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Two single mutations commonly cause qualitative change of nonspecific carboxylesterases in insects

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ABSTRACT

Carboxylesterases provide key mechanisms of resistance to insecticides, particularly organophosphates (OPs), in insects. One resistance mechanism is a qualitative change in the properties of a carboxylesterase. Two mutant forms, G151D and W271L, have been observed, mostly in dipteran species, to affect substrate specificity of enzymes. But whether these two single mutations can commonly change character of insect carboxylesterases is unknown. In our study carboxylesterase genes from seven insects distributed among four orders were cloned, mutated at position 151 or 271 and expressed in *Escherichia coli*. The kinetics of the purified recombinant proteins was examined towards an artificial carboxylester and two OP insecticides. The G/A151D and W271L mutation significantly reduced carboxylesterase activity in 87.5% and 100% cases, respectively, and at the same time conferred OP hydrolase activities in 62.5% and 87.5% cases, respectively. Thus, the change at position 271 is more effective to influence substrate specificity than that at position 151. These results may suggest that these two mutations have the potential to cause insecticide resistance broadly in insects.

1. Introduction

Carboxylesterases (EC 3.1.1.1), or carboxyl/cholinesterases, are ubiquitous among animals, plants and bacteria (Oakeshott et al., 1999; Marshall et al., 2003), and have a variety of physiological functions, such as degradation of neurotransmitters, metabolism of specific hormones and pheromones, detoxification, defense and behavior (Vogt et al., 1985; Taylor and Radic, 1994). Mammalian carboxylesterases play a critical role in the hydrolytic biotransformation of a vast number of structurally diverse drugs (Satoh and Hosokawa, 1998). In insects, carboxylesterases are key components of defense against xenobiotic compounds, including insecticides (Oakeshott et al., 2005). Phylogenetic analyses suggest that the majority of insect carboxylesterase lineages have evolved since insects diverged from the lines leading to nematodes and vertebrates (Oakeshott et al., 1999, 2005). Genomes of an individual insect species may contain different members of the carboxylesterase gene family. For example, 35, 51, 54, 24, 41, 49 and 76 carboxylesterase-encoding genes have been identified in the genomes of *Drosophila melanogaster*, *Anopheles gambiae*, *Aedes aegypti*, *Apis mellifera*, *Nasonia vitripennis*, *Tribolium castaneum* and *Bombyx mori*, respectively (Oakeshott et al., 2005, 2010; Claudianos et al., 2006; Strode et al., 2008; Yu et al., 2009).

Even though considerable divergence can be observed between members of the carboxylesterase family (e.g., in some cases exhibiting as little as 20% pairwise identity in amino acid sequences), their tertiary structures are believed to be very similar, and key residues are highly conserved. The basic structure of all members of the family is believed to be the α/β hydrolase fold, which is comprised of an 11-strand β-sheet with 14 α-helices in loops situated between the strands. Catalytically active members of the carboxylesterase family have a catalytic triad of Ser/His/Glu (or Asp). Other key features include the nucleophilic elbow surrounding the nucleophile serine (GXSXG), the leaving group pocket, the acyl pocket, and the oxyanion hole. The latter three sites accommodate the alcohol, acid, and oxyanion moieties of the substrate, respectively (Oakeshott et al., 1999).

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Table 1
Information for the selected carboxylesterases studied in this work. Mutated nucleotides are underlined. The bracketed accession number refers to the sequence used for primer design. F, forward primer. R, reverse primer.

Insect	Primers for CaE amplification	Mutation at 151	Accession number in NCBI database
Spodoptera litura	F: ATGGTGCAAGTGAGAGTGAATGA	G→D	EU783914
(Lepidoptera)	R: TCACAATACATATTTTTCATACAATTT	ggt→gat	(DQ445461)
Bombyx mori	F: ATGTTATTTGCGATTATTATATG	G→D	EU783913
(Lepidoptera)	R: TTATTTTTGATACTTCTCATATATC	ggt→gat	(NM_001046709)
Aphis gossypii	F: ATGGAAGTCGTCATTGAACAAG	A→D	EU783916
(Homoptera)	R: CTAAACAATGGATTCAGATAATTCTT	gcc→gac	(AY485216)
Nilaparvata lugens	F: ATGGCTGTGAAGTGGGAAATG	G→D	EU783912
(Homoptera)	R: TTATGCTCCTGGGAAGTTCTTCT	ggt→gat	(AF302777)
Tribolium castaneum	F: ATGGGCCAACCTGTCGTTAA	G→D	EU783911
(Coleoptera)	R: TCATAATTTATTGTTCGGGTTG	ggt→gat	(AJ850289)
Harmonia axyridis	F: ATGATATCTTTGAAAGTGAAGAGTG	G→D	EU783917
(Coleoptera)	R: TCAATAGGTGTCAAACGGTGAG	gga→gac	(AB201553)
Apis mellifera	F: ATGAACAAACAAATAGTAACTGTAAA	A→D	EU783915
(Hymenoptera)	R: TTAAGATAATTTGGATGATAAAAGA	gca→gac	(XM_001121897)

Resistance to a large and important class of insecticides, the organophosphates (OPs), can occur through carboxyl/cholinesterases. One well documented mechanism is through acetylcholinesterase, a cholinesterase with high specificity. Resistance can occur through a mutation to a form of acetylcholinesterase that is less sensitive to inhibition by OPs (Fournier and Mutéro, 1994). Alternatively, resistance can occur in nonspecific carboxylesterases, which exhibit high activity (as wild-type enzymes) towards substrates such as α- or β napthyl-acetate. In the case of the nonspecific esterases, there are two mechanisms of resistance. One, a quantitative mechanism, is characterized by the overproduction of carboxylesterase proteins through gene amplification or transcriptional up-regulation. In this mechanism, the over-expressed esterase proteins work like "sponges" to sequester insecticide molecules rather than hydrolyzing them (Chevillon et al., 1999). Lower dipterans, such as the mosquitoes *Culex pipiens*, *Culex tarsalis* and *Culex tritaeniorhynchus*, and sap-sucking insects, such as *Myzus persicae* and *Schizaphis graminum* have been shown to use this strategy to survive OP treatments (Field and Devonshire, 1997; Raymond et al., 1998; Hemingway and Karunaratane, 1998; Karunaratne et al., 1998). The second mode of resistance, a qualitative mechanism, results from changes in the enzymatic properties of esterases, specifically, increased activity towards OPs and decreased activity towards generic substrates such as naphthyl acetate. The same mutant form, G137D (denoted as G151D in this paper), has been found in field-resistant populations of several higher dipterans, *Lucilia cuprina*, *Lucilia sericata*, *Cochliomyia hominivorax* and *Musca domestica* and is known to be able to cause the characteristic shift in substrate specificity from napthyl acetate to OPs (Newcomb et al., 1997; Claudianos et al., 1999; Hartley et al., 2006; Carvalho et al., 2006). Another variant, W251L (denoted as W271L in this paper), was observed in malathion-resistant populations of *L. cuprina* and the mutation is known to decrease naphthyl acetate hydrolysis activity and increase activity towards OPs (Campbell et al., 1998). A change at position 251 was also found in a malathion-resistant strain of the parasitoid wasp *Anisopteromalus calandrae*, but with a different substitution, W251G (Zhu et al., 1999). Although these mutations have not been found in OP-resistant natural populations of the mosquito *C. pipiens* and the fruitfly *D. melanogaster*, mutagenesis *in vitro* proved that their mutant esterases, at least W251L, also showed a change in enzymatic properties similar to their counterparts in *L. cuprina* (Devonshire et al., 2003; Cui et al., 2007).

As stated as above, point mutation at 151 and 271 equivalent positions in carboxylesterases confers OP resistant have been reported mostly in species of dipterans except one case (the parasitoid wasp *A. calandrae*). Whether mutations in positions 151 and 271 in carboxylesterases are restricted to the shifts in the substrate specificity toward OPs in dipterans or it is a more common feature in insects is unknown. Here, we address this question at a biochemical level by examining the kinetics of hydrolysis of β-naphthyl acetate and two OP insecticides (paraoxon and chlorfenvinphos) of recombinant wild-type and mutant carboxylesterases from seven insect species from four orders.

2. Materials and methods

2.1. Carboxylesterase cloning

Total RNA was extracted from two lepidopteran species (*Spodoptera litura*, *B. mori*), two homopteran species (*Aphis gossypii*, *Nilaparvata lugens*), two coleopteran species (*T. castaneum*, *Harmonia axyridis*) and one hymenopteran species (*A. mellifera*) with an RNeasy Mini kit and then treated with DNase I (Qiagen, Valencia, CA, USA). Up to 5 ug of DNA-free total RNA was reverse-transcripted into cDNA with SuperScriptTM III first strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). Primers for amplifying the open reading frame (ORF) of each carboxylesterase from the seven insects were designed based on the sequences deposited in the NCBI database (Table 1). The 50 μl RT-PCR reaction was comprised of 0.25 μg of template cDNA, 0.25 μM of each primer, 0.2 mM of each dNTP, 2.5 Units of Taq polymerase in a 1× reaction buffer (Expand High Fidelity PCR Kit, Roche, Basel, Switzerland). The PCR was performed on a thermocycler (Eppendorf, Westbury, NY, USA) with a denaturing step at 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 s, 55 °C for 30 s, 68 °C for 2 min, and a final step of 7 min at 68 °C. PCR products with an adenine at 3′ terminus were connected with the pGEM-T easy vector (Promega, San Luis Obispo, CA, USA) and transfected into DH5α cells. Three independent clones for each carboxylesterase were sequenced to ensure that cDNA sequences are correct. The obtained sequences were deposited in the NCBI database as new entries due to differences (from 2 to 88 amino acid residues) from the corresponding sequences used in primer design (Table 1).

2.2. Sequence alignment, phylogenetic analysis and localization of mutagenesis sites

The deduced amino acid sequences of carboxylesterases from the seven insect species were aligned, along with homologues E3 from *L. cuprina* (AAB67728), E7 from *M. domestica* (AAD29685), αE7 from *D. melanogaster* (NP_524261), EstB1 from *C. pipiens*

(AAA28289), and AChE from *D. melanogaster* (**1DX4_A**) using the CLUSTALW (www.ebi.ac.uk/Tools/clustalw2/index.html). The amino acid residues corresponding to the mutations G137D and W251L found in *L. cuprina* carboxylesterase in insecticide resistant field populations were G111, W225 in *T. castaneum*, G131, W243 in *H. axyridis*, G135, W248 in *N. lugens*, A108, W221 in *A. mellifera*, A107, W219 in *A. gossypii*, G126, W239 in *B. mori* and G107, W219 in *S. litura*. These two positions are here designated as G151 & W271, following the sequence of *D. melanogaster* AChE, as it is the only insect member of the carboxylesterase family whose tertiary structure has been determined (Harel et al., 2000).

To categorize the seven esterases, the sequences were first aligned, using ClustalW, along with selected members of the 14 major clades of insect carboxylesterases described in Oakeshott et al. (2005). Next, a phylogenetic tree was constructed among a total of 49 esterases using the neighbor-joining method (complete deletion and the Poisson-correction model) in MEGA 3.1 software. Bootstrap analysis (1000 replicates) was applied to evaluate the internal support of the tree topology.

Deduced amino acid sequences were analyzed with the SignalP 3.0 server (Nielsen et al., 1997; Bendtsen et al., 2004) for the possible presence of signal peptides for extracellular secretion.

2.3. Mutagenesis

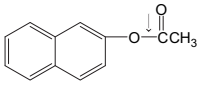
The cloned ORF of each carboxylesterase was inserted into the multiple cloning sites of the pUC18 vector by PCR by incorporating two restriction endonuclease recognition sites into the two ends of ORFs. The amplicons were then digested with the corresponding endonucleases and ligated into pUC18 using T4 DNA ligase (NEB, Ipswich, MA, USA). All of the seven carboxylesterases were inserted between *Sac*I and *Sal*I sites, except *N. lugens* carboxylesterase, which was inserted between *Sac*I and *Pst*I sites.

Mutations were introduced into carboxylesterase ORFs contained in the pUC18 vector by site-directed mutagenesis with the QuikChange™ kit (Stratagene) according to the manufacturer's protocols. One or two bases were changed at the 151 or 271 sites to obtain target single mutants (ggt→gat or gga→gac for G151D; gcc→gac or gca→gac for A151D; tgg→ttg for W271L, Table 1). All mutants were verified by sequencing.

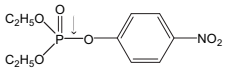
2.4. Expression of recombinant proteins

The mutated and wild-type carboxylesterase ORFs (encoding the mature enzymes, without signal peptide sequences in those enzymes where they exist) were transferred to the expression vector pET28a via PCR cloning by incorporating two restriction endonuclease recognition sites into the ends of ORFs. The amplicons were then digested with the corresponding endonucleases and ligated into pET28a using T4 DNA ligase. *T. castaneum* carboxylesterase was inserted between *Nde*I and *Sal*I sites, *S. litura* between *Sac*I and *Xho*I, and *N. lugens* between *Nde*I and *Not*I. The other four carboxylesterases were inserted between *Nhe*I and *Xho*I. After sequencing to rule out possible PCR-introduced errors, the recombinant plasmids were transformed to *Escherichia coli* BL21 (DE3) to produce recombinant proteins with histidine tags at both termini for purification. For each carboxylesterase, two single mutants (G/A151D and W271L) and the wild-type were expressed and assayed.

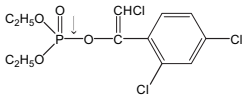
One colony from freshly transformed cells was used to inoculate 2 ml Luria-Bertani (LB) medium containing 50 μg/ml kanamycin and incubated overnight at 37 °C. A 1 ml cell suspension was used to inoculate 500 ml LB medium containing 50 μg/ml kanamycin, and induced with 1 mM IPTG for 24 h at 18 °C at 200 rpm agitation when cell growth attained the mid-exponential-phase ($OD_{600} = 0.5$).



β-naphthyl acetate



paraxon



chlorfenvinphos

Fig. 1. Chemical structures of substrates used. Arrow shows the cleavage site.

2.5. Purification of expressed carboxylesterases and immune reaction with an anti-carboxylesterase antibody

Wild-type and mutant carboxylesterases were expressed in *E. coli* and purified using His Bind Kits (Novagen) as described in Cui et al. (2007). Fractions from the supernatant, cell pellets and purified target proteins were analyzed on a 12% SDS-PAGE gel stained with Coomassie Blue R250 solution (Fazekas de St. Groth et al., 1963). Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard (Bradford, 1976). The purified target proteins were verified via western blot analysis with an anti- *C. pipiens*' carboxylesterase B1 polyclonal rabbit antibody and visualized in x-ray exposure films. The *C. pipiens*' carboxylesterase B1 was expressed in BL21 (DE3) and purified in previous studies (Cui et al., 2007). The purified proteins were sent to Beijing Protein Innovation (Beijing, China) as antigens to produce polyclonal antibody in rabbits.

2.6. Assays of enzymatic activity

Carboxylesterase activities were assayed using artificial substrate β-naphthyl acetate (Fig. 1) in 0.2 M phosphate buffer (pH 7.0). An appropriate amount of purified enzyme solution was added into a range of concentrations of substrate solutions spanning the Km (based on preliminary tests), to a final volume of 3.0 ml. After incubating the mixture for 5 min at 37 °C, 0.5 ml freshly prepared diazo blue SDS reagent (0.3% fast blue B salt in 3.5% aqueous SDS) was added. The colour developed as a result of β-naphthol formation was measured at 555 nm in a Beckman DU-800 spectrophotometer (USA) and quantified using a β-naphthol standard curve. The lower limit of detection was around 6 μM of substrate solution.

The degradation rates for two OPs, paraoxon and chlorfenvinphos (Fig. 1) were measured using gas chromatography to determine the OP hydrolase activity of the purified recombinant proteins. An appropriate amount of purified enzyme was added into a range of concentrations of substrate solutions in 0.2 M potassium-phosphate buffer (pH 7.0) spanning the Km (based on preliminary tests) to a final volume of 15 ml, and incubated at 37 °C for 45 min. A half milliliter of sample was taken out and mixed with 0.5 ml of petroleum ether, and then dried with anhydrous sodium sulfate. The product was extracted with redistilled hexane and analyzed on a Hewlett–Packard 5890 series II GC with a nitrogen phosphorous detector (NPD), 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 each assay. Injector, column and detector temperatures were set at 250 °C, 200 °C and 300 °C, respectively. Concentration of each OP insecticide was determined by comparing the peak area to a standard curve. The lower limits of detection for paraoxon and chlorfenvinphos were around 7 nM and 5 nM, respectively. Three to five replicates of

assays were performed out and two technique repeats were done in each assay. Six to eight concentrations were prepared for each substrate to estimate the enzymatic kinetic constants, V_{max} and K_m, using HYPER software based on the Lineweaver–Burk method. k_{cat} and k_{cat}/K_m values were also calculated for all substrates. Differences between the wild-type and mutants or between the two mutants of one carboxylesterase were evaluated statistically by *t*-test using SPSS 11.0 software.

3. Results

3.1. Sequence alignment and phylogenetic analysis of carboxylesterases cloned from seven insects

Carboxylesterases were cloned from cDNAs from 7 species belonging to four insect orders. Pairwise identities of the predicted amino acid sequences among the 7 species ranged from 23% to 76%. Forty-three amino acid residues (~2.2%) were identical across the 7 species in this study and 5 previously reported insect carboxylesterases, such as the catalytic triad (S238, E367 and H480) and the targeted mutagenesis residue W271. The other targeted position, 151, was Ala in the enzymes from *A. mellifera*, and *A. gossypii*, Asp in the enzyme from insecticide resistant *M. domestica*, and Gly for the other species. The carboxylesterases from *N. lugens*, *B. mori* and *H. axyridis* were predicted to have a secretory signal peptide in the N-terminus of proteins (Fig. 2).

In Fig. 3, we highlight the 7 carboxylesterases selected for this study in the context of the phylogenetic tree of Oakeshott et al. (2005). Through sequence alignment with members of each clade, followed by phylogenetic analysis, it can be observed that six carboxylesterases cloned from *S. litura*, *B. mori*, *A. gossypii*, *T. castaneum*, *H. axyridis* and *A. mellifera*, respectively, belonged to clade A. This clade is believed to comprise mainly intracellular catalytically active proteins and have more general dietary and/or detoxification functions (Oakeshott et al., 2005). The *N. lugens* carboxylesterase is placed in clade E, as an ortholog of dipteran β-esterases. All of the seven esterases are considered nonspecific and selected with an intention to supplement the currently available information on OP resistance in higher dipterans.

3.2. Solubility of E. coli expressed recombinant carboxylesterases and verification via western blot

The wild-type and the mutated carboxylesterases were expressed in *E. coli* BL21 (DE3) cells. In the cases of recombinant carboxylesterases of *T. castaneum*, *B. mori*, *A. gossypii*, *H. axyridis* and *N. lugens*, the majority of the expressed enzyme was insoluble after sonication of the *E. coli* cells, whilst the soluble and insoluble part of recombinant carboxylesterases of *S. litura* and *A. mellifera* was nearly equivalent. In each case only enzyme from the sonication supernatants (i.e., soluble enzyme) was used for purification, enzymatic studies, and beyond. This solubility provides an initial

Fig. 2. Comparison of protein sequences from 7 species in this study and 5 previously reported insect carboxylesterases. The sequences used included *C. pipiens* esterase B1 (AAA28289), *M. domestica* E7 (AAD29685), *L. cuprina* E3 (AAB67728), *D. melanogaster* αE7 (NP_524261) and *D. melanogaster* AChE (1DX4_A). Catalytic triad residues are boxed. The mutation positions G/A151D and W271L are indicated with arrows. The secretory signal peptide is shaded in yellow. "*" means that the residues are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed. "." means that semi-conserved substitutions are observed.

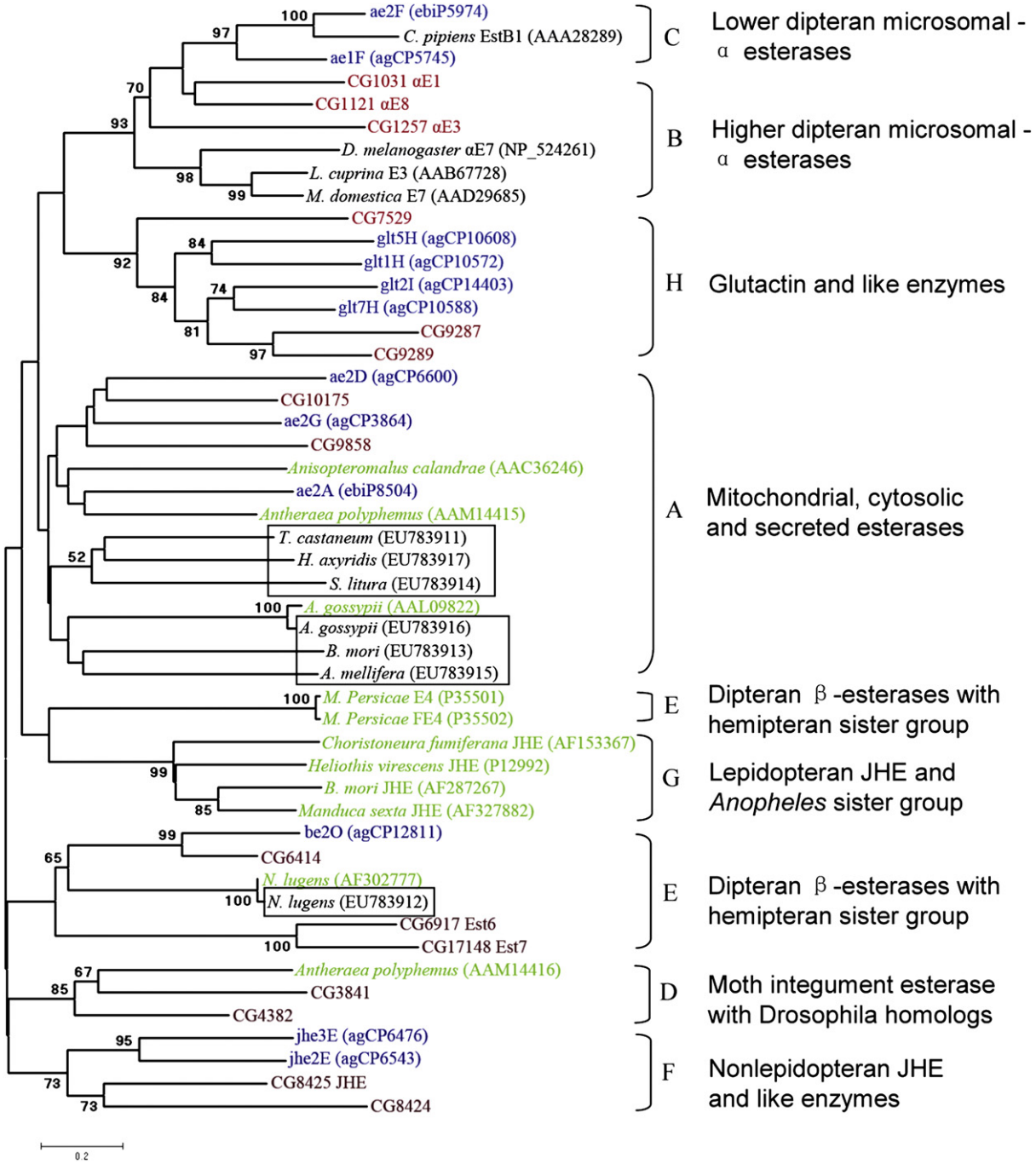


Fig. 3. An unrooted distance neighbor-joining tree showing phylogeny of catalytic classes of insect carboxylesterases. Sequences from the *D. melanogaster* and *A. gambiae* genomes (red and blue, respectively), and from other insects (green) are as used in Oakeshott et al. (2005). Nodes with >50% bootstrap support (1000 pseudoreplicates) are indicated. Clades are designated according to the nomenclature adopted by Oakeshott et al. (2005). Only clades A-H are shown. The studied seven carboxylesterases in this experiment are highlighted with boxes.

basis for believing that the molecules were properly folded. The purity of most samples looked more than 90% after affinity chromatography (Supplementary Fig. S1), and the target proteins were immunoreactive to the anti- *C. pipiens*' carboxylesterase B1 antibody (Fig. 4).

3.3. Hydrolysis activity of wild-type and mutant carboxylesterases towards an artificial carboxyl-ester

Activities of the purified recombinant wild-type and mutant esterases towards β-naphthyl acetate are presented in Table 2,

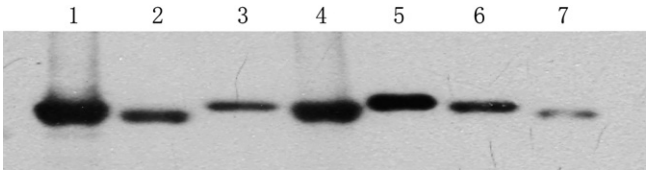


Fig. 4. The immunoreaction of the purified target proteins towards anti- *C. pipiens*' carboxylesterase B1 antibody in western blotting. The samples are wild-types of carboxylesterases of *T. castaneum* (1), *H. axyridis* (2), *A. mellifera* (3), *N. lugens* (4), *S. litura* (5), *B. mori* (6) and *A. gossypii* (7). The immunoreactions of the mutant carboxylesterases are similar to their wild-type counterparts (data not shown).

Table 2
Catalytic efficiencies of recombinantly expressed wild-type (WT) and mutant carboxylesterases towards several substrates.

Insect	Enzyme	β-naphthyl acetate			Paraoxon			Chlorfenvinphos	
		K_m(SE) (μM)	k_{cat}(SE) (min^{-1})	k_{cat}/K_m(SE) (min^{-1} μM^{-1})	K_m(SE) (μM)	k_{cat}(SE). (min^{-1})	k_{cat}/K_m(SE) (min^{-1} μM^{-1})	K_m(SE) (μM)	k_{cat}(SE) (min^{-1})	k_{cat}/K_m(SE) (min^{-1} μM^{-1})
A. gossypii	WT	79.1(8.5)	2902(301)	36.7(7.8)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	A151D	91.5(13.1)	3324(95)	37.5(6.4)	12.1(0.8)	1.9(0.1)	0.16(0.02)	4.1(0.1)	0.71(0.01)	0.17(0.01)
	W271L	n.d.	n.d.	n.d.	6.2(0.1)[c]	1.1(0.01)[c]	0.18(0.01)[c]	3.2(0.1)[c]	0.56(0.02)[c]	0.18(0.01)
A. mellifera	WT	176.2(7.1)	9507(427)	53.9(4.6)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	A151D	n.d.	n.d.	n.d.	3.3(0.1)	0.66(0.01)	0.20(0.01)	1.9(0.1)	0.37(0.01)	0.20(0.02)
	W271L	660(17.1)[c]	133.8(1.7)[c]	0.20(0.01)[c]	3.8(0.3)	0.55(0.03)[c]	0.15(0.02)[c]	1.9(0.1)	0.29(0.01)[c]	0.15(0.01)[c]
S. litura	WT	230.5(13.8)	27703(2827)	119.6(19.4)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	G151D	31.5(3.0)[c]	130.8(9.5)[c]	4.2(0.7)[c]	6.3(0.3)	1.1(0.04)	0.18(0.02)	3.0(0.1)	0.53(0.005)	0.18(0.01)
	W271L	n.d.	n.d.	n.d.	5.5(0.2)	1.1(0.03)	0.20(0.01)[c]	4.3(0.2)[c]	0.76(0.02)[c]	0.18(0.01)
T. castaneum	WT	131.3(7.0)	31803(811)	243.2(19.2)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	G151D	n.d.	n.d.	n.d.	7.0(0.9)	0.78(0.04)	0.12(0.02)	n.d.	n.d.	n.d.
	W271L	1073(35)[c]	363.6(11.6)[c]	0.34(0.02)[c]	4.5(0.2)[b]	0.90(0.02)[b]	0.20(0.01)[c]	4.0(0.1)	0.67(0.01)	0.17(0.01)
B. mori	WT	9.1(1.2)	815.8(33.9)	93.5(16.2)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	G151D	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.6(0.1)	0.30(0.01)	0.19(0.02)
	W271L	n.d.	n.d.	n.d.	2.4(0.1)	0.50(0.01)	0.21(0.01)	1.0(0.1)[c]	0.28(0.01)	0.27(0.04)[c]
H. axyridis	WT	1235(190)	711.1(114.2)	0.57(0.2)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	G151D	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	W271L	n.d.	n.d.	n.d.	3.1(0.1)	0.67(0.01)	0.21(0.01)	1.0(0.05)	0.28(0.01)	0.28(0.02)
C. pipiens[a]	WT	26.3(2.5)	717(18)	26.8(3.2)	–	–	–	n.d.	n.d.	n.d.
	A151D	n.d.	n.d.	n.d.	–	–	–	n.d.	n.d.	n.d.
	W271L	n.d.	n.d.	n.d.	–	–	–	5.5(0.2)	0.20(0.01)	0.04(0.003)
N. lugens	WT	411.4(54.6)	159.8(16.4)	0.39(0.1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	G151D	809.8(70.7)[b]	241.8(14.4)[b]	0.30(0.04)[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	W271L	1114(180)[b]	203.6(29.2)	0.18(0.1)[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., no activity detectable.
[a] data from Cui et al. (2007).
[b] , $p < 0.05$.
[c] , $p < 0.01$; the statistic *t*-test was done for the pair comparison between WT and one of mutants for β-naphthyl acetate hydrolysis, and between the two mutants for OP hydrolysis.

along with results from our previous analysis of a carboxylesterase from *C. pipiens*. The wild-type enzyme from *T. castaneum* exhibited the highest catalytic efficiency (kcat/Km) towards β-naphthyl acetate, followed by the wild-type enzymes from *S. litura*, *B. mori*, *A. mellifera*, *A. gossypii*, *C. pipiens*, *H. axyridis* and *N. lugens* in turn. The biggest difference in catalytic efficiencies was around 600 fold (between enzymes from *T. castaneum* and *N. lugens*).

The mutations in 151 and 271 position have suppressing effects on enzymatic activity toward β-naphthyl acetate in most cases (13 out of the total 16 mutants). The exceptions were A151D in *A. gossypii*, G151D and W271L in *N. lugens*, where either no significant or only less than 3-fold decreases in enzymatic activity were observed. Furthermore, mutations at these two positions contribute differently to the enzymatic activities. W271L (all of the 8 cases) has a more consistent negative influence on catalytic activity toward β-naphthyl acetate than that of G/A151D (7/8 cases).

3.4. Activity of wild-type and mutant carboxylesterases towards OPs

Catalytic efficiencies of the wild-type and mutant enzymes for two OPs (paraoxon and chlorfenvinphos) are shown in Table 2, including results from our previous analysis of a carboxylesterase from *C. pipiens*. None of the eight wild-type enzymes could hydrolyze paraoxon or chlorfenvinphos at detectable levels. The mutation G/A151D conferred hydrolysis activity towards both or one of OPs in 5 out of 8 cases, with exceptions in *H. axyridis*, *C. pipiens* and *N. lugens*; whereas the mutation W271L conferred OP hydrolysis activity in 7 out of 8 cases, with one exception in *N. lugens*. So position 271 is more sensitive to mutation than that of 151 regarding the OP catalytic activities. A151D and G151D in *A. gossypii* and *S. litura* carboxylesterases showed 220-and 23-fold higher efficiency toward β-naphthyl acetate than that of OPs, respectively. Interestingly, G151D mutants in carboxylesterases from *T. castaneum* and *B. mori* only acquired activities toward one of the OPs. On the other hand, W271L mutants in carboxylesterases from *A. mellifera* and *T. castaneum* that retained reduced activities toward β-naphthyl acetate showed little difference in efficiency between artificial substrate and OPs.

The hydrolysis efficiencies towards chlorfenvinphos of one or both of the mutants of the carboxylesterases from seven insect species are much higher than that of the mutants of blowfly esterase E3 expressed *in vitro*. The values of kcat/Km of blowfly E3 mutants G151D and W271L towards chlorfenvinphos were 230 M^{-1} S^{-1} (or 0.013 μM^{-1} min^{-1}) and 11 M^{-1} S^{-1} (or 6.8×10^{-4} μM^{-1} min^{-1}), respectively (Campbell et al., 1998). As reported in this paper, the kcat/Km of the G/A151D mutants from *A. gossypii*, *A. mellifera*, *S. litura* and *B. mori* ranged from 0.17 μM^{-1} min^{-1} to 0.2 μM^{-1} min^{-1} (Table 2), 13-fold–15-fold higher than E3 G151D. The kcat/Km of the W271L mutants from seven species (not including *N. lugens*) ranged from 0.04 μM^{-1} min^{-1} to 0.28 μM^{-1} min^{-1} (Table 2), 59-fold–410-fold higher than E3 W271L. Furthermore, unlike the case of blowfly E3 where the G151D substitution enhanced the enzyme's hydrolysis activity towards diethyl OPs (e.g. chlorfenvinphos) or analogs (e.g. diethyl 7-hydroxycoumaryl phosphate) more than did the W271L substitution (Campbell et al., 1998; Devonshire et al., 2003), the G/A151D mutants from seven species, in the current work, did not increase hydrolysis efficiency more than the W271L mutants towards either of the two diethyl OPs.

4. Discussion

We investigated whether point mutations at 151 and 271 equivalent positions in carboxylesterases that confer OP hydrolysis is a characteristic limited to Dipterans or a more general feature in insects. The results from this study support the later hypothesis. The results are consistent with the observation that species other than dipterans also

show that mutation at 271 equivalent position in a carboxylesterase from *A. calandrea* confers OP resistance (Zhu et al., 1999).

The seven carboxylesterases examined in this study displayed a rather wide range of activities towards the generic substrate β-naphthyl acetate. This is perhaps not unexpected, since the enzymes were selected from a wide range within the phylogenetic tree for the carboxylesterase family. The differences in enzyme efficiencies might not be related to whether the carboxylesterase is cytosolic or secretory types. The predicted cytosolic wild-type carboxylesterases (5 out of 8 carboxylesterases in this study) have about 9-fold differences in efficiencies toward β-naphthyl acetate (between *T. castaneum* and *C. pipiens*). If one looks at the predicted secretory carboxylesterases namely the *H. axyridis*, *B. mori*, and *N. lugens*, the efficiencies of the wild-type recombinant proteins toward β-naphthyl acetate also varies widely (between *B. mori* and *N. lugens* there is a 239-fold difference). It may have something to do with the differences in the active site of each enzyme. Because the carboxylesterases are considered as nonspecific, the active site of each enzyme may differ in its accommodations to varied natural substrates. Thus, each carboxylesterase may have different efficiency toward β-naphthyl acetate regardless the cellular localization.

The mutation G/A151D and W271L have different effects on substrate specificity in carboxylesterases. In the cases of G/A151D mutation, 4/7 or 57% cases increased hydrolysis toward paraoxon and 4/8 or 50% cases toward chlorfenvinphos. So less than 60% will result in hydrolysis of OPs. On the other hand, in the cases of W271L mutations, 6/7 or 85.7% cases increased hydrolysis toward paraoxon and 7/8 or 87.5% cases toward chlorfenvinphos. Thus more than 85% will lead to hydrolytic activity toward OPs. However, in the study of *L. cuprina*, *L. sericata*, *M. domestica* and *D. melanogaster*, both of the two mutants decreased the enzymes' carboxylesterase activity and increased their OP hydrolase activity (Newcomb et al., 1997; Campbell et al., 1998; Claudianos et al., 1999; Devonshire et al., 2003; Hartley et al., 2006). One possible explanation for this discrepant observation is the enzymes from the above four Dipteran insects are orthologs of αE7 of the α-esterase cluster (clade B in Fig. 3) while the seven carboxylesterases investigated in this study belong to different clades (clades A and E in Fig. 3).

What is really striking is the carboxylesterase from *N. lugens*. There are no reports to associate OP resistance with the carboxylesterases examined in this study except the one from *N. lugens*, whose activity was elevated through gene amplification in an OP resistant strain (Small and Hemingway, 2000). In this study this enzyme had very low activity toward β-naphthyl acetate, and mutations at G151D and W271L neither largely changed the catalytic activity toward β-naphthyl acetate (1.3- and 2.2-fold, respectively) nor conferred hydrolysis of OPs. Was it due to the fact that it is clade E or is it because it has very low catalytic activity toward β-naphthyl acetate to start with and its substrate(s) and binding cavity different from clades A, B and C which other carboxylesterases belongs to? May be this exceptional carboxylesterase (*N. lugens*) means that metabolic resistance to OPs by hydrolysis is restricted to certain clades of carboxylesterases. Therefore, future study should be conducted with other clades of carboxylesterases to see if this hypothesis is true.

It has been assumed that the mutation G151D, located in the oxyanion hole of the enzyme's active site, alters the orientation of the nucleophilic attack by a water molecule. This new orientation facilitates its attack on a tetrahedral phosphorylated serine rather than a planar acylated serine (Newcomb et al., 1997; Campbell et al., 1998). The mutation W271L, sitting in the acyl pocket of the active site, is thought to create more space to accommodate substrates with bulky acid groups and reduce the steric obstruction to the inversion that must occur around phosphorus during hydrolysis of OPs (Heidari et al., 2004). Our results indicate that the greater space created by the mutation at 271 position seems to have more effects on substrate specificity than the altered orientation of the nucleophilic attack from the mutation at 151 position in carboxylesterases.

In summary, the results from this study suggest that G/A151D or W271L mutation could be a common mechanism in the development of OP resistance in insects. Therefore, this resistance mechanism should be carefully looked for in widely divergent field populations of insects.

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Appendix. Supplementary material

Supplementary material associated with this paper can be found, in the online version, at. doi:10.1016/j.ibmb.2010.09.004

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