Comparisons of Properties of Acetylcholinesterase from Two Field-Collected Populations of *Oxya chinensis* Thunberg (Orthoptera: Acrididae) and the Role of Acetylcholinesterase in the Susceptibility to Malathion

WU Hai-hua, YANG Mei-ling, GUO Ya-ping and MA En-bo

*College of Life Science and Technology, Shanxi University, Taiyuan 030006, P.R.China*

Abstract

In this study, acetylcholinesterase (AChE) was extracted from two field-collected populations of *Oxya chinensis* (Xinxiang City, Henan Province and Changzhi City, Shanxi Province). AChE activities were decreased when concentrations of ATC increased, showing a characteristic phenomenon of substrate inhibition at high concentration in both populations. Such inhibition occurred at relatively low concentration for AChE from Xinxiang population but relatively high for AChE from Changzhi population. The kinetic study showed that there were no significant differences between the two populations in the $K_m$ values. The $K_m$ value in Changzhi population was only 1.09-fold higher than that in Xinxiang population. However, significant differences were observed between the two populations in $V_{max}$ values. The $V_{max}$ value in Changzhi population was 1.32-fold higher than that in Xinxiang population. The inhibition study *in vitro* showed that the AChE from both populations exhibited similar rank order in sensitivity to inhibition by three OPs, as determined by comparison of their bimolecular rate constants ($k$), from the most potent inhibition to the least was chlopyrifos-oxon $>$ paraoxon $>$ demeton-s-methyl for AChE from the two populations and that the $k$ values in Xinxiang population were lower than those in Changzhi population. The $I_{50}$ values of AChE from Xinxiang population were 4.84-, 2.66-, and 1.92-fold less sensitive to inhibition by paraoxon, chlopyrifos-oxon, and demeton-s-methyl. These results were consistent with the results in bioassay. It is inferred that AChE insensitivity to OP insecticides plays an important role in the differences of insusceptibility of *Oxya chinensis* to malathion between the two populations.

Key words: Acetylcholinesterase, Enzyme inhibition, Enzyme kinetics, *Oxya chinensis*

INTRODUCTION

*Oxya chinensis* Thunberg (Orthoptera: Acrididae), a major insect pest in rice, occurs in all rice-growing areas of China. This widespread species mainly inhabits low-lying grasslands, rice fields and their surrounding banks. It primarily feeds on leaves of rice and other gramineous crops. Nymphs and adults of *Oxya chinensis* have caused considerable damage to rice in parts of China[1]. These bring about great losses for our agricultural production. The easiest, most rapid and economical method for controlling *Oxya chinensis* in general is usually by the use of insecticides. However, the frequent use of insecticides resulted in the control difficulty to *Oxya chinensis*. It has been noticed that the susceptibility of *Oxya chinensis* to some insecticides has declined. This phenomenon may evolve into the so-called resistance to some insecticides under the long-term insecticide selection pressure. The methods often used to monitor insecticide resistance are bioassay and biochemical assay. Bioassays are absolutely essential to confirm the presence of resistance in populations and to quantify the levels of resistance associated with...
particular mechanisms. Biochemical monitoring techniques enable researchers to obtain very accurate assessments of resistance.

Resistance to organophosphorus insecticides is conferred by a limited number of mechanisms in all insects analyzed to date. These mechanisms predominantly involve either metabolic detoxification of the insecticide before it reaches its target site, or changes in sensitivity of the target site so that it is no longer susceptible to insecticide inhibition. The most common metabolic resistance mechanisms involve esterases, glutathione S-transferases or monooxygenases.

In the previous study, it is found that *Oxya chinensis* from Changzhi, Shanxi Province was more susceptible to malathion than that from Xinxiang, Henan Province by bioassays. Biochemical studies indicated that the general esterase activities of *Oxya chinensis* from Xinxiang population were higher than those from Changzhi population and the GST activities were similar between the two populations. From these results, it is inferred that elevated esterase activities appeared to play a major role and GST activities may not play a role in the decrease of susceptibility of *Oxya chinensis* to malathion in Xinxiang population.

Resistance to organophosphorus insecticides is often due to altered acetylcholinesterase (AChE) in many other insects. Acetylcholinesterase (AChE, EC3.1.1.7) functions at cholinergic synapses by terminating the chemical impulse of the neurotransmitter acetylcholine (ACh). AChE is a remarkably efficient enzyme that hydrolyzes ACh into acetate and choline at a rate approaching the upper limit set by the diffusion of substrate[2]. AChE is also the target site for carbamate and organophosphate insecticides. These compounds react in an analogous way to ACh, forming a complex and then, respectively, carbamylating or phosphorylating the active site of the enzyme. The inhibition of AChE by carbamate or organophosphate compounds occurs via a reversible complex formation followed by carbamylation or phosphorylation of AChE[3]. In a number of insect species, altered AChE has been found with a reduced sensitivity to inhibition by organophosphate and carbamate insecticides[4-7]. Less sensitivity of AChE to these compounds originates from point mutations in the AChE gene that modify either the inhibitor affinity or the inhibition rate constants or both[8-10]. Knowledge of AChE kinetics is a prerequisite for understanding the effects of mutation in resistant insects.

To examine whether or not reduced sensitivity of AChE was involved in malathion insusceptibility in *Oxya chinensis*, AChE from two field collected populations (Xinxiang and Changzhi) in China was characterized. Thus, the objects of this study were to (1) study the kinetic and inhibitory properties of AChE to substrates and inhibitors in the two populations; (2) compare the sensitivity of AChE to three selected OP compounds in the two populations.

**MATERIALS AND METHODS**

**Insects**

Fifth-instar nymphs of *Oxya chinensis* were collected from Xinxiang City, Henan Province and Changzhi City, Shanxi Province. The habitat of Xinxiang population is a field of rice where insecticides have been used frequently. *Oxya chinensis* in Changzhi population inhabits grasslands in the surroundings of reservoirs where insecticides are seldom used.

**Chemicals**

Bicinchoninic acid solution (BCA), acetylthiocholine iodide (ATC), 5, 5’-dithio-bis (2-nitrobenzoic acid) (DTNB), and paraoxon (diethyl 4-nitrophenyl phosphate, 90% pure) were purchased from Sigma Chemical (St. Louis, MO). Bovine serum albumin (BSA) protein assay standard was purchased from Bio-Rad Laboratories (Hercules, CA). Chlopyrifos-oxon (O, O-diethyl O-(3, 5, 6-trichlorl-2-pyridinyl) phosphate, 97% pure) and demeton-s-methyl (S-2-ethylthioethyl O, O-dimethyl phosphorothioate, 95% pure) were purchased from Chem Service (West Chester, PA).

**Enzyme preparation**

For comparing total activities and specific activities of AChE in different populations, enzymes were extracted by homogenizing heads. For each population, 32 female and 32 male heads were divided into eight groups randomly and were homogenized in 0.1M ice-cold phosphate buffer (pH 7.5) containing 0.3% (v/v) TritonX-100 at a ratio of 1:10 (w/v). All homogenates
Comparisons of Properties of Acetylcholinesterase from Two Field-Collected Populations of *Oxya chinensis* Thunberg

were centrifuged at 15 000 x g for 30 min at 4 °C. Supernatants were transferred to fresh tubes and used as enzyme sources. For comparing AChE kinetics and inhibitions in the two populations, the enzymes homogenized previously were mixed together.

**AChE assays**

AChE activities in different populations were determined based on the method of Ellman et al.\[11\] as modified by Zhu and Clark\[12\] using ATC as substrate. Briefly, 20 µL of enzyme preparation was incubated in a final reaction volume of 200 µL in 0.1 M phosphate buffer (pH 7.5) containing 0.25 mM ATC and 0.4 mM DTNB. The AChE activity was determined for 2 min at 405 nm with a V_{max} kinetic microplate reader and SOFTmax computer software (Molecular, Devices Menlo Parl, CA).

**Determination of kinetic parameters**

Kinetic activities were determined based on the method of Ellman et al.\[11\] as modified by Zhu and Clark\[12\] using substrate ATC. The AChE activity at 12 substrate concentrations from 3.9 µM to 8 mM was determined based on the reaction in 0.1 M phosphate buffer (pH 7.5) for 2 min, whereas the initial velocity was determined based on the reaction for 30 s at room temperature and pH 7.5 using a V_{max} kinetic microplate reader and SOFTmax computer software (Molecular Devices Corp Menlo Park, CA) at 405 nm. The enzyme kinetic parameters, Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were determined using Hanes transformations.

**In vitro** inhibition of AChE

AChE inhibition by three OP compounds (paraoxon, chlorpyrifos-oxon, demeton-s-methyl) was carried out by the method of Zhu and Clark\[13\] as modified by Gao et al.\[14\]. Briefly, 10 µL of the AChE preparation and 10 µL of appropriately diluted OP inhibitor were mixed rapidly in a 96-well microplate with a multichannel pipette. After the mixture was preincubated at room temperature (24 °C) for 2 min, the inhibition reaction was stopped by the addition of 180 µL of the ATC/DTNB mixture. The residual AChE activity was determined for 2 min under the same conditions with the microplate reader at 405 nm. The final concentrations of ATC and DTNB on the reaction mixture were 0.50 and 0.04 mM, respectively. The bimolecular rate constant (k_i) was determined by calculating the slope of the linear regression of the log percentage residual AChE activity against the organophosphate concentration by the method of Aldridge and Davison\[15\].

**Protein assay**

Protein concentration of each enzyme preparation was determined according to Smith et al.\[16\] using BSA as the standard. The measurement was performed with the microplate reader at 560 nm.

**Data analysis**

K_{m}, V_{max}, k_i and I_{50} values of AChE between the two populations were subjected to Student's t-test.

**RESULTS**

Comparisons of kinetic parameters

Fig. 1 shows the effect of the concentrations of the model substrate ATC on the activities and Hanes plots (insert) for kinetics of AChE from the Changzhi and Xinxiang (XX) populations. Each point represents the mean of four determinations (n=4). Vertical bars indicate SD of the mean. The secondary plots (inserted) are Hanes plots of \([s]/v\) vs \([s]\) for AChE hydrolyzing ATC.

![Fig. 1 Effect of substrate concentration on the hydrolysis of ATC by AChE from Xinxiang (XX) and Changzhi (CZ) populations](image-url)
Xinxiang populations. AChE activities were decreased when concentrations of ATC increased, showing a characteristic phenomenon of substrate inhibition at high concentration in both of populations. The significant differences lie in the fact that the phenomenon of substrate inhibition appeared in the relatively high concentration in Changzhi population (≥ 8 mM), while it appeared in the relatively low concentration in Xinxiang population (≥ 4 mM). The affinities ($K_m$) and hydrolyzing efficiencies ($V_{max}$) of AChE in the two populations were determined by kinetic analysis. Table 1 showed the $K_m$ and $V_{max}$ values. There were no significant differences between the two populations in the $K_m$ values. The $K_m$ values in the two populations were similar. The $K_m$ value in Changzhi population was only 1.09-fold higher than that in Xinxiang population. However, significant differences were observed between the two populations in $V_{max}$ values. The $V_{max}$ value in Changzhi population was 1.32-fold higher than that in Xinxiang population.

### Inhibition of AChE by organophosphates

Three selected OP compounds were used to compare the sensitivity levels of AChE from Xinxiang and Changzhi populations of *Oxya chinensis* (Fig.2). Table 2 summarizes various inhibition constants determined from the inhibition reaction of three organophosphorus insecticides on AChE from the two populations. The AChE from both populations exhibited similar rank order in sensitivity to inhibition by three OPs, as determined by comparison of their bimolecular rate constants ($k_i$), from the most potent inhibition to the least.
was chlopyrifos-oxon > paraoxon > demeton-s-methyl for AChE from the two populations. There have no significant differences in $k_i$ values between the two populations, but the $k_i$ values in Xinxiang population were lower than those in Changzhi population. However, significant differences were observed in $I_{50}$ values between the two populations. The $I_{50}$ values of AChE from Xinxiang population were 4.84-, 2.66-, and 1.92-fold less sensitive to inhibition by paraoxon, chlopyrifos-oxon, and demeton-s-methyl. From Fig. 3, it is shown that the changing trends of AChE activities in the two populations as the concentrations of inhibitors changed were similar, but the speed of inhibition in Changzhi population was faster than that in Xinxiang population.

### DISCUSSION

AChE is the target-site for organophosphate and carbamate pesticide in the central nervous system, and its role in cholinergic synapses is essential for life[8]. Substrate inhibition at higher concentrations is a typical phenomenon for AChE and is probably due to the binding of excess substrate to the peripheral anionic site to form an enzyme-substrate-substrate complex[17,18]. The substrate inhibition is likely influenced by changing the con-
formation of aromatic residues near the lip of the catalytic gorge of AChE, the probable location of the peripheral anionic site. From Fig. 1, ATC at higher concentration (≥ 8 mM for the Changzhi population and ≥ 4 mM for the Xinxiang population) showed a significant inhibition of the enzyme by the substrate. Such inhibition occurred at relatively low concentration for AChE from the Xinxiang population but relatively high for AChE from the Changzhi population. These results indicated that AChE for Xinxiang and Changzhi populations of *Oxya chinensis* were significantly different in response to different substrate concentration.

In this study, a significant reduction in *V*<sub>max</sub> values was observed for the Xinxiang population. This loss of AChE activity is consistent with increased insensitivity in most cases, although there have been cases of unaltered or increased AChE activity in resistant strains. This hypothesis was supported by the result of inhibition in vitro. The results of inhibition in vitro indicated that the *I*<sub>50</sub> values of three OP inhibitors on the AChE of Xinxiang were higher than on those of Changzhi population. This result was consistent with the results of bioassays in vivo using malathion. In the precious study, the results showed that the LD<sub>50</sub> value in Xinxiang population was 3.31-fold higher than that in Changzhi population. All the results showed that AChE insensitivity to OP insecticides plays an important role in the differences of insusceptibility of *Oxya chinensis* to malathion between the two populations.

In our results, the mean specific activities were 8.38 and 11.94 mmol min<sup>-1</sup> mg<sup>-1</sup> protein in Xinxiang and Changzhi populations, respectively. This was reverse to the results of bioassays in vivo using malathion. Byrne and Devonshire had reported the decreasing activity of AChE in the resistant *Bemisia tabaci*. But in some cases, the rising activity of AChE in resistant strain was also found. Didier Fournier et al. thought resistance of organophosphate pesticides correlated with the mount of AChE in the central nervous system and the strain with higher activity of AChE shows more resistant. While Zhu and Brindley thought there is no correlation between AChE activity and resistance in some insects. However, a lot of reports showed that the insensitivity of AChE was a critical characteristic for the target resistance to OP and carbamate insecticides. With the obvious decrease of the AChE sensitivity, it could conclude that AChE insensitivity is involved in the insusceptibility of *Oxya chinensis* to malathion. Most of the insensitivity of AChE to OP was caused by the altered AChE. Therefore, future focus of this work is to isolate the relevant AChE from each population and obtain amino acid sequence information allowing for the isolation of the respective genes. Knowledge of the molecular basis of resistance allows for the development of molecular diagnostic probes, management of genes within *Oxya chinensis* populations, and possible reversion of resistance with antiresistant compounds.

Furthermore, from the results of inhibition in vitro using three inhibitors, it is implicated that in the future management of *Oxya chinensis*, chlopyrifos-oxon may be efficient and demeton-s-methyl need to be used seldom as possible as we can.

**Acknowledgments**

The authors thank Professor Zhu Kunyan (Kansas State University, USA) for providing instructions in the course of experiments and Professor Zhang Feng (Shanxi University, P.R.China) for help in analyzing of data. This work was supported by National Natural Science Foundation of China (30170612), and Science and Technology Commission of Shanxi Province (041005) to Ma Enbo.

**References**


[6] Tripathi R K, O’Brien R D. Insensitivity of acetylcholinesterase as a factor in resistance of houseflies to...
Comparisons of Properties of Acetylcholinesterase from Two Field-Collected Populations of *Oxya chinensis* Thunberg


(Edited by SUN Lu-juan)