solution was centrifuged at about 1000 *g* and the absorbancy of β-naphthol formation was measured at 555 nm. The enzymatic activity was calculated from a β-naphthol standard curve. One unit (U) enzyme activity was defined as the amount of enzyme that produced 1 μmol β-naphthol from the substrate per min at 37 °C and pH 7.0. Enzyme was expressed as either specific activity (U mg^{-1} protein) or volumetric activity (U ml^{-1} medium). The effect of pH on CbE E4 activity was measured from pH 4 to 9 in either 20 mM sodium acetate (pH 4–6), KH_2PO_4/KOH (pH 6–7) or Tris/HCl (pH 7–9) buffers. The optimum temperature for the enzyme activity was determined in 20 mM KH_2PO_4/KOH buffer (pH 7.0) from 30 to 70 °C. An initial hydroxylation velocity of CbE E4 was determined in 20 mM KH_2PO_4/KOH buffer (pH 7.0) at 30 °C over the substrate concentration range from 0.08 to 5 mM β-naphthyl acetate. *Km* values of CbE E4 were determined by Lineweaver-Burk plots.

Pesticide degradation tests and analytical methods

Measurement of the Carbaryl degradation was performed spectrophotometrically by monitoring the concentration of α-naphthol which was produced during the hydrolysis of Carbaryl (Devonshire 1977, Hayatsu & Nagata 1993). Experiments were performed as follows. A reaction mixture contained, in a total volume of 20 ml, 10 mM KH_2PO_4/KOH buffer (pH 7.5), 130 μM Carbaryl, and 5 ml enzyme preparation (107 U ml^{-1}), and was incubated at 37 °C. Carbaryl solutions without CbE E4 served as controls. Samples (2 ml) were taken at different intervals. Then 0.5 ml Diazo Blue coupling reagent (DBLS), which was freshly prepared by dissolving 45 mg Fast Blue B salt in 4.5 ml water and adding 10.5 ml 5% (v/v) SDS to the samples. After the Fast Blue B salt had reacted with α-naphthol, which resulted from the hydrolysis of the Carbaryl, the A_{590} was measured.

Carbaryl degradation was calculated by formation of α-naphthol.

The degradation of other pesticides (Dichlorovos, Parathion, Pirimicarb, Malathion Deltamethrin) by the CbE E4 was determined according to the method described by Leng & Qiao (1986). Pesticides were dissolved in 15 ml 10 mM KH_2PO_4/KOH (pH 7.5) and then 5 ml enzyme (107 U ml^{-1}) was added. A total reaction volume was 20 ml. Pesticide solutions without enzyme served as controls. The mixtures were incubated at 37 °C and 1 ml samples were taken from the flask at different intervals, to which 1 ml petroleum ether was added. The samples were then dried with anhydrous Na_2SO_4 and the products extracted with redistilled hexane and analyzed by gas chromatograph (GC). GC analysis was performed using electron-capture (ECD) and nitrogen-phosphorus detectors (NPD) with N_2 as the carrier gas at 1 ml min^{-1}. Splitless injections of 1 μl (purge off time: 1 min) were performed on a fused silica capillary column (0.53 mm i.d. × 30 m × 0.5 μm film thickness, Supelco Corp. USA). Dichlorovos, Parathion, Pirimicarb and Malathion were detected using NPD. Injector, column and detector temperatures were set at 300 °C, 220 °C and 300 °C, respectively. Deltamethrin was analyzed by ECD on the same column. Injector, column and detector temperatures were set at 300 °C, 270 °C and 300 °C, respectively. Pesticide concentrations were determined by comparing their peak areas with that of standard curves.

Results and Discussion

Cloning the CbE E4 genes from peach–potato aphid

The CbE E4 genes with and without signal peptide sequence (the intact CbE E4 gene or the abridged CbE E4 gene) were respectively cloned from peach–potato aphid by RT-PCR. Sequencing results show that the ORF of intact CbE E4 gene contains 1656 base pair (bp) nucleotides encoding 552 amino acids. The abridged CbE E4 gene is shorter than the intact CbE E4 gene, which lacks of signal peptide sequence of 69 bp nucleotides, and encodes 529 amino acids.

Expression and purification of the recombinant CbE E4

The recombinant plasmids of pET28b-intact E4 and pET28b-abridged E4 were each transformed into *E. coli* BL21 (DE3). Although the product of the intact CbE E4 gene was not detected, the abridged CbE E4 gene was expressed successfully after induction (Figure 2). We supposed that the signal peptide sequence in the intact CbE E4 gene affected its expression efficiency. From SDS-PAGE analysis, the molecular weight of the expressed product was about 60 kDa, which matched the theoretical molecular weight calculated from its sequence. The final biomass was 8.2 g dry cells l^{-1}, and the amount of the CbE E4 present in total cell lysates, estimated by densitometric scanning of SDS-PAGE, was 28% of total cellar protein. The recombinant CbE E4, being expressed as a His-tagged fusion protein, was purified using Ni-NTA agarose affinity chromatography and yielded 4.5 mg protein/ml^{-1}. Theoretically, 2.3 g CbE E4 could be obtained from 1 l culture, however, only 170 mg CbE E4 was purified totally with a recovery of 7% (Figure 2).

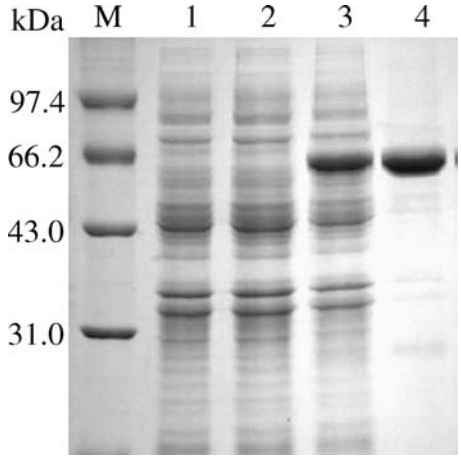

Fig. 2. SDS-PAGE analyses of the CbE E4 production in *E. coli* BL21 (DE3) after IPTG induction. Total proteins produced by the cells harboring different plasmids were separated by SDS-PAGE (10% polyacrylamide) to detect the production of the CbE E4. The samples loaded were: Lane M, protein marker (in kDa); lane 1, BL21 (DE3)/pET28b; lane 2, BL21 (DE3)/pET28b-intact E4; lane 3, BL21 (DE3)/pET28b-abridged E4; lane 4, purified recombinant CbE E4.

Enzymatic activity and characterization of the recombinant CbE E4

The purified CbE E4 had specific activities of 24 U mg^{-1} protein or 108 U ml^{-1} medium. The Michaelis constant (*Km*) was 2.4 mM β-naphthyl acetate, and the maximal initial rate (*V*max) was 280 μmol min^{-1} mg^{-1}. The optimal temperature and pH were 37 °C and 7.0, respectively. The CbE E4 was stable at 37 °C but rapidly inactivated above 50 °C, with a half-life of about 0.5 h at 50 °C. CbE E4 was stable in the neutral pH range, exhibiting almost 100% of its total activity between pH 7.0 and 7.5 (data not shown).

Degradation of pesticides by the recombinant CbE E4

The degradation of Carbaryl is shown in Figure 3. The recombinant CbE E4 hydrolyzed 64% Carbaryl (yielding α-naphthol) within 2.5 h. Pirimicarb and Deltamethrin were not, however, degraded. Thus not all pesticides containing carboxyl esters can be degraded by the CbE E4.

Although a number of bacteria with Carbaryl hydrolase activity have been investigated (Hayatsu *et al.* 1999, Karpouzas *et al.* 2000), enzymes for degrading Carbaryl have not been reported in insects. This is the first report to our knowledge on the production, purification and properties of a Carbaryl hydrolase from insects.

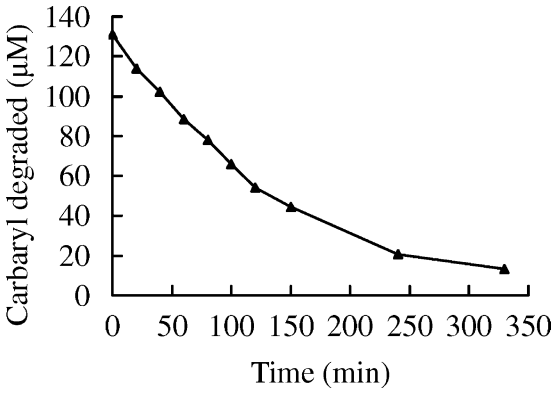

Fig. 3. Degradation of Carbaryl by CbE E4. The reaction was performed in a 100 ml flask containing 5 ml enzyme, 130 μM Carbaryl, KH_2PO_4/KOH buffer (10 mM, pH 7.5), at 37 °C. The total reaction volume was 20 ml.

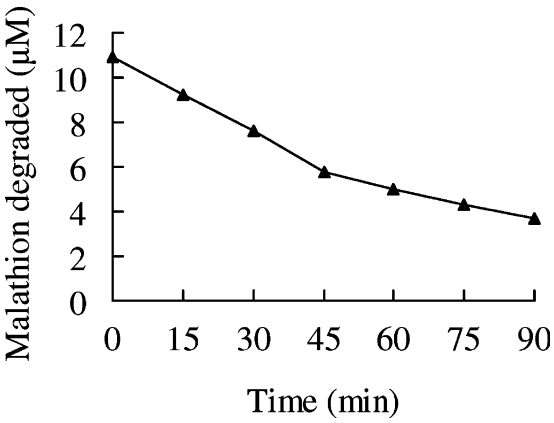

Fig. 4. Degradation of Malathion by CbE E4. The reaction was performed as given in Fig. 3 using 10.9 μM Malathion.

CbE E4 also hydrolyzed 80% Malathion within 1.25 h (Figure 4). Malathion is probably detoxified through the hydrolysis of one or both of its two ethyl carboxylester groups (see Figure 1) particularly in view of non-esterified organophosphorus pesticides Dichlorovos and Parathion, not being attached by recombinant CbE E4. The carboxylesterase that hydrolyzes Malathion is widely distributed in mammals but only sporadically in insects, where in some cases it is responsible for insecticide resistance (Haubruge *et al.* 2002).

In conclusion, from pesticide-resistant peach–potato aphid, we successfully isolated the CbE E4 gene and obtained its recombinant product with catalytic activities for Carbaryl and Malathion. Our work showed that the CbE E4 could be considered as an attractive candidate for the development of a biocatalyst in future practical use. The elucidation of the detailed pesticide degradation mechanisms of the enzyme is under progress.

Acknowledgements

This research was supported by the National High Technology Research and Development Program of China (863 Program), Resource and Environment Technology Field (No. 2002AA601160).

References

Blain PG (1990) Aspects of pesticide toxicology. *Adverse Drug React. Acute Poisoning Rev.* **9**: 37–68.

Brown P, Charlton A, Cuthbert M, Barnett L, Ross L, Green M, Gillies L, Shaw K, Fletcher M (1996) Identification of pesticide poisoning in wildlife. *J. Chromatogr. A.* **754**: 463–478.

Devonshire AL (1977) The properties of a carboxylesterase from the peach–potato aphid, *Myzus Persicae* (*Sulz.*), and its role in conferring insecticide resistance. *Biochem. J.* **167**: 675–683.

Devonshire AL, Moores GD (1982) A carboxylesterase with broad substrate specificity causes organophosphorus, carbamate and pyrethroid resistance in peach–potato aphids (Myzus Persicae). *Pestic Biochem. Physiol.* **18**: 235–246.

Haubruge E, Amichot M, Cuany A, Berge JB, Arnaud L (2002) Purification and characterization of a carboxylesterase involved in malathion-specific resistance from *Tribolium castaneum* (Coleoptera: Tenebrionidae). *Insect Biochem. Mol. Biol.* **32**: 1181–1190.

Hayatsu M, Hirano M, Nagata T (1999) Involvement of two plasmids in the degradation Carbaryl by *Arthrobacter* sp. strain RC 100. *Appl. Environ. Microbiol.* **65**: 1015–1019.

Hayatsu M, Nagata T (1993) Purification and characterization of Carbaryl hydrolase from *Blastobacter* sp. M501. *Appl. Environ. Microbiol.* **59**: 2121–2125.

Kao LR, Motoyama N, Dauterman WC (1984) Studies on hydrolases in various house fly strains and their role in Malathion resistance. *Pestic Biochem. Physiol.* **22**: 86.

Karpouzas DG, Morgan JA, Walker A (2000) Isolation and characterization of 23 carbofuran-degrading bacteria from soils from distant geographical areas. *Lett. Appl. Microbiol.* **31**: 353–358.

Leng XF, Qiao CL (1986) The biodegradation of the Decis, Acetofenate (7504) and DDT by the perfusion isolated rat cells (in Chinese). *Sci. Bull.* **19**: 1505–1508.

Sogorb MA, Vilanova E (2002) Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. *Toxicol. Lett.* **128**: 215–228.

Van Asperen K (1962) A study of housefly esterases by means of a sensitive colorimetric method. *J. Insect Physiol.* **8**: 401–416.