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Oxo-Type Organophosphate-Resistant Acetylcholinesterase from Organophosphate-Unsusceptible *Culex tritaeniorhynchus*

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A highly resistant strain of *Culex tritaeniorhynchus* was employed to clarify the mechanisms of the acquired resistance of mosquitoes to organophosphorus and carbamate insecticides by an enzymological approach. Carboxylesterase (CE) activity was higher in the abdomen than in the head and thorax, and was higher in every part of the body of the resistant strain as compared with the susceptible strain. Acetylcholinesterase (AChE) activity was high in the head, and was higher in every part of the body in the susceptible strain as compared with the resistant strain.

Greater inhibition of CE by oxo-type organophosphorus insecticides was observed in the susceptible strain (11-, 8.2- and 22.3-fold greater inhibitions by fenitrooxon, malaoxon and dichlorvos, respectively) than in the resistant strain. Much stronger inhibition of AChE was observed in the susceptible strain (1227-, 76.5- and 183-fold inhibitions by fenitrooxon, malaoxon and dichlorvos, respectively) than in the resistant strain. These results suggest that both AChE and CE are involved in the development of the acquired resistance to organophosphorus and carbamate insecticides, and that the former enzyme plays the major role.

Keywords——*Culex tritaeniorhynchus*; insecticide resistant; acetylcholinesterase; carboxylesterase; oxo-type organophosphate inhibition

In 1983, Kamimura and Maruyama[1] reported that *Culex tritaeniorhynchus* captured in Toyama Prefecture was extremely unsusceptible to both organophosphorus and carbamate insecticides, and suggested that this unsusceptibility was a result of acquired resistance to these insecticides. Later, Watanabe and Kamimura,[2] and Yasutomi and Takahashi,[3] by examining the resistance of *Culex tritaeniorhynchus* captured from various parts of Japan, reported that the mosquitoes generally seemed to have acquired a strong resistance to these insecticides.

As to the mechanisms of the acquired resistance, direct detoxication of insecticides by carboxylesterase in the case of *Culex pipiens quinquefasciatus*[4] and *Culex tarsalis*,[5] and an indirect effect arising from acetylcholinesterase not being inactivated by the insecticide in the case of *Anopheles albimanus*[6] have been reported. We attempted to clarify the mechanisms of the acquired resistance of *Culex tritaeniorhynchus* to organophosphorus and carbamate insecticides by an enzymological approach.

Materials and Methods

Chemicals——Fenitrothion, malathion, fenthion and dichlorvos were purchased from Wako Pure Chemical Co., Ltd. Fenitrooxon and malaoxon were kindly supplied by Sumitomo Chemical Co., Ltd. Substrates and other reagents for the measurement of acetylcholinesterase (AChE) and carboxylesterase (CE) activity were of analytical

TABLE I. LC_{50} Values of Organophosphorus Insecticides for the Larvae of R and S *Culex tritaeniorhynchus*

Insecticides	LC_{50} Values (ppm)		R/S
	R	S	
Malathion	27.0	0.011	2500
Fenitrothion	23.2	0.0044	5300
Fenthion	34.1	0.0052	6200

reagent grade.

Mosquitoes——Organophosphorus-resistant and susceptible strains of the vector of Japanese encephalitis virus, *Cx. tritaeniorhynchus*, were used for the present study. A resistant strain Toyama (R) was bred by Yasutomi from a field population collected at a cow shed in a paddy field area in Ohyama, Toyama Prefecture. A susceptible strain *re-e-ae* (S) was brought from Pakistan by Kamimura in 1983. Larval susceptivities of R and S strains to malathion, fenitrothion and fenthion are shown in Table I.

Enzyme Assay——One hundred female mosquitoes from 3 to 8 d after emergence or their separated heads, thoraxes or abdomens were homogenized in 2.0 ml of 0.01 M Tris–HCl buffer solution (pH 8.0) containing 0.1% Triton X-100 in a glass homogenizer with a Teflon pestle in an ice-cold bath. The homogenates were centrifuged at 12500 × *g* for 15 min and the supernatants were used as the crude enzyme.

AChE activity was determined by the method of Ellman *et al.*[7] with acetylthiocholine chloride as the substrate. A mixture of 0.2 ml of the enzyme solution, 0.2 ml of distilled water, 0.5 ml of 2 mM acetylthiocholine and 0.1 ml of 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was incubated at 30°C and the absorbance at 412 nm was recorded for about 10 min with a spectrophotometer (Gilford 250). The activity was calculated by applying the molecular extinction coefficient of 2-nitro-5-mercaptobenzoic acid.

CE activity was determined by the method of Gomori[8a] and van Asperen and Oppenoorth[8b] with β-naphthyl acetate as the substrate. The enzyme solution (0.5 ml) was incubated at 37°C for 15 min with 0.5 ml of 0.5 mM β-naphthyl acetate solution containing 1% acetone, then 0.15 ml of a mixture of 1% naphthanil diazo blue B salt and 5% sodium lauryl sulfate (ratio 2 : 5) was added, then the absorbance at 540 nm was measured after incubation for a further 30 min. A calibration curve was obtained with various amounts of β-naphthyl acetate solution.

Inhibition Test——A mixture of the enzyme and an inhibitor was preincubated at 30°C for 15 min and the activity was determined as mentioned above. I_{50} values represent the inhibitor concentration in the preincubation mixture causing 50% inhibition. A stock solution of inhibitor was prepared in ethanol and diluted with water before use.

Results and Discussion

AChE and CE Activities and Their Distribution in the Mosquito Body

The highest AChE activity, 4.27 μmol/min/mg protein, was observed in the head of strain S, while only one-tenth (0.426 μmol/min/mg) as much activity was observed in the abdomen of the same strain. The same tendency was observed in strain R, though the activity ratio of head/abdomen (1.8) was not as high as that (10) in strain S (Table II). The R/S activity ratio in each part of the body was always smaller than one. When the crude preparation obtained from strain R was preincubated together with that from strain S, the activity was exactly the sum of the activities of strains R and S. This result suggests that no activating or inhibiting factor is present in the preparations.

The highest CE activity, 1.62 μmol/min/mg protein, was observed in the abdomen of strain R. The R/S ratio of the CE activity was always more than one in each part of the body, the highest ratio (3.0) being recorded in the thorax (Table II).

Inhibition of AChE and CE Activities by Oxo-Type Organophosphorus Insecticides

The I_{50} values of these insecticides were measured as shown in Table III, using crude preparations from the head and thorax of strains R and S. Based on the I_{50} values on AChE, R/S inhibition ratios of 1227, 76.5 and 183 were observed with fenitrooxon, malaoxon and

TABLE II. Distribution of Acetylcholinesterase and Carboxylesterase in the Body of R and S *Culex tritaeniorhynchus*

Esterase	Part of mosquito body	Specific activity (μm/min/mg protein)		R/S
		R	S	
Acetylcholinesterase	Whole	0.284	1.78	0.167
	Head	0.597	4.27	0.143
	Thorax	0.383	1.22	0.333
	Abdomen	0.338	0.426	0.769
Carboxylesterase	Whole	0.727	0.535	1.36
	Head	0.307	0.214	1.44
	Thorax	0.961	0.314	3.06
	Abdomen	1.62	0.941	1.72

TABLE III. I_{50} Values of Organophosphorus Insecticides on Acetylcholinesterase and Carboxylesterase from R and S *Culex tritaeniorhynchus*

Insecticides	I_{50} (M) Acetylcholinesterase		I_{50} (M) Carboxylesterase	
	R	S	R	S
Fenitrooxon	2.7×10^{-5}	2.2×10^{-8}	2.1×10^{-6}	2.0×10^{-7}
Malaoxon	2.6×10^{-4}	3.4×10^{-6}	7.6×10^{-7}	9.3×10^{-8}
Dichlorvos	3.3×10^{-5}	1.8×10^{-7}	7.8×10^{-6}	3.5×10^{-7}

dichlorvos, respectively. These values indicate that the sensitivity of AChE from strain R to organophosphorus insecticides is very much lower than that of the enzyme from strain S. In addition, no factors affecting the inhibitory power of fenitrooxon in strain R were observed, because the inhibitory power of this compound on AChE from strain S was not reduced by preincubation with homogenate obtained from strain R. When using larvae as a crude enzyme preparation, the I_{50} values of fenitrooxon were 56 and 0.057 μM in strains R and S, respectively. This R/S ratio is 9825, which roughly corresponds to the value of the R/S ratio (5300) of lethality by fenitrothion (Table I).

In addition, R/S ratios calculated from I_{50} values on CE activity show 11-, 8.2- and 22.3-fold stronger inhibitions by fenitrooxon, malaoxon and dichlorvos, respectively, in strain S (Table III). These results indicate that CE from strain S is more sensitive to organophosphorus insecticides than the enzyme from strain R. Considering the finding already mentioned that CE activity itself was higher in strain R than in strain S, this enzyme may play some role in the development of resistance to organophosphorus insecticides.

Our data lead to the presumption that two processes relating to AChE and CE are involved in the development of resistance to organophosphorus insecticides. The first one may be enhanced resistance of AChE to organophosphorus insecticides in strain R. The second one may be an enhanced detoxification mechanism *via* increased CE activity in strain R. Similar suggestions have already been made in the cases of *Nephotettix cincticeps*,[9] *Musca domestica*,[10a,b] *Anopheles albinanus*[6a,b] and *Tetranychus urticae*.[11] In *Cx. tritaeniorhynchus*, inhibition of AChE from strain R by fenitrooxon was surprisingly weaker than that from strain S. Such an extreme difference in inhibition has never been found previously. The R/S ratios of inhibition (1230 in adults, 9820 in larvae) almost correspond to the R/S ratios of LC_{50} (5300 in larvae). Therefore, the low sensitivity of AChE to organophosphorus

insecticides seems to be important in the development of acquired resistance. We hope to clarify further the precise mechanisms of resistance by investigating differences in enzyme molecular species of AChE between susceptible and resistant strains.

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