Molecular characterization and inhibition analysis of the acetylcholinesterase gene from the silkworm maggot, *Exorista sorbillans*

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Several organophosphorus (OP) insecticides can selectively kill the silkworm maggot, *Exorista sorbillans* (*Es*) (Diptera: Tachinidae), while not obviously affecting the host (*Bombyx mori*) larvae, but the mechanism is not yet clear. In this study, the cDNA encoding an acetylcholinesterase (AChE) from the field *Es* was isolated. One point mutation (Gly353Ala) was identified. The *Es*-353G AChE and *Es*-353A AChE were expressed in baculovirus-insect cell system, respectively. The inhibition results showed that for eserine and Chlorpyrifos, *Es*-353A AChE was significantly less sensitive than *Es*-353G AChE. Meanwhile, comparison of the I_{50} values of eserine, dichlorvos, Chlorpyrifos and omethoate of recombinant *Es* AChEs with its host (*Bombyx mori*) AChEs indicated that, both *Es* AChEs are more sensitive than *B. mori* AChEs. The results give an insight of the mechanism that some OP insecticides can selectively kills *Es* while without distinct effect on its host, *B. mori*. [BMB reports 2010; 43(8): 573-578]

INTRODUCTION

Acetylcholinesterase (AChE; EC 3.1.1.7) is a key enzyme in the cholinergic system that regulates the levels of the neurotransmitter acetylcholine (ACh) and terminates nerve impulses by catalyzing the hydrolysis of ACh in the synaptic cleft (1). Insect AChEs have been of great interest because of their critical functions in neurotransmission, and they are the primary targets of organophosphorus (OP) and carbamate (CB) insecticides.

In 1986, the first insect acetylcholinesterase gene (*ace*) was cloned from the fruit fly, *Drosophila melanogaster* (2), and it was then classified into the *ace2* group. A second *ace* locus, in a different linkage group, has been found in some insect spe-

cies (3). It is now acknowledged that most insects have two AChEs encoded by two *aces* (4). These insect AChEs are classified into two types according to their homology to *Drosophila* AChE. AChEs homologous to *Drosophila* AChE are *Ace*-orthologous AChE (AO-AChE) or *ace2s*. AChEs with less similarity to *Drosophila* AChE are *Ace*-paralogous AChE (AP-AChE) or *ace1s*. Nevertheless, within the order Diptera, the Aristoceran flies, including the fruit fly, *D. melanogaster*, Australian blow fly, *Lucilia cuprina*, olive fruit fly, *Bactrocera oleae*, and the house fly *Musca domestica*, possess only one type of *ace* gene, the *ace2*.

The silkworm maggot (*Exorista sorbillans, Es*), a well-known larval endoparasitoid of the silkworm and saturniid silkworms in all silk producing areas of Asia and causes severe damage to the silk industry accounting for 15-20% of yield loss. Several OP insecticides could be directly sprayed on the diseased silkworm to control *Es* in silk production. Lower concentrations of some OP insecticides such as dimethoate can selectively kill *Es* without distinct toxicity to its host, the silkworm, but the mechanism is not yet clear. In this study, the gene encoding AChE from the endoparasitoid fly was cloned and expressed, a point mutation associated with reduced sensitivity of the expressed AChE to OP insecticides was identified, and the inhibition kinetics of OP insecticides with respect to *Es*-AChE and the host *Bm*-AChEs were compared.

RESULTS

Amplification and sequence analysis

The length of amplified cDNA was 2,195 bp, containing an open reading frame of 2,103 bp to encode a 701-amino acid polypeptide (GenBank Accession No. HM028669) (Supplementary figure). *Es*-AChE is longer than the AChEs from the fruit fly *Drosophila melanogaster* (648 aa) and the house fly *Musca domestica* (691 aa) but is seven residues shorter than that from the blowfly *Lucilia cuprina* (708 aa). We amplified and sequenced 10 *Es-ace* clones from different *Es* adults and identified a point mutation in amino acid 353 (Gly353Ala) (GenBank Accession No. HM028670). Among ten clones, four had this mutation.

A putative signal peptide and a GPI anchoring sequence were predicted at the N- and C-termini of the deduced amino acid sequences respectively. Alignment of amino acid sequences from different flies and the silkworm showed that most major structural and functional motifs, including the catalytic triad, acyl binding pocket, anionic subsite, oxyanion holes, peripheral anionic subsite, and omega loop are highly conserved (Fig. 1). The sequences differ mainly in the N-region (amino acids 1-94) and C-region (amino acids 658-701). The *Es*-AChE is highly homologous to AChEs from other Aristoceran flies in Diptera, particularly to those of the house fly and the blowfly, for which the sequences are more than 83% identical, while the sequence identities between *Es*-AChE and the host (*B. mori*) AChEs, Bm-AChE2 and Bm-AChE1, are much lower, only 58% and 36%, respectively (Fig. 1, Supplementary Table 1).
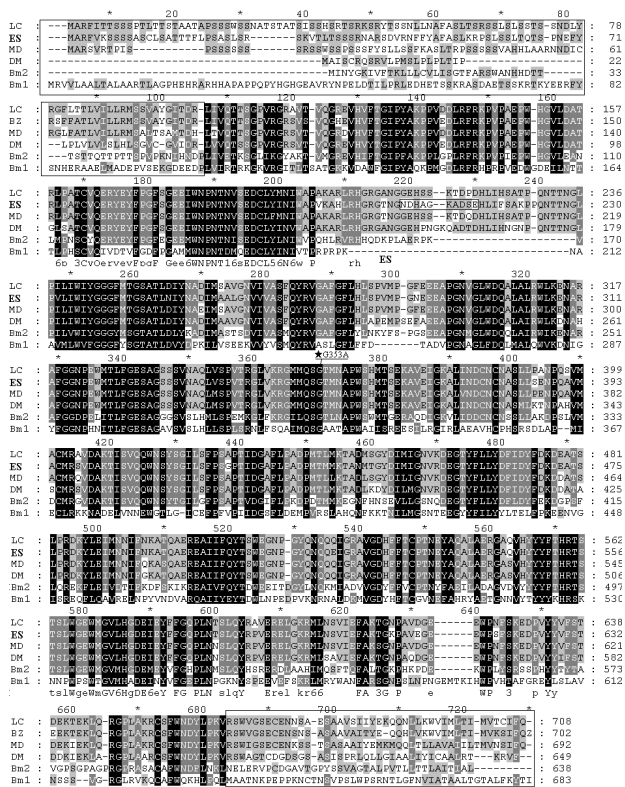

Fig. 1. Alignment of deduced amino acid sequences of *Es*-AChE with its host, *Bombyx mori*, and other Aristoceran fly homologues. All insect sequences were retrieved from GenBank (Dm, *Drosophila melanogster*; Lc, *Lucilia cuprina*; Es, *Exorista sorbillans*; Md, *Musca domestica*; Bm2, *Bombyx mori* AChE2; Bm1, *Bombyx mori* AChE1). The alignment was determined using Clustal X software. The black shading shows identity across all six sequences, while boxing shows the main differences among the six sequences. A five-pointed star indicates the amino acid mutation (Amino acids: G to A).

Expression of *Es*-353A and *Es*-353G *ace* in insect cells

Tn-5B1-4 cells were infected with the two recombinant viruses at a MOI of 10 and harvested at 96 h post-infection. SDS-PAGE analysis demonstrated that both the Es-353G-AChE and *Es*-353A-AChE were highly expressed in the Tn-5B1-4 cells, with a yield of about 0.4 g L^{-1}. The expressed products were approximately 65 kDa (Fig. 2). Both AChEs were determined to be secretory proteins, with efficient secretion into the serum-free medium shown in the SDS-PAGE analysis (Fig. 2).

AChE activity and kinetic parameter comparisons

K_m values of *Es*-353Gly-AChE for the substrates ATC, BTC, and PTC were 62.1 ± 5.2, 68.5 ± 10 and 80.2 ± 2.7 µmol/L while the K_m values of *Es*-353Ala-AChE were 63.2 ± 5.1, 63.1 ± 4.4 and 81.7 ± 3.4 µmol/L respectively. The data showed that K_m values of the two mutants for the three substrates had no distinct differences. However, the V_{max} values of the purified *Es*-353A-AChE for the substrates ATC, BTC, and PTC were 28.1 ± 1.9, 13.9 ± 0.9 and 13.6 ± 0.15 µmol/mg_protein/min showed slightly higher than those of *Es*-353G-AChE in hydrolyzing the three substrates, the V_{max} values of the purified *Es*-353G-AChE for ATC, PTC, and BTC were 22.4 ± 0.65, 10.4 ± 0.36, and 10.6 ± 0.07 µmol/mg_protein/min respectively.

Inhibition of the AChE by four inhibitors

The median inhibition concentrations (I_{50}) of eserine, dichlorvos, chlorpyrifos, and omethoate for *Es*-353A-AChE were 29.6, 58.8, 9232 and 4163 10^{-9}mol, and were 2.6, 1.3, 1.4 and 1.4 times higher than those of *Es*-353G-AChE, respectively. Statistical analysis showed that I_{50}s of eserine and omethoate for *Es*-353A-AChE were significantly higher than those for *Es*-353G-AChE (P < 0.05). The bimolecular reaction constants (K_i) of *Es*-353G-AChE with the four insecticides were 2.4, 1.4, 3.5 and 1.6 fold those of *Es*-353A-AChE, respectively (Fig. 3, Supplementary Table 2). These data indicated that
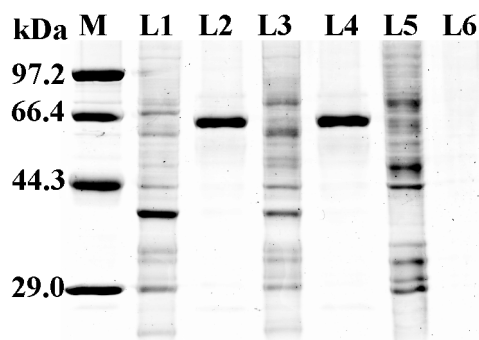

Fig. 2. Expression of *Es*-353G-AChE and *Es*-353A-AChE in insect cells. M, Marker; L1, *Es*-353Gly-AChE in Tn-5B1-4 cells; L2, *Es*-353G-AChE in medium; L3, *Es*-353A-AChE in Tn-5B1-4 cells; L4, *Es*-353A-AChE in medium; L5, Tn-5B1-4 cells infected with Bacmid vector (control); L6, medium of Tn-5B1-4 cells infected with Bacmid vector (control).
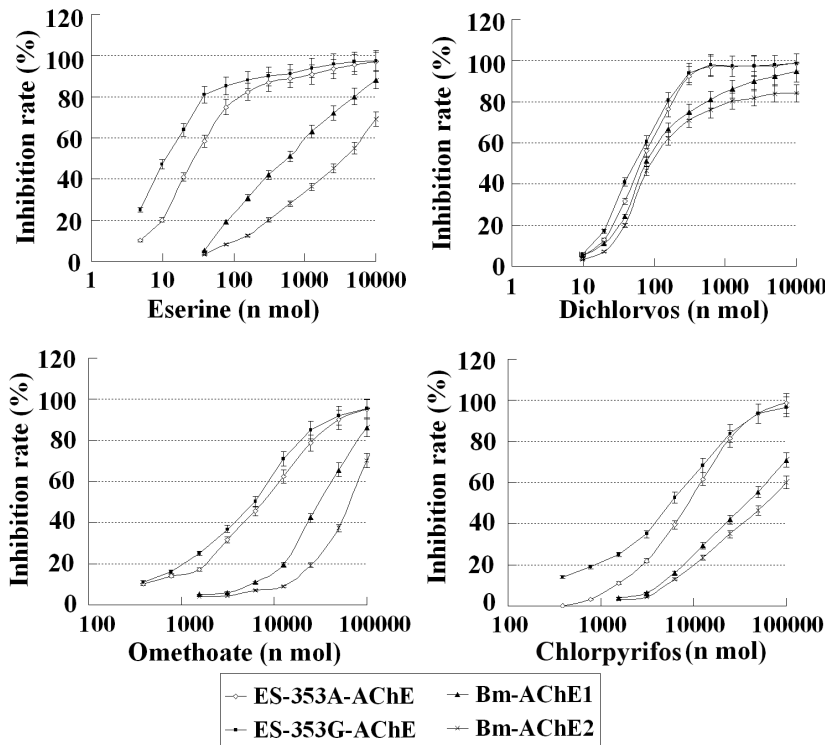
Fig. 3. Inhibition of *Es*-353G-AChE, *Es*-353A-AChE, *Bm*-AChE1 and *Bm*-AChE2 by four inhibitors. (A) Eserine, (B) Dichlorvos; (C) Omethoate, (D) Chlorpyrifos.

Es-353G-AChE was more sensitive to the four insecticide than was *Es*-353A-AChE.

Comparison of the AChE inhibition of *Es* with that of its host
Previously, the cDNAs encoding two AChEs (*Bm*-AChE1 and *Bm*-AChE2) from the *Bombyx* were cloned, and the corresponding proteins were heterologously expressed to compare their enzymatic properties and interactions with OP and CB inhibitors *in vitro* (5).

In this study, the inhibitory properties of recombinant *Bm*-AChEs (5) and *Es*-AChEs were compared. The median inhibition concentrations (I_{50}) of eserine, dichlorvos, chlorpyrifos and omethoate for *Bm*-ace1 AChE were 62, 1.2, 2.6 and 4.5 times higher than those of *Es*-353A-AChE (Fig. 3), while the I_{50} of *Bm*-ace2 AChE for the four inhibitors were 93-, 4.1-, 5.2- and 7.2-fold higher than those of *Es*-353A-AChE (Fig. 3). Statistical analysis showed that I_{50}s of *Es*-353A-AChE or *Es*-353G-AChE were significantly lower than those for *Bm*-ace1 or *Bm*-ace2 AChE (P < 0.05). The data indicated that *Es*-AChE is more sensitive than are *Bm*-AChEs to the four inhibitors.

DISCUSSION

Many insect species have been shown to possess two different *ace* genes (4-6), but the Aristoceran flies, such as the fruit fly (2), the house fly (7), the blowfly (8), and olive fruit fly (9),

have only one type of AChE. *Es* is also an Aristoceran fly and is thought to possess only one type of AChE.

OP insecticides targeting AChE have been widely used to control the silkworm maggot (10). However, the mechanism of the selective killings is unclear. The differences in detoxification systems and AChEs between the host silkworm and the endoparasitoid maggot might partially explain the selectivity. The silkworm possesses two types of AChEs while the silkworm maggot may have only one type of AChE. The AChE amino acid sequences of the parasite and the host are quite different, with the identity between *Es*-AChE and *Bm*-AChE2 being 58% and the identity between *Es*-AChE and *Bm*-AChE1 being only 36%. The amino acid sequence differences might cause subtle changes in enzyme 3D structures, resulting in their different responses to inhibitors. Our result showed that the median inhibition concentrations (I_{50}) of the three OP insecticides, dichlorvos, chlorpyrifos and omethoate, for *Bm*-AChEs are much higher than for *Es*-AChEs, indicating that *Es* AChEs are more sensitive to the inhibitors. This result gives an insight into the mechanism by which lower concentrations of some OP insecticides can selectively kill *Es* without distinct effects on its host, *B. mori*. Noticeably, *Es*-AChE shows much higher sensitivity to a non-OP inhibitor, eserine, than do *Bm*-AChEs, implying the possibility of developing more selective insecticides other than OPs for silkworm maggot control.

In this study, a mutation (G353A) of *Es*-AChE was identified

in four out of ten flies studied. The G353A mutation was also found in AChE from the fruit fly (G324A) (7), the house fly (G262A) (8), *Lucilia cuprina* (G303A) (11), and *Plutella xylostella* AChE1 (G324A) (12). Initial modeling of these mutations within the *Drosophila* structure revealed that G324A is located close to the active-site triad at the base of the gorge, with G324A likely to interact with gorge residues, which affects the orientation of the catalytic serine (ser-200 in Torpedo) and possibly plays a role in influencing inhibitor sensitivity (13, 14). In this study, the efficiency of *Es*-Gly353-AChE in substrate hydrolysis is lower than that of *Es*-Gly353-AChE (G353A), a result similar to AChEs from other insects (4, 15). The G353A mutation of the *Es*-AChE resulted in a low level of insensitivity to four of the compounds *in vitro* (Table 2), with a similar phenomenon observed for the fruit fly and the housefly. The intensive use of OP insecticides in *Es* control has resulted in the resistance of *Es* to the insecticides. The link of the mutation G353A with the resistance phenotype needs further investigation.

MATERIALS AND METHODS

Insects
The pupae of the silkworm maggot (*Exorista sorbillans, Es*) were collected from Chunan, Zhejiang Province, China. One day after emergence, the adults were immediately frozen and stored at -80°C prior to mRNA extraction.

Chemicals
Acetylthiocholine iodide (ATC), propionylthiocholine iodide (PTC), butyrylthiocholine iodide (BTC) and 5, 5'-Dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. Other analytical grade chemicals were purchased from commercial sources.

Molecular cloning and characterization of the *ace* gene
Total RNA was extracted from whole bodies of adult of *Es* using Trizol reagent according to the manufacturer's protocol (Shanghai Sangon Co., China). First-strand cDNA was synthesized from total RNA using reverse transcriptase with oligo-dT as the primer. Degenerate oligo-nucleotide primers were designed based on the conserved regions of aces from Brachyceran flies. First, a 1277-bp nucleic acid was cloned using degenerate primers JYJB-ACHE-F1 (5'TACTTCCC(T/C)GGCTT(T/C)TCC GG3') and JYJB-ACHE-ZR2 (5'CCCATCCATTC(A/G)CCCCA (T/C)AA3'). The 5' noncoding region prior to the initiation codon was cloned using degenerate primer JYORFqian10 (5'G (C/G)(G/C)(T/G)(T/C)(T/G)(A/T)(T/C)(C/A/T)(C/A)(T/C)TA(G/T) TATGGCTCG3') and nondegenerate primer JYZR797 (5'ACCT GGCATAACCGGTGAAAGATGT3'). The 3' region was cloned using the primer JYZF1565 (5'CACAAATGAATATGCCCAGG C3') and the degenerate primer JYJB-R (5'TTTTATTGAAAAAT GCTTTTGACCATT3'). The full-length AChE gene was then cloned using the primers JY-F (5'-GGATCCATGGCTCGTTTTG TAAAGTCATC; BamHI site underlined) and JY-R (5' TTTTATT

G(A/G)AAAAT GC(A/T)(A/T)(T/G)TGACC3').

The primers JY-F and JY-QR (5'-TCTAGATTAGTTTTCACAT TCAGAACCAAC; *XbaI* site underlined) were used to amplify C-terminal truncated fragments of *Es*-353G ace and *Es*-353A ace for construction of expression vectors. The 99 bp deleted from the 3' terminus encode a glycolipid anchor that tethers the AChE to cell membrane, and its deletion may facilitate the secretion of the expressed AChE into the medium (16).

PCR was initiated by an initial denaturation step of 3 min at 94°C, followed by 31 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 2 min, with a final extension step at 72°C for 10 min. PCR products were purified and then cloned into the pMD-18T vector (Takara Co., China) and sequenced.

Bioinformatics analysis of the *ace* gene
The amino acid sequences of the *Es*, fruit fly, house fly and blow fly *ace* proteins were aligned using ClustalX and edited with GeneDoc software. Identity values were calculated using BLAST (http://blast.ncbi.nlm.nih.gov/). Locations of signal peptide cleavage sites were predicted using Signal P 3.0 (http://www.cbs.dtu.dk/services/signalP/).

Expression of *Es-353G-AChE* and *Es-353A-AChE* in Tn-5B1-4 cells
The C-terminal truncated fragments of the *Es*-353G and *Es*-353A *aces* were inserted into pFastBac1 vectors and the recombinant plasmids were transformed into competent DH10 Bac to obtain recombinant bacmid DNA. Transfection of the constructed bacmid DNA into Tn-5B1-4 cells was mediated by lipofectin. Five days post-transfection, the supernatant containing recombinant virus was harvested by centrifugation at 5,000 g to remove cell debris for 5 min and stored at 4°C for sequential infection and gene expression. Healthy Tn-5B1-4 cells were infected with the recombinant baculovirus; cells infected with the non-recombinant bacmid were used as a control.

AChE secreted into the supernatant was purified by Sepharose affinity chromatography on a computer-monitored fast performance liquid chromatography system (AKTA, GE Healthcare). The affinity column was prepared using our described method (17) with ECH Sepharose 4B as a matrix and procainamide as a ligand. Protein concentrations were determined by the Bradford method using a bovine serum albumin (BSA) as a standard and measuring OD at 595 nm, using a microplate reader.

AChE activity assays
AChE activity was evaluated by measuring the product of its reaction with the substrate acetylthiocholine iodide (ATC). The product, thiocholine, was evaluated according to the method presented in Ellman (18) with some modifications. The reaction mixture contained 180 μl of 0.1 M phosphate buffer (pH 8.0), 0.4 mM ATC, 0.1 mM DTNB (5,5-dithio-bis (2-nitrobenzoic acid)) and 20 μl of enzyme. The reaction mixture was incubated at room temperature for 5 min, and AChE activity

was determined by measuring the OD at 405 nm with a multi-functional microplate reader (Tecan, Genios).

Determination of substrate specificity and kinetic parameters

Substrate specificity and the effect of substrate concentration on the expressed *Es*-353G-AChE and *Es*-353A-AChE were determined for 11 concentrations of ATC, BTC, and PTC ranging from 7.8 to 8,000 μM at 28°C, pH 8.0.
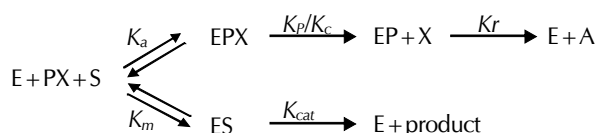
The Michaelis-Menten constant (K_m) and maximal reaction velocity (V_{max}) were determined for the three substrates using a Lineweaver-Burke plot. AChE activity was converted to nanomoles of acetylthiocholine hydrolyzed per minute. The final concentrations of ATC and DTNB were 0.1 mM and 0.4 mM, respectively.

Inhibition of *Es*-353G-AChE and *Es*-353A-AChE by four insecticides

Four insecticides were used as cholinesterase inhibitors, and the median inhibition concentration (I_{50}) for each inhibitor was calculated based on the log of concentration versus probit regression. Differences of I_{50}s were estimated by using DPS© Data Processing System (19). The inhibition of *Es*-353G-AChE and *Es*-353A-AChE with insecticides were analyzed following Zhu *et al*. (20), with several modifications. Each of the eleven different concentrations of insecticides (5 μl) was mixed with each enzyme (20 μl) using a multichannel pipette and incubated for 2 min at room temperature. The inhibition of AChE activity was measured immediately after adding 180 μl of ATC and DTNB solution to the inhibition mixture.

The data were analyzed according to the reactions depicted in the following scheme (21):

K_a (micromolar), K_p or K_c (minutes^{-1}), and K_i ($K_i = K_p$(or K_c)/

$$E+PX+S \quad \overset{K_a}{\underset{K_m}{\rightleftharpoons}} \quad \begin{matrix} EPX \xrightarrow{K_P/K_c} EP+X \xrightarrow{Kr} E+A \\ \\ ES \xrightarrow{K_{cat}} E+product \end{matrix}$$

K_a(molar^{-1}minutes^{-1})) were obtained based on the method of Main *et al*. (22) by applying the following equation:

A straight line was obtained with the double-reciprocal plot of 1/[inhibitor] versus Δt/ (2.303Δlog v), where the slope repre-

$$\frac{1}{[i]} = \frac{K_p \text{ or } K_c}{K_a} \left(\frac{\Delta t}{2.303 \, \Delta \log v} \right) - \frac{1}{K_a}$$

sents the bimolecular reaction constant (K_i).

Acknowledgements

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