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Cloning and Site-Directed Mutagenesis of *Musca domestica* Acetylcholinesterase for Enhancing Sensitivity to Organophosphorus and Carbamate Insecticides

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Abstract Mature acetylcholinesterase (AChE) gene (*gm*, 1,836 bp) was cloned from the housefly and successfully expressed in the *E. coli* CodonPlus (DE3) RIL system (GM-E, 72 kDa) with a yield of 1,630 mU/g fresh cells. Using the *gm*, 10 kinds of mutants were constructed and expressed for enhancing sensitivity to insecticides. The sensitivity of these mutants to five kinds of organophosphate (OP) and three carbamate insecticides was investigated by measuring the apparent bimolecular inhibition constant (k_i=k_2/K_d). Surprisingly, the sensitivity of quadruple mutant IGFT was enhanced as much as 7-fold for acephate, 164-fold for demeton-S-methyl, 484-fold for dichlorvos, 523-fold for edifenphos, 30-fold for ethoprophos, 30-fold for benfuracarb, 404-fold for carbaryl, and 107-fold for furathiocarb, compared with that of GM-E, although the sensitivity of each single point mutant was slightly increased. These mutational studies indicated that (i) contradictory to Walsh *et al.* [39], the residue 327 is the important key residue for enhancing sensitivity as much as the residue 262, (ii) the residue 82 and additional residues of 234, 236, and 585 are also important, and (iii) sensitivity was cooperatively accelerated as the number of strategic mutations increased.

Key words: Acetylcholinesterase, housefly, insecticide sensitivity, mega-primer-based site-directed mutagenesis, apparent bimolecular rate constant of inhibition (k_i)

Acetylcholinesterase (AChE, EC 3.1.1.7) is a serine-type carboxylesterase that catalyzes the hydrolysis of the neurotransmitter acetylcholine in neuromuscular junctions and brain cholinergic synapses in insects [6, 33]. Insect AChE is also a target of organophosphorus (OP) and carbamate insecticides, which are analogs of the substrate acetylcholine, because these insecticides phosphorylate or carbamoylate, the serine residue in the AChE active site [26], leading to the paralysis and death of insects.

Intensive use of insecticides is highly responsible for the resistance of various insects, especially for resistance to acetylcholinesterase. To eliminate these resistant insects, more toxic and excessive insecticides have been used for inhibiting the AChE of insects, to cultivate vegetables. This intensive use of insecticides results in a problem of residual insecticides remaining in vegetables as well as in soil, water, and air. Furthermore, these residual insecticides have an extremely harmful effect on human health. For example, a total forwarding amount of insecticides in the Republic of Korea in the year 2000 was as much as over 6,000 tons, and the World Health Organization (WHO) estimated annual world-wide insecticide-related deaths to be a total of 220,000 persons [36].

The detection of these harmful residual insecticides in vegetables has been chemically analyzed by the combination of gas chromatography and mass spectrometry. However, this technique is expensive and time consuming. Furthermore, when harmful residual insecticides are found in vegetables, these have already been taken in human stomach. Therefore, the technique is not suitable in field or home. Consequently, a necessity for an alternative simple, low cost, and rapid detection method has come to the front. This is the reason of why the residual insecticides detection kit using AChE as a biosensor has been developed. In addition to its rapidity and low cost, the AChE biosensor-based kit has two other advantages. First, AChE allows to detect low concentrations of insecticides in the same sample compared with the original method. Second, it also permits the identification of insecticide by using several electrodes bearing different AChEs [1].

In the present study, in order to develop an OP and carbamate insecticides detection kit using AChE as an enzyme source, the housefly AChE gene (*gm*) was cloned and mutated for enhancing sensitivity to OP and carbamate insecticides. All mutants were expressed in *E. coli* RIL strain and

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purified. Sensitivity to insecticides was calculated by measurement of the apparent bimolecular inhibition constant ($k_i=k_2/K_d$) to five kinds of OP (acephate, demeton-S-methyl, dichlorvos, edifenphos, and ethoprophos) and three carbamate (benfuracarb, carbaryl, and furathiocarb) insecticides. Using these results, the relationships between primary structures of AChE near its active site gorge and the extent of its contribution to sensitivity to OP and carbamate insecticides were examined.

MATERIALS AND METHODS

Houseflies, Plasmids, *E. coli* Strains, and Media

Houseflies, a gift from Gyeongsang National University of Korea, were reared under a 16 L/8 D regime at 25±1°C and 60±5% relative humidity. Adult houseflies were frozen in liquid nitrogen and stored at −70°C for RNA isolation.

The pGEM-T EASY vector (Promega) and the expression vector pRSET B (Invitrogen) were used for cloning and expression of the AChE gene, respectively. *E. coli* TOP10F' (Invitrogen) was used for gene manipulation and *E. coli* BL21 CodonPlus (DE3) RIL (Stratagene) for AChE expression. *E. coli* TOP10F' was grown in Luria-Bertani (LB) medium containing 1% tryptone, 0.5% yeast extract, and 1% NaCl, supplemented with 100 μg/ml ampicillin (Sigma), and 34 μg/ml chloramphenicol (Sigma) was additionally added for the *E. coli* CodonPlus (DE3) RIL strain.

RNA Isolation and Polymerase Chain Reaction (PCR) for Mature AChE Gene Cloning

Total RNA and mRNA were isolated from the heads of houseflies according to the method previously described [19]. For AChE gene cloning, a single-stranded cDNA was prepared from 1 μg of mRNA using the oligo dT-3 sites adapter primer in the 3'-Full RACE Core Set (Takara). 3'-RACE with the 3'-Full RACE Core Set was used to identify the 3'-region of the gene.

The primers used in AChE gene cloning are listed in Table 1. The first PCR was amplified with 0.2 μM each of gene-specific primer MD5 and 3-sites adapter primer, and nested PCR was performed with gene-specific primers 3mature-f and 3mature-r2 to obtain a 3'-end fragment of the gene. The PCR product was cloned into the pGEM-T EASY vector, sequenced, and analyzed.

PCR analysis was performed on the 5'-region of the AChE gene as follows. First, a single-strand cDNA was synthesized with an avian myeloblastosis virus (AMV) reverse transcriptase using an AChE gene-specific primer MD3, followed by hydrolysis of the mRNA with RNase H. After purification of the first strand cDNA by affinity chromatography on a silica matrix, first PCR was performed using gene-specific primers 5up-f and 5mature-r2. Subsequently, nested PCR was performed using gene-specific primers 5mature-f and 5mature-r to obtain a 5'-end fragment of the gene. The PCR product was cloned into the pGEM-T EASY vector, sequence, and analyzed.

Mega-Primer-Based Site-Directed Mutagenesis

Mega-primer-based site-directed mutagenesis was carried out according to Ke and Madison [18].

To prepare a single point replacement mutant, a M82I mega-primer in the case of the M82I mutant was synthesized in the first amplification reaction using the substituted primer, M82I-r and 5mautre-f, at a low annealing temperature using pT-Gm as template. The second PCR was performed using M82I mega-primer (252 bp) and 5mature-r at a high annealing temperature. The resulting PCR product (855 bp) was subcloned into pGEMT EASY, named as pTM82I-855, and sequenced. Subsequently, pT-Gm and pTM82I-855 were digested with KpnI/HindIII, and a 831-bp fragment from pTM82I-855 was inserted into the KpnI/HindIII-digested pTGm (pTGm-M82I).

In the case of A262G mutant, a mega-primer was synthesized in the first amplification reaction using a substituted primer, A262G-f and 5mature-r, at a low annealing temperature (96°C 1 min, 60°C 30 sec, 72°C 30 sec) using pT-Gm as a template. Second PCR was preformed using the A262G mega-primer (72 bp) and 5mature-f at a

Table 1. Synthetic oligonucleotides for AChE gene cloning and its mutagenesis.

Primers	Oligonucleotide Sequences (5'→3')	Notes (underlines)
3-sites adaptor	CTGATCTAGAGGTACCGGATCC	
3mature-f	GGTAAAGCTTTGGTAAATGACTGTAACTGTAATGC	HindIII in the AChE gene
3mature-r	TCTAGACCTTGGAAAATGCTATTCACCATTG	Synthesized XbaI
3mature-r2	GAATTCAATTTGGAAAATGCTATTGACCATTC	Synthesized EcoRI
5mature-f	GGTACCATGACAGATCATCTAACGGTTCAAACG	Synthesized KpnI
5mature-r	CAAAGCTTTACCAATTTCAACCGC	HindIII in the AChE gene
5mature-r2	TACCAAAGCTTTACCAATTTCAACCGCCAGAG	HindIII in the AChE gene
5up-f	CGTTATACTGCTGCGCATGTCAGCG	
MD-5	GGGATCAGGCCTTGGCCTTGC	
MD3	CATAGAGCAGAAAATATGTGCCTTCATC	

high annealing temperature. The resulting PCR product (855 bp) was subcloned into a pGEMT EASY vector (named as pTA262G-855) and sequenced. Subsequently, pT-Gm and pTA262G-855 were digested with KpnI/HindIII restriction enzymes, and a 831-bp fragment from pTA262G-855 was inserted into the KpnI/HindIII-digested pTGm (pTGm-A262G).

A similar procedure was used to synthesize the Y327F mutant with the first PCR primers, 3mature-f and Y327F-r, and the second PCR primers Y327F mega-primer (152 bp) and 3mature-r. The second PCR product of 1,005 bp was ligated into pGEMT EASY, and named as pTY327F-1005. Digestion of pT-Gm and pTY327F-1005 with HindIII/XbaI and insertion of the 995 bp fragment from pTY327F-1005 into the HindIII/XbaI-digested pTGm produced a mature AChE carrying the Y327F mutation (pTGm-Y327F).

A585T mutant was prepared by using pT-Hm [19], which was cloned from another housefly provided by Korean National Institute of Health; pT-Hm and pT-Gm were digested with HindIII/XbaI, and a 995-bp fragment from pT-Hm was then inserted into the HindIII/XbaI-digested pT-Gm (pTGm-A585T).

The double mutant (GF, A262G/Y327F) was generated by combining the A262G mutant gene with the Y327F mutant. The pTGm-A262G and pTGm-Y327F were digested with HindIII and XbaI, and the 995-bp fragment from pTY327F was inserted into the HindIII/XbaI-digested pTA262G (pTGm-GF).

Another double mutant (IG, M82I/A262G) was made by PCR using pTGm-A262G as a template. Thus, the pTGm-A262G was amplified with the M82I mega-primer, and 5mature-r (pTIG-855), pT-Gm, and pTIG-855 were then digested with KpnI/HindIII restriction enzyme and a 831-bp fragment from pTIG-855 was inserted into the KpnI/HindIII-digested pTGm (pTGm-IG). The other mutant (IF, M82I/Y327F) was made by the digestion of pTGm-M82I and pTGm-Y327F (pTGm-IF).

The triple mutant (IGF, M82I/A262G/Y327F) was made by the digestion of pTGm-IG and pTGm-Y327F with HindIII/XbaI, and a 995-bp fragment from pT-Y327F was then inserted into the HindIII/XbaI-digested pTGm-IG (pTGm-IGF).

Quadruple mutant (IGFT, M82I/A262G/Y327F/A585T) was made using PCR. The pT-Hm was amplified with 3mature-f and Y327F-r by the first PCR, and the second PCR was performed by Y327F mega-primer (152 bp) and 3mature-r. The second PCR product of 1,005 bp was ligated into pGEMT EASY, and named as pTHm-Y327F-1005. Digestion of pTGm-IGF and pTHm-Y327F-1005 by HindIII/XbaI, and insertion of the 995-bp fragment from pTHm-Y327F-1005 into the HindIII/XbaI-digested pTGm-IGF, produced a mature AChE carrying the IGFT mutation (pTGm-IGFT).

The resistant mutant (double mutant, GS, E234G/A236S) was constructed by using another strain of AChE gene, *hm* (pT-Hm) [19]. pT-Hm was digested with KpnI/HindIII, and a 831-bp fragment from pT-Hm was inserted into the KpnI/HindIII-digested pT-Gm (named pTGm-GS).

Expression of AChE in *E. coli*

The cloned AChE gene, its mutated genes, and the expression vector pRSET B were digested with KpnI and EcoRI. Each gene (1,836 bp) was inserted into the KpnI/EcoRI-digested pRSET B, which was then transformed into *E. coli* CodonPlus (DE3) RIL strain, respectively. A single transformant was inoculated into 5 ml of LB medium at 37°C. When optical density (OD) at 600 nm reached 1.0, isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) was added to a final concentration of 2 mM, and the cells were incubated additionally for 16 h at 30°C and then harvested by centrifugation at 4°C for 5 min at 2,760 ×*g* [21]. The harvested cells were resuspended in 1 ml of 50 mM Tris-HCl buffer (pH 8.0) and sonicated for 2 min. After centrifugation (1 h, 15,000 ×*g*, and 4°C), the supernatant obtained was used for the measurement of AChE activity.

Purification of AChEs

The preparation of AChE affinity resin was carried out according to the procedure described by Cuatrecasas *et al.* [4], Cuatrecasas [2], and Cuatrecasas and Anfinsen [3]. Spacers of 1,6-hexane diamine (2 g) and succinic anhydride (0.3 M) were attached to the CNBr-activated Sepharose 4B (3 g) swollen in 1 mM HCl. The succinylated resin was aminated by 1,6-hexane diamine (2 g) with the aid of carbodiimide, and resuccinylated by succinic anhydride. For completion of the reaction, trimethyl (*m*-aminophenyl) ammonium hydrochloride was covalently attached to the end of the spacer on the resultant succinylated resin, which was then packed into a column (1×10 cm).

Each harvested supernatant was applied (2 ml/min) onto an AChE affinity column equilibrated with 50 mM Tris-HCl (pH 8.0). Unspecifically bound proteins were desorbed by washing with an equilibration buffer containing 0.4 M NaCl. Bound AChE was eluted with an equilibration buffer containing 0.4 M NaCl and 0.2 M tetraethylammonium iodide (Sigma). At the re-affinity chromatography step, AChE was eluted by a competitor of tetraethylammonium iodide (0.2 M) containing acetylthiocholine iodide (ATCh, 0.2 M), which was then removed by dialysis against 100 mM sodium phosphate buffer (pH 8.0) to obtain an active recombinant AChE. Finally, AChE was concentrated by ultrafiltration (Amicon) on ice using a 10-kDa ultrafiltration membrane (Millipore).

Enzyme Assay

AChE activity was measured at 25°C according to the method of Riddles *et al.* [32] using acetylcholine iodide

as a substrate. The reaction mixture with an extinction coefficient of 14,150 $M^{-1}cm^{-1}$ at 412 nm contained 0.5 mM acetylthiocholine iodide (ASCh) and 0.5 mM 2,2'-dinitro-5,5'-dithiobenzoic acid (DTNB) in 0.1 M sodium phosphate buffer, pH 8.0. The reaction rate was monitored spectrophotometrically by increase of absorbance at 412 nm for 5 min after the addition of AChE. Protein concentration was determined with the Bradford protein assay kit (Bio-Rad).

Determination of Kinetic Parameters

The initial velocity (V_0) of each mutant, Michaelis-Menten constant (K_m), and maximal velocity (V_{max}) were determined at various low substrate (ATCh) concentrations (3 μM–20 mM) in 0.1 M sodium phosphate buffer (pH 8.0) by measuring the amount of acetylthiocholine iodide (ATCh) hydrolyzed. The turnover number of AChE, k_{cat}, was also determined.

Inhibitory Studies

If OP or carbamate insecticide concentration is at least 10-fold higher than that of substrate ATCh, the inhibitory reaction of AChE with OP or carbamate insecticide is bimolecular, but follows pseudo-first-order kinetics. This progressive inhibition of AChE hydrolysis with insecticide is described by the following reaction scheme:

$$\begin{array}{ccccc} & & & k_2 & \\ & & \mathbf{E.P\text{-}X} & \rightarrow & \mathbf{E\text{-}P + X} \\ & K_d \nearrow \swarrow & & & \\ \mathbf{E + P\text{-}X + S} & & & & \\ & K_m \searrow \nwarrow & & & \\ & & \mathbf{E\text{-}S} & \rightarrow & \mathbf{E + products} \\ & & & k_{cat} & \end{array}$$

where E is the free enzyme, P-X is an OP or carbamate insecticide, E.P-X is the Michaelis complex, E-P is the phosphorylated or carbamylated enzyme, and X is the leaving group.

K_d ($=k_1/k_{-1}$) is the apparent dissociation constant for the enzyme-inhibitor Michaelis complex:

$$K_d=K_m[P\text{-}X]/(K_m+[S])(V_C/V_0-1)$$

where V_C is the velocity of a control reaction carried out in the absence of inhibitor but at the same [S] as in the inhibition reaction, and V_0 is the velocity of a reaction carried out in the presence of inhibitor.

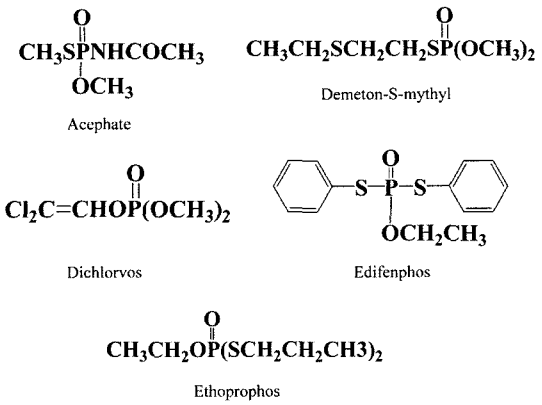
k_2 is the phosphorylation or carbamoylation rate constant.

$$k_2=(\Delta \ln v/\Delta t)/\{1+K_d/[P\text{-}X](1-\alpha)\}$$

where

$$\ln v=\ln(v_1/v_2)=\ln\{([E]_0-[E\text{-}P_1])/([E]_0-[E\text{-}P_2])\}$$

A. OP insecticides



B. Carbamate insecticides

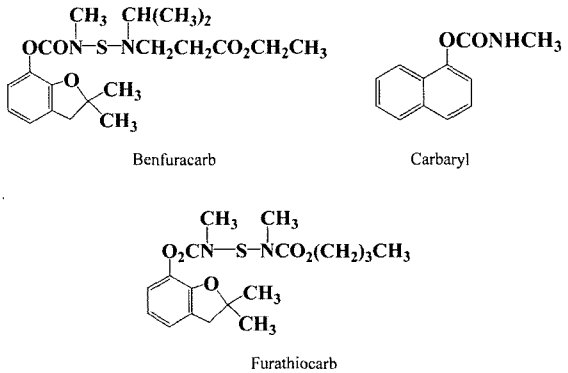


Fig. 1. The chemical structures of OP and carbamate insecticides tested.
A. OP insecticides; **B.** Carbamate insecticides.

$$\alpha=[S]/(K_m+[S]).$$

k_i ($=k_2/K_d$) is the apparent bimolecular rate constant of inhibition. The values were measured for each enzyme at various time points by incubation with the inhibitor [12, 25].

Organophosphate (OP) insecticides such as acephate, demeton-S-methyl, dichlorvos, edifenphos, and ethoprophos (Fig. 1A), and carbamate insecticides such as benfuracarb, carbaryl, and furatiocarb (Fig. 1B), were purchased from Sigma. Inhibitory activity of each mutant AChE was measured in the presence of 0.5 mM ATCh at eight concentrations of OP or carbamate insecticide. A control reaction was performed without any insecticides. Progressive inhibition activity of each mutant AChE over time was continuously recorded for 5 min. The residual activity of AChE at every 30-sec interval was measured for fitting the inhibition curve.

The average sensitivity of each mutant AChE to OP and carbamate insecticides was calculated by the use of following formula:

$$_e[\{\Sigma(k_i \text{ of each mutant AChE}/k_i \text{ of GM-E})\}/n]$$

RESULTS AND DISCUSSION

Cloning of Mature AChE Gene

About 800 mg of mRNA was prepared from 500 housefly heads and used as a template for cDNA synthesis. Using 5'-RACE, an 849-bp fragment including the 5'-region of the AChE gene was amplified and cloned into the pGEMT EASY vector (pT5G). In addition, a 1,006-bp fragment harboring the 3'-region of AChE gene was amplified by 3'-RACE and cloned into the pGEMT EASY vector (pT3G). Both pT5G and pT3G were digested with HindIII/SacI. A 1,046-bp fragment from pT3G was ligated into the HindIII/SacI-digested pT5G, and the resulting construct was designated as pT-Gm, which consisted of a mature AChE gene (*gm*) and the pGEMT EASY vector.

The open reading frame of *gm* (GenBank accession number AF533335) consisted of a 1,836-bp mature protein sequence (Fig. 2). The catalytic triad (★ in Fig. 2; S235, E364, and H477), choline-binding site (W83 in Fig. 2), four potential N-linked glycosylation sites (# in Fig. 2), intra-disulfide linkages (C66-S-S-C93, C289-S-S-C309, and C439-S-S-C557 in Fig. 2), and inter-disulfide linkage site (C575 in Fig. 2) were found to be conserved in *gm*. When the deduced amino acid sequence of the *gm* was

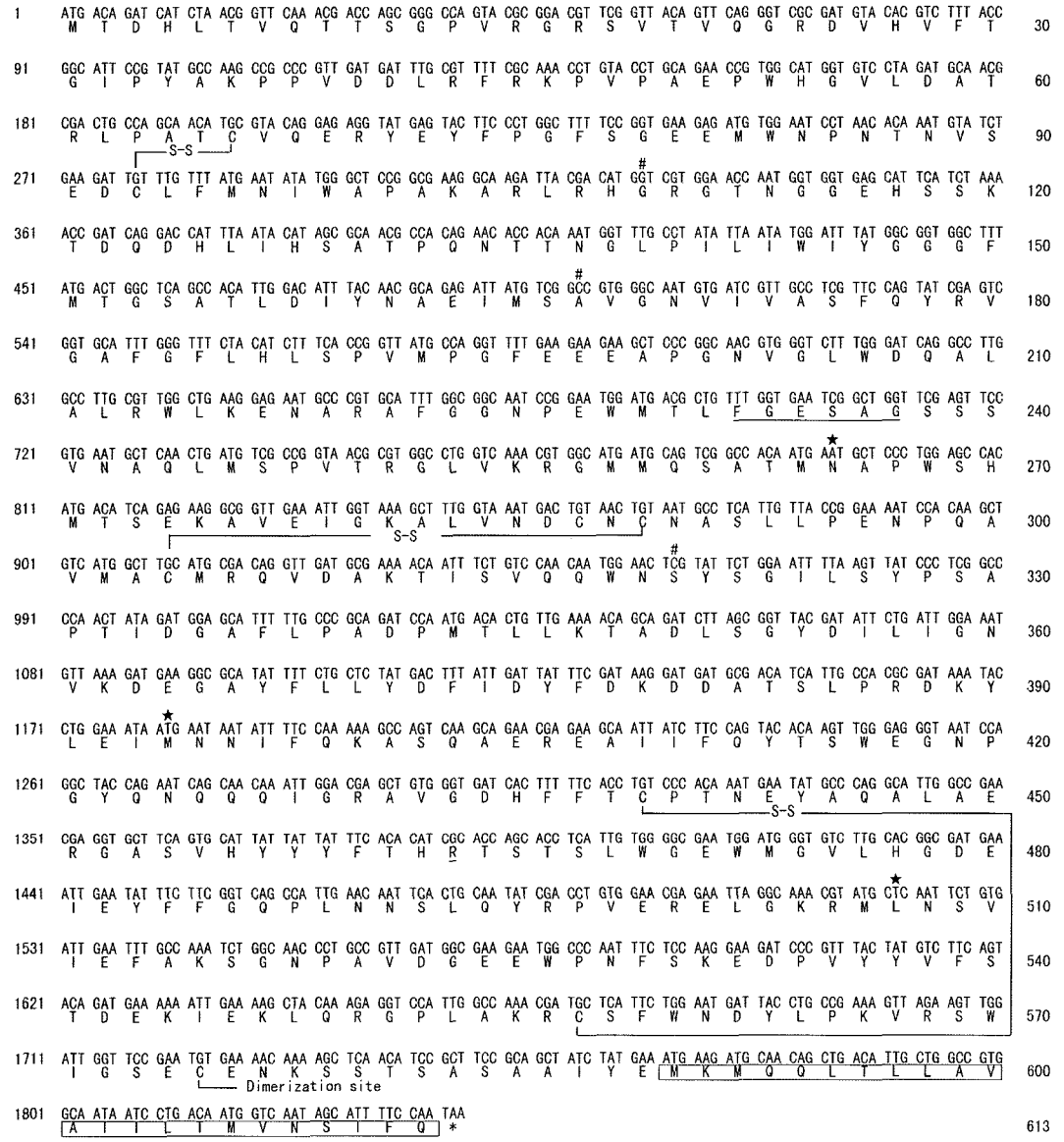


Fig. 2. DNA sequence of the *M. domestica* AChE cDNA, *gm*, and its deduced amino acid sequence.
Various symbols are as follows: for the catalytic triad, S235, E364, and H477; # for the four potential N-linked glycosylation sites. The intra-disulfide linkages are indicated at C66-S-S-C93, C289-S-S-C309, and C439-S-S-C557, and inter-disulfide linkage site is C575. The glycolipid anchor is shown in the box. This sequence and deduced amino acid sequences have been deposited in the GenBank with accession number AF533335.

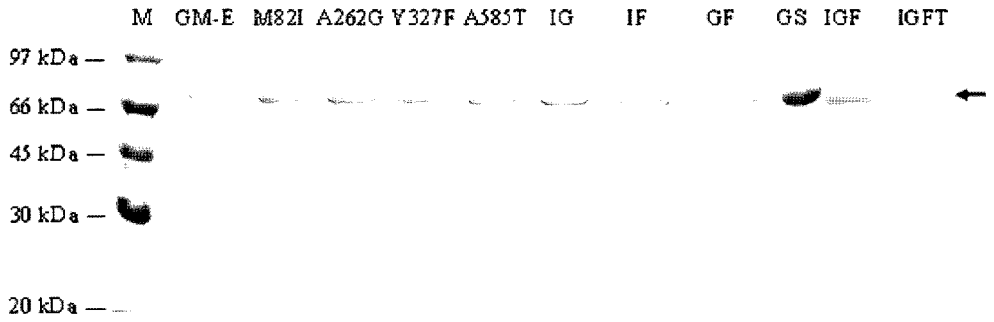


Fig. 3. SDS-PAGE (10%) after purification of wild-type AChE (GM-E) and its mutants expressed in *E. coli*. M is the molecular weight marker and the arrow indicates the purified AChEs.

compared with other *M. domestica* genes, *gm* showed high sequence homologies with two other houseflies (99% identity) [19,22].

Functional Expression and Purification of Wild-Type AChE in *E. coli*

The open reading frame of *gm* was subcloned into pRSET digested with KpnI/EcoRI, and the resulting construct (pRGm) was transformed into the *E. coli* BL21 CodonPlus (DE3) RIL strain. AChE activity was detected with a yield of 1,630 mU/g of fresh cells. The yield of GM-E (1,630 mU/g cells, 163 mU/ml) was 15-fold higher than that of Heim *et al.* [13]. In addition, the yield was increased 5.8-fold (28.0 mU/ml) [19] and 3.5-fold (46.0 mU/ml) [13] compared with that of AChEs expressed in *Pichia pastoris*.

The high purity of the GM-E has not been achieved using His-tagged affinity chromatography under various conditions [17, 20, 21]. Thus, in the present study, the expressed enzyme (GM-E) was purified by AChE affinity column chromatography. AChE activity was detected only in the fraction eluted with 0.2 M tetraethylammonium iodide solution. The purified GM-E showed 53.4-fold increase of specific activity with a yield of 20%, but with low purity. Re-chromatography of AChE was performed for further purification, resulting in 920.2-fold increase of specific activity with a yield of 6.4%. Ultrafiltration to concentrate the enzyme preparation resulted in 990.8-fold increase without any loss of AChE. This GM-E was then subjected to 10% SDS-PAGE and resulted in over 95% purity with an estimated molecular weight of about 72 kDa (Fig. 3).

Site-Directed Mutagenesis, Expression of Mutated AChEs in *E. coli*, and Purification

The construct of each single mutation was named aspTGm-M82I, pTGm-A262G, pTGm-Y327F, and pTGm-A585T. Double mutant constructs were pTGm-GF, pTGm-IG, pTGm-IF, and pTGm-GS. Triple construct was pTGm-IGF, and quadruple mutant was pTGm-IGFT. All mutants were subcloned into pRSEC B and were transformed into the *E. coli* BL21 CodonPlus (DE3) RIL strain, respectively. All mutated AChEs were successfully expressed as a functionally active form (soluble form) such as wild-type (GM-E). Expressed AChEs were abbreviated as follows: M82I for pRGm-M82I, A262G for pRGm-A262G, Y327F for pRGm-Y327F, A585T for pRGm-A585T, GF for pRGm-GF, IG for pRGm-IG, IF for pRGm-IF, GS for pRGm-GS, IGF for pRGm-IGF, and IGFT for pRGm-IGFT (Table 2). Each expressed mutant was purified by AChE affinity chromatography and purified AChEs were

Table 2. Kinetic parameters of AChEs expressed in *E. coli* and its mutants.

Mutants	K_m±SD (μM)	V_{max}±SD (nM/min)	k_{cat}±SD ($\times 10^3$ min^{-1})
Wild-type (GM-E)	29.2±0.5	9.3±0.8	40.7±9.3
M82I	28.9±0.2	8.8±0.4	38.6±4.6
A262G	22.1±0.4	14.4±0.5	62.5±5.6
Y327F	43.7±0.7	22.5±0.5	97.8±3.5
A585T	29.3±0.5	8.8±0.6	38.4±6.8
IG (M82I+A262G)	21.6±0.9	20.6±0.9	89.4±7.7
IF (M82I+Y327F)	40.3±0.5	28.2±0.6	122.6±2.6
GF (A262G+Y327F)	15.8±0.5	32.9±0.5	143.0±5.2
GS (E234G+A236S)	35.2±0.3	8.4±0.4	36.3±1.6
IGF (M82I+A262G+Y327F)	12.1±0.2	33.6±0.5	146.2±3.6
IGFT (M82I+A262G+Y327F+A585T)	11.6±0.2	40.0±1.5	147.7±5.3

analyzed on 10% SDS-PAGE (Fig. 3). Fig. 3 shows that each mutant was successfully expressed in *E. coli* such as wild-type and purified by AChE affinity chromatography. The molecular weight of each purified mutant was estimated as 72 kDa, similar to wild-type, GM-E.

Determination of Kinetic Parameters of AChEs

The kinetic constants, K_m and V_{max}, were determined using Lineweaver-Burk plots (Table 2). The K_m of GM-E was 29.2 μM. In most cases, the point mutations had an effect on both K_m and V_{max} values of the enzymes (Table 2). In the case of single point mutations, the K_m values of M82I (28.9 μM) and A585T (29.3 μM) were similar to the affinity of the enzyme for the substrate ATCh compared with that of GM-E. The K_m value of A262G (22.1 μM) decreased by 24%, whereas that of Y327F (43.7 μM) was increased by 50%. Furthermore, the K_m values of double mutant GF, triple mutant IGF, and quadruple mutant IGFT were

Table 3. Dissociation constants (K_d), biomolecular rate constants ($k_i=k_2/K_d$), and sensitivity ratios of recombinant AChEs expressed in *E. coli* to OP insecticides, acephate, demeton-S-methyl, dichlorvos, edifenphos, and ethoprophos.

Mutant	Acephate			Demeton-S-methyl		
	K_d±SD (10^{-6} M)	k_i±SD ($\times10^6$ $M^{-1}min^{-1}$)	Sensitivity ratio (fold)	K_d±SD (10^{-6} M)	k_i±SD ($\times10^6$ $M^{-1}min^{-1}$)	Sensitivity ratio (fold)
WT	34.3±2.0	0.006±0.001	1.0	4.126±0.205	0.052±0.005	1.0
M82I	35.7±0.2	0.006±0.001	1.1±0.4	4.302±0.204	0.045±0.002	0.9±0.1
A262G	26.1±0.4	0.008±0.001	1.4±0.4	2.956±0.424	0.096±0.003	1.9±0.3
Y327F	15.6±0.7	0.014±0.001	2.7±0.4	2.862±0.753	0.087±0.012	1.7±0.4
A585T	33.5±0.5	0.006±0.001	1.1±0.2	4.052±0.522	0.051±0.002	1.0±0.1
IG	24.6±0.9	0.008±0.001	1.4±0.4	2.742±0.942	0.072±0.021	1.5±0.6
IF	15.3±0.5	0.014±0.001	2.5±0.6	2.553±0.525	0.095±0.003	1.9±0.3
GF	6.9±0.8	0.031±0.004	5.5±1.6	0.446±0.008	0.562±0.042	11.0±1.9
GS	142.2	0.001	0.2±0.6	30.409±1.90	0.005±0.001	0.1
IGF	4.3±0.3	0.053±0.004	9.2±2.2	0.057±0.002	7.041±0.286	137.2±18.8
IGFT	5.1±0.4	0.043±0.004	7.5±1.9	0.052±0.002	8.402±0.479	164.0±25.0

Mutant	Dichlorvos			Edifenphos		
	K_d±SD (10^{-6} M)	k_i±SD ($\times10^6$ $M^{-1}min^{-1}$)	Sensitivity ratio (fold)	K_d±SD (10^{-6} M)	k_i±SD ($\times10^6$ $M^{-1}min^{-1}$)	Sensitivity ratio (fold)
WT	1.765±0.091	0.129±0.006	1.0	0.396±0.014	0.571±0.032	1.0
M82I	1.793±0.133	0.123±0.008	1.0±0.1	0.422±0.201	0.791±0.398	1.4±0.8
A262G	0.173±0.042	1.393±0.003	10.8±0.5	0.325±0.192	1.040±0.614	1.9±1.2
Y327F	0.275±0.304	0.837±0.021	6.5±0.5	0.245±0.105	0.855±0.434	1.6±0.9
A585T	1.822±0.225	0.105±0.008	0.8±0.1	0.398±0.051	0.703±0.087	1.3±0.3
IG	0.172±0.052	1.403±0.464	11.1±0.1	0.315±0.008	0.668±0.002	1.2±0.1
IF	0.192±0.053	1.277±0.645	10.1±0.5	0.296±0.015	1.613±0.082	2.8±0.3
GF	0.155±0.016	2.289±0.374	18.0±3.8	0.270±0.002	13.244±0.911	23.4±3.0
GS	3.955±0.403	0.046±0.001	0.4	0.844±0.021	0.261±0.003	0.5
IGF	0.013±0.001	47.360±2.181	368.8±34.1	0.003	166.714±7.07	293.0±28.9
IGFT	0.0121	62.348±0.889	484.7±29.4	0.002	297.362±7.85	523.2±43.1

Mutant	Ethoprophos		
	K_d±SD (10^{-6} M)	k_i±SD ($\times10^6$ $M^{-1}min^{-1}$)	Sensitivity ratio (fold)
WT	34.25±6.96	0.006±0.001	1.0
M82I	34.39±3.21	0.006±0.001	1.1±0.3
A262G	28.92±3.62	0.008±0.001	1.4±0.4
Y327F	26.67±4.77	0.008±0.001	1.4±0.4
A585T	34.57±3.53	0.006±0.001	1.1±0.3
IG	24.76±2.96	0.008±0.001	1.4±0.4
IF	21.3±5.75	0.009±0.001	1.6±0.4
GF	4.48±0.43	0.048±0.002	8.3±1.7
GS	36.14±12.26	0.006±0.001	1.1±0.3
IGF	1.12±0.02	0.196±0.006	33.8±6.6
IGFT	1.21±0.06	0.174±0.007	30.0±6.2

gradually reduced with increasing number of point mutations, whereas that of resistant mutant GS was increased by 21%.

The catalytic activities per active site for mutant AChEs (k_{cat}) were also examined (Table 2). The turnover number (k_{cat}) of GM-E was estimated to be 40.7×10^3 min^{-1}, which was about one third of *M. domestica* AChE (123.8×10^3 min^{-1}) [15] and *D. melanogaster* (107×10^3 min^{-1}) [9]. The k_{cat} values of M82I (38.6×10^3 min^{-1}) mutant and A585T (38.4×10^3 min^{-1}) with single amino acid replacement were similar to that of GM-E, whereas those of A262G and Y327F were enhanced by 54% and 140%, respectively. The double mutant GF, triple IGF, and quadruple IGFT showed average 3.5-fold higher k_{cat} values than GM-E. The GS mutant showed slightly lower turnover efficiency than the GM-E.

Inhibitory Studies

The inhibition studies of AChE from GM-E and its mutants with the OP insecticides and carbamate insecticides are summarized in Table 3 and 4, respectively. The pseudo-first-order kinetics was observed for the progressive inhibition of AChE with over 10-fold excess of insecticides, compared with the substrate ATCh.

Studies on Inhibition of OP Insecticides

Table 3 shows the apparent bimolecular rate constant of inhibition ($k_i=k_2/K_d$) of acephate, demeton-S-methyl, dichlorvos, edifenphos, and ethoprophos by each mutant AChE.

First of all, the K_d values of single point mutants M82I (35.7×10^{-6} M) and A585T (33.5×10^{-6} M) for acephate were similar to that of wild-type GM-E (34.3×10^{-6} M). However, those of A262G (26.1×10^{-6} M) and Y327F (15.6×10^{-6} M) decreased significantly, indicating that both A262G and Y327F mutations enhanced the affinity of A262G mutant and Y327F to acephate. The double mutant GF ($6.9\pm0.8\times10^{-6}$ M), triple mutant IGF ($4.3\pm0.3\times10^{-6}$ M), and quadruple mutant IGFT ($5.1\pm0.4\times10^{-6}$ M) reduced their K_d values significantly, enhancing their affinities to acephate. However, there was less variation for k_2 values among all mutants. The k_i value was used to estimate the sensitivity levels of each mutant expressed in *E. coli*, compared with that of GM-E. The k_i values of single point mutant M82I and A585T were identical to that of wild-type GM-E (0.006×10^6 $M^{-1}min^{-1}$). However, other single point mutants A262G and Y327F showed 33% and 133% higher k_i values than GM-E. The k_i value of triple point mutant IGF

Table 4. Dissociation constants (K_d), biomolecular rate constants ($k_i=k_2/K_d$), and sensitivity ratios of recombinant AChEs expressed in *E. coli* to carbamate insecticides, benfuracarb, carbaryl, and furathiocarb.

Mutant	Benfuracarb			Carbaryl		
	K_d±SD (10^{-6} M)	k_i±SD ($\times10^6$ $M^{-1}min^{-1}$)	Sensitivity ratio (fold)	K_d±SD (10^{-6} M)	k_i±SD ($\times10^6$ $M^{-1}min^{-1}$)	Sensitivity ratio (fold)
WT	0.107±0.007	2.521±0.196	1.0	0.218±0.011	1.245±0.072	1.0
M82I	0.122±0.003	2.160±0.243	0.6±0.5	0.215±0.014	1.266±0.082	1.0±0.1
A262G	0.093±0.011	2.641±0.310	1.1±0.2	0.203±0.008	1.210±0.087	1.0±0.1
Y327F	0.78±0.006	3.508±0.270	1.4±0.2	0.189±0.012	1.259±0.080	1.0±0.1
A585T	0.119±0.004	2.363±0.082	1.0±0.1	0.227±0.004	1.222±0.020	1.0±0.1
IG	0.093±0.008	3.456±0.297	1.4±0.2	0.195±0.004	1.201±0.024	1.0±0.1
IF	0.087±0.013	3.445±0.514	1.4±0.3	0.167±0.012	1.415±0.101	1.2±0.2
GF	0.026±0.002	13.041±1.170	5.2±0.9	0.029±0.005	26.651±2.704	21.6±3.4
GS	0.392±0.015	0.572±0.021	0.2	0.057±0.036	0.41±0.034	0.3
IGF	0.006±0.001	77.253±6.344	31.0±4.9	0.002±0.001	353.10±18.91	285.4±32.0
IGFT	0.006±0.001	75.541±11.581	30.5±7.0	0.002±0.001	499.46±33.90	404.1±50.1

Mutant	Furathiocarb		
	K_d±SD (10^{-6} M)	k_i±SD ($\times10^6$ $M^{-1}min^{-1}$)	Sensitivity ratio (fold)
WT	0.355±0.046	0.640±0.008	1.0
M82I	0.361±0.006	0.637±0.009	1.0
A262G	0.329±0.002	0.631±0.022	1.0±0.1
Y327F	0.311±0.012	0.720±0.033	1.1±0.1
A585T	0.348±0.002	0.688±0.016	1.1
IG	0.298±0.006	0.792±0.016	1.2
IF	0.304±0.004	0.803±0.010	1.3
GF	0.014±0.004	17.21±0.006	26.9±0.3
GS	0.481±0.002	0.567±0.031	0.9±0.1
IGF	0.006±0.001	77.254±6.342	120.9±11.4
IGFT	0.004±0.003	68.532±0.654	107.1±2.4

was the highest among all mutants tested, indicating that it was the most sensitive to acephate. The quadruple mutant IGFT showed the next highest value. However, just as predicted, resistant mutant GS showed a lower k_i value than GM-E. The high K_d value of GS mutant was responsible for decreasing its k_i value.

In the case of demeton-S-methyl, the K_d and k_2 values showed patterns similar to those towards acephate. The k_i value of quadruple mutant IGFT (8.402×10^6 $M^{-1}min^{-1}$) to demeton-S-methyl showed the highest value among all mutants tested. The k_i value of GS mutant to demeton-S-methyl was only one-tenth of that of GM-E.

For dichlorvos, the k_i value of quadruple mutant IGFT (62.348×10^6 $M^{-1}min^{-1}$) was dramatically increased (484-fold), compared with that of GM-E. However, the k_i value of GS mutant was reduced to 36% of that of GM-E.

For edifenphos, the K_d and k_2 values showed patterns similar to other OP insecticides. The k_i value of quadruple mutant IGFT (297.36×10^6 $M^{-1}min^{-1}$) to edifenphos showed the maximal value among all OP insecticides tested (523-fold). Such high k_i value might be due to the structure of edifenphos, which is similar to that of acetylcholine. The k_i value of GS mutant was reduced to 46% compared with that of GM-E (Table 3).

For ethoprophos, the K_d and k_2 values showed similar patterns. However, the k_i value of triple mutant IGF ($0.196\pm0.006\times10^6$ $M^{-1}min^{-1}$) to ethoprophos was 33.8-fold higher than that of IGFT (30-fold).

Inhibitory Studies on Carbamate Insecticides

Table 4 shows the apparent bimolecular rate constant of inhibition of each mutant AChE towards benfuracarb, carbaryl, and furathiocarb.

For benfuracarb, the K_d values of both single point mutants M82I and A585T were similar to that of wild-type GM-E. Those of A262G and Y327F were slightly decreased, indicating that both of these A262G and Y327F mutations enhanced the affinity between these mutants and benfuracarb. The triple mutant IGF and quadruple mutant IGFT revealed significantly reduced K_d values, indicating enhancement of their affinities to benfuracarb. There was little variation among all mutants for k_2 values. The k_i values of single point mutant M82I and A585T were 14% lower than that of wild-type GM-E (2.521×10^6 $M^{-1}min^{-1}$). However, single point mutants A262G and Y327F showed 17% and 40% higher k_i values. The k_i values of double point mutants IG (37%), IF (36%), and GF (5.2-fold) were enhanced more than that of the single point mutants. The k_i value of triple point mutant IGF (77.253×10^6 $M^{-1}min^{-1}$) was the highest among the mutants tested, except for IGFT. The k_i value of resistant mutant GS was reduced (0.571×10^6 $M^{-1}min^{-1}$).

In the case of carbaryl, the K_d and k_i values showed patterns similar to benfuracarb. The k_i value of quadruple mutant IGFT (499.46×10^6 $M^{-1}min^{-1}$) was dramatically (404-fold) increased, compared with that of GM-E. The k_i value of GS mutant was reduced by 33%, compared with that of GM-E (Table 4).

For furathiocarb, the K_d and k_2 values showed similar patterns. The k_i value of quadruple mutant IGFT (68.532×10^6 $M^{-1}min^{-1}$) was dramatically increased, compared with that of GM-E, and the k_i value of GS mutant was reduced by 11% compared with that of GM-E (Table 4).

Sensitivity Ratio of AChEs

The k_i value of each mutant AChE was divided by the k_i of wild-type, GM-E, to calculate the relative ratio. This ratio indicates that the sensitivity of each mutant's AChE is enhanced or reduced (*i.e.*, how many fold), when GM-E's value is set at 1.

Sensitivity Ratio of AChEs to OP Insecticides

The replacements of amino acids associated with sensitivity to OP insecticides showed various sensitivity ratio patterns to OP insecticides. Each mutant displayed a sensitivity ratio that was positive and larger than zero to acephate, demeton-S-methyl, dichlorvos, edifenphos, and ethoprophos tested (Table 3).

The single amino acid replacement mutant Y327F showed the following sensitivity ratios: to acephate (2.7-fold), demeton-S-methyl (1.7), dichlorvos (6.5), edifenphos (1.6), and ethoprophos (1.4), compared with GM-E. A262G showed a slight increase of sensitivity ratio to acephate (1.4), demeton-S-methyl (1.9), dichlorvos (10.8), edifenphos (1.9), and ethoprophos (1.4). Other single mutants M82I and A585T showed a similar sensitivity ratio, ranging from 0.9 to 1.4, to acephate, demeton-S-methyl, dichlorvos, edifenphos, and ethoprophos, compared with that of GM-E.

In the case of double amino acids replacement, the sensitivity ratio of each double mutant was generally higher than those of single mutants. The sensitivity ratio of IG mutant was as follows: acephate (1.4-fold), demeton-S-methyl (1.5), dichlorvos (11.1), edifenphos (1.2), and ethoprophos (1.4), compared with GM-E. For IF, the ratio was as follows: acephate (2.5), demeton-S-methyl (1.9), dichlorvos (10.1), edifenphos (2.8), and ethoprophos (1.6). For GF, the ratio was as follows: acephate (5.5), demeton-S-methyl (11.0), dichlorvos (18.0), edifenphos (23.4), and ethoprophos (8.3).

Generally, quadruple mutant IGFT showed the highest sensitivity ratio: acephate (7.5-fold), demeton-S-methyl (164.0), dichlorvos (484.7), edifenphos (523.2), and ethoprophos (30). However, in some cases, IGF showed the highest sensitivity ratio to acephate (9.2) and ethoprophos (33.8) instead of IGFT.

On the other hand, only the resistant mutant GS showed reduced sensitivity ratio: acephate (0.2-fold), demeton-

S-methyl (0.1), dichlorvos (0.4), edifenphos (0.5), and ethoprophos (1.1).

Sensitivity Ratio of AChEs to Carbamate Insecticides

The replacement of amino acid showed various sensitivity ratio patterns to carbamate insecticides. Each mutant displayed a sensitivity ratio that was positive and larger than zero to benfuracarb, carbaryl, and furathiocarb (Table 4).

The 327F mutant showed the following sensitivity ratio: to benfuracarb (1.4-fold), carbaryl (1.0), and furathiocarb (1.1); M82I, A262G, and A585T mutants showed about one sensitivity ratio to carbamate insecticides.

In the case of double amino acids replacement, the average sensitivity ratio of double mutants was generally higher than that of single mutants. The sensitivity ratio of IG mutant was as follows: benfuracarb (1.4), carbaryl (1.0), and furathiocarb (1.2), compared with GM-E. For IF, the ratio was as follows: benfuracarb (1.4), carbaryl (1.2), and furathiocarb (1.3). The sensitivity ratio of GF mutant was significantly increased as follows: benfuracarb (5.2), carbaryl (21.6), and furathiocarb (26.9).

The triple mutant IGF showed the highest sensitivity ratio to benfuracarb (31.0-fold) and furathiocarb (120.9-fold). However, IGFT showed the highest sensitivity ratio for carbaryl (404.1-fold).

On the other hand, the resistant mutant GS demonstrated significantly reduced sensitivity ratios to benfuracarb (0.2-fold), carbaryl (0.3), and furathiocarb (0.9).

Enhancement of AChE Sensitivity to OP and Carbamate Insecticides Using Site-Directed Mutagenesis

Comparison of primary and tertiary structures of proteins provides valuable information about a protein's unique function. Although AChEs from *Torpedo californica* [34] and *Drosophila melanogaster* [11] show low sequence identity (36%), they share conserved residues at the active site and the mutated sites. In particular, these conserved and mutated residues have important implications for the function of AChE. It has generally been known that intensive use of insecticides is strongly responsible for resistance of various insects to insecticides and results in resistant AChE-containing spontaneous mutations. The AChE gene from a strain of *Musca domestica* was initially cloned to search for more sensitive AChE; however, it was found later that AChEs from G strain (GM) are insensitive (resistant) to insecticides. Sequence alignment of its *gm* with those from other species, including *T. californica* and *D. melanogaster*, pinpointed several mutation sites in GM (Met-82, Val-180, Ala-262, and Tyr-327) [19], where the corresponding residues in the sensitive strain are Ile-82, Val-180, Gly-262, and Phe-327 (residue numbers are based on those in the *gm*). These mutated residues were then proposed to be critical in determining enzyme sensitivity.

Preliminary studies on the mutations found in *Musca domestica* (housefly) identified two mutations in common with those in *Drosophila*, as well as two novel ones whose influence on the catalytic properties of the enzyme have not yet been established [5, 16], as mentioned above.

Walsh *et al.* [39] have identified four point mutations (V180L, G262A, F327Y, and G365A) in the housefly gene encoding AChE that are associated with phenotypes [5, 16]. G262A and F327Y, which have previously been reported for *Drosophila* AChE [29], cause resistance of AChE to insecticides and they have been predicted to be the highly conserved mutations that are responsible for target site insensitivity to different classes of insecticide across a wide range of species [7]. Initial modeling within the *Torpedo* structure revealed that both G262A and F327Y are located in the active site triad at the base of the gorge, with G262A likely to affect the orientation of the catalytic serine, and F327Y to decrease the available space within the acyl-binding pocket [29]. The V180L mutation has little effect on the properties of the AChE. G365A is adjacent to the glutamate of the catalytic triad and decreases the affinity of the enzyme to acetylthiocholine. These changes are responsible for resistance by restricting the binding of insecticides within the active site (Fig. 4).

Among these mutated sites, previous results on mutagenesis helped us narrow down our candidate residues to Gly-262 and Tyr-327. For example, M82I and V180L substitutions exert no or little effect on the sensitivity of AChE to fenitroxon or bendicarb [5]. Mutation of Gly-262 into alanine or valine perturbs the space opposite to the catalytic

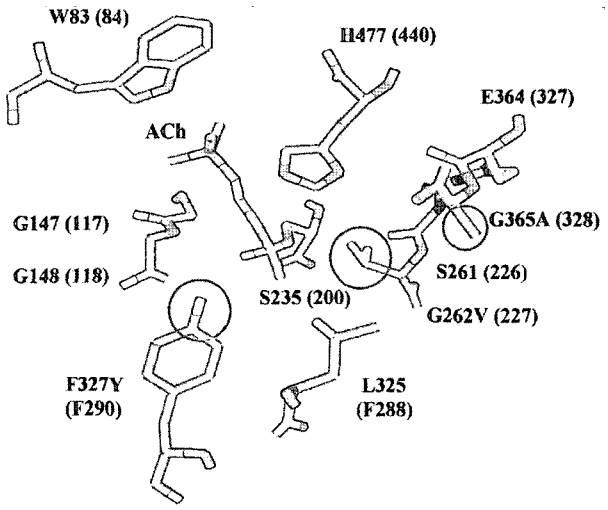


Fig. 4. Location of mutation residues within the active site gorge responsible for resistance to OP and carbamate insecticides. Mutations are modeled on the X-ray structure of *Drosophila* AChE [11]. Residues are numbered in accordance with the housefly sequence; the corresponding numbering for *Torpedo* AChE (rather than *Drosophila*) is given in parentheses. Additional side groups from the mutated residues are circled.

triad by increasing its side chain, decreasing in enzyme sensitivity to fenitroxon [22]. These effects are more clearly obvious in other cases; 4.3-fold increased insensitivity (resistance) to dichlorvos in G262A, and to dichlorvos by 58-fold and to bendicarb by 100-fold in G262V [39]. However, mutation at residue 327 (F327Y) showed only minor effect on insensitivity to bendiocarb, dichlorvos, azamethiphos, and malaoxon [39].

Contrary to the above, however, our results strongly suggest that M82I, A262G, Y327F, and A585T mutations are key residues for sensitivity to OP and carbamate insecticides, and that they react cooperatively with each other.

Although Walsh *et al.* [39] proposed that mutation of the residue 327 toward resistance (F327Y) showed only minor effect on insensitivity, our result on restoration mutation (Y327F) shows that the residue 327 has as much an important role reported in enzyme sensitivity to insecticides as the residue 262, which is contradictory to the Y327F mutation reported by Walsh *et al.* [39]. Our results show that sensitivity ratios of Y327F mutant to OP insecticides acephate (2.7-fold), demeton-S-methyl (1.7), dichlorvos (6.5), edifenphos (1.6), and ethoprophos (1.4) are similar to those of A262G to acephate (1.4), demeton-S-methyl (1.9), dichlorvos (10.8), edifenphos (1.9), and ethoprophos (1.4). Furthermore, sensitivity ratios of Y327F mutant to the carbamate insecticides tested also do, indicating that the residue 327 has as much major positive effects on sensitivity to OP insecticides as the residue 262.

Although Devonshire *et al.* [5] proposed that M82I mutation does not show any sensitivity to insecticides, our result revealed that single M82I mutation slightly increased sensitivity to OP insecticides (Table 3). When combined with other residues (especially with A262G and Y327F), its sensitivity to OP and carbamate insecticides was dramatically increased (Table 3). As shown in Fig. 4, because this residue at 82 (Met or Ile) is located next to the main component of the amino acid W-83 [39], this is another indication that the residue 82 has an effect on recognizing the choline moiety of the substrate acetylcholine.

Although single A585T mutation had little effect on sensitivity, A585T mutation cooperatively increased sensitivity when combined with 82, 262, and 327 residues. However, the exact function of residue 585 is not yet clear.

As mentioned earlier, resistant mutant GS (E234G+ A236S) has amino acid sequence FGGSSG instead of FGESAG (underlined residues 232–237 in Fig. 2) [19], which are conserved throughout all AChEs. In our previous study, we suggested that GS mutation might be related with resistance to insecticides [19]. In this study, residues 234 and 236 amino acids in the conserved sequence FGESAG were suggested to play a main role in modulating sensitivity to OP and carbamate insecticides, because GS mutant showed from 1- to 10-fold resistance to the OP and carbamate insecticides tested.

When the number of mutations was increased, the sensitivity ratio was cooperatively accelerated to OP and carbamate insecticides (Tables 3 and 4). Specifically, the sensitivity ratio of quadruple mutant IGFT was increased dramatically by as much as 7-fold for acephate, 164-fold for demeton-S-methyl, 484-fold for dichlorvos, 523-fold for edifenphos, 30-fold for ethoprophos, 30-fold for benfuracarb, 404-fold for carbaryl, and 107-fold for furathiocarb, compared with that of GM-E. These results indicate that each residue has an effect on other residues and reacts cooperatively.

In conclusion, these mutational studies ranging from single to quadruple amino acid substitutions indicated that the residue 327 is an important key residue to enhance as much sensitivity to OP and carbamate insecticides as the residue 262. The residue 82 or 585 itself had relatively little effect on sensitivity. Interestingly, the residues 234 and 236 were also found to be important for the change of sensitivity. Furthermore, although the single point mutation had little or no effect on sensitivity to OP and carbamate insecticides, the sensitivity was increased cooperatively, when the number of strategic mutations increased.

Function of *ace-2* Type Gene

Since the first cloning of the *Drosophila melanogaster* AChE gene (*ace*) [10], more than one hundred *ace* genes have been cloned (http://bioweb.ensam.inra.fr/ESTHER/general?what=index) from vertebrates and invertebrates. Recently, this *ace* has been classified into *ace-1* and *ace-2*: the original *ace* gene reported was *ace-2* type gene. The *ace-2* genes from Dipteran species had been known to be responsible for the resistance to OP and carbamate insecticides through various mutational studies of *ace-2* genes [10, 22, 29, 38, 39]. However, some later cloned *ace-2* genes from other insects [24, 28, 31, 35, 37] did not show any resistance to insecticides, although they showed sequence homology with those of Diptera species. These other *ace-2* type genes may not be concerned with resistance to insecticides. Since 2002, a new type of genes [8, 14, 23, 30, 40] have been cloned (*ace-1*). These *ace-1* genes do not show any sequence homology with *ace-2* genes, but they have been suggested to be concerned with resistance to insecticides, based on the mutational studies of two *ace-1* genes from two mosquitoes [40, 41]. Referring to these mosquitoes mutational results, some papers suggest that only *ace-1* genes are responsible for the resistance to OP and carbamate insecticides, and mutational study of *ace-2* type genes from Dipteran species may possibly hamper the test of resistance to OP and carbamate insecticides.

However, our mutational study using *ace-2* type gene (*gm*) showed results contradictory to those mentioned by Weill *et al.* [40] and Weill *et al.* [41]. They showed that 5 mutations occurring in *Drosophila melanogaster* resistant

strains provide a low resistance ratio and this situation hampers the design of a molecular test. However, our mutational studies on enhancing sensitivity indicated that the sensitivity to OP and carbamate insecticides was accelerated cooperatively when the number of mutations was increased (Tables 3 and 4). For example, the sensitivity of IGFT to insecticides was increased dramatically by as much as 168-fold for demeton-S-methyl, 483-fold for dichlorvos, 520-fold for edifenphos, 401-fold for carbaryl, and 107-fold for furathiocarb. These results indicate that the *ace-2* gene is also responsible for resistance and/or sensitivity to insecticides.

If so, what is the exact function of *ace-1* and *ace-2*? Why do Dipteran species have only the *ace-2* type gene, whereas other insects have both ace-*1* and -*2* genes? Is only the *ace-2* gene concerned with AChE resistance to insecticides, or is it only *ace-1* The answers to these questions are? still not clear.

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