Increased activity and reduced sensitivity of acetylcholinesterase associated with malathion resistance in a field population of the oriental migratory locust, *Locusta migratoria manilensis* (Meyen)

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Abstract

The susceptibility to malathion, and the activity and sensitivity of acetylcholinesterase (AChE, EC 1.1.1.7) were compared between two populations of the oriental migratory locust, *Locusta migratoria manilensis* (Meyen) collected from Wudi County of Shandong Province in East China and Huangliu County of Hainan Province in South China. Huangliu population showed 8.5-fold resistance to malathion compared with Wudi population. AChE from Huangliu population showed 4.8-fold higher activity than that from Wudi population toward the model substrate acetylthiocholine (ATC). Kinetic studies indicated that AChE from Huangliu population had 2.6-fold lower affinity, but 5.0-fold higher catalytic activity toward ATC than AChE from Wudi population. Significantly increased activity of AChE in Huangliu population was also confirmed by non-denaturing polyacrylamide gel electrophoresis. Inhibition kinetics revealed that AChE from Huangliu population was 9.8-, 2.4-, 8.0- and 7.7-fold less sensitive to inhibition by paraoxon, malaoxon, chlopyrifos oxon, demeton-S-methyl, respectively, than that from Wudi population. Our studies revealed that a mild resistance to malathion in Huangliu population was associated with reduced sensitivity and increased catalytic activity of AChE. Our results suggest that alterations of AChE may play an important role conferring or contribute to malathion resistance in Huangliu population of the locust.

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1. Introduction

The oriental migratory locust, *Locusta migratoria manilensis* (Meyen) (Orthoptera: Acrididae) is one of the most serious agricultural pests responsible for significant yield losses in many regions of the world. In China, infestations of *L. migratoria manilensis* are common and sometimes become extremely destructive [1]. In massive locust outbreak areas, the density of the nymph can reach as high as about 5000 individuals per square meter [2]. Organophosphorus (OP)\(^1\) insecticides have been used continuously for more than twenty years as the major control measure for *L. migratoria manilensis*. This situation may lead to high levels of resistance to OPs in some locust populations in China [3–5]. However, we know very little about insecticide resistance and resistance mechanisms in locust popula-

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\(^1\) Abbreviations used: OP, organophosphate; AChE, acetylcholinesterase; ATC, acetylthiocholine iodide; BCA, bicinechonic acid solution; BSA, bovine serum albumin; DTNB, 5,5'-dithio-bis (2-nitrobenzoic acid); HL, Huangliu population; PAGE, polyacrylamide gel electrophoresis; WD, Wudi population.
tions although the massive outbreak of locusts frequently became problematic in recent years around the world.

The OP and carbamate insecticides exert their neurotoxic effects by inhibiting acetylcholinesterase (AChE, EC 2.1.1.7), a critical enzyme involved in nerve impulse transmission [6]. Consequently, decreased sensitivity of AChE to inhibition by these insecticides has been implicated in insecticide resistance in many insect and other arthropod species [7–9]. Many studies indicated that decreased sensitivity of AChE to inhibition by OPs and/or carbamates is due to altered AChE associated with increased AChE activity in several insect species, including housefly (Musca domestica) [10], green rice leafhopper (Nephotettix cincticeps) [11], fruitfly (Drosophila melanogaster) [12] and greenbug (Schizaphis graminum) [13].

Huangliu of Hainan Province is a locust frequently infested area in China, and malathion is a major insecticide used to control the locust. However, infestation frequency of the locust in Wudi of Shandong Province in China was lower than that in Huangliu. Therefore, malathion was seldom used to control the locust. To examine whether or not AChE was involved in OP resistance in the field populations of L. migratoria manilensis, we (1) assessed the susceptibility to malathion in two field-collected populations (Huangliu and Wudi) in China; (2) compared AChE specific activities, AChE kinetics, and AChE activity staining on non-denaturing polyacrylamide gel; and (3) examined the sensitivity levels of AChE to four OP compounds in the two locust populations.

2. Materials and methods

2.1. Insects

The fifth-instar nymphs of L. migratoria manilensis were collected from Huangliu County of Hainan, a seacoast province in South China (E108°31′ N18°41′), and Wudi County of Shandong, a seacoast province in East China (E117°88′ N38°01′), in 2006. All the locust samples were stored at −80 °C until use.

2.2. Chemicals

Bicinchoninic acid solution (BCA), acetylthiocholine iodide (ATC) and 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Malathion (O,O-dimethyl-S-1,2-di(carboethoxy)ethyl phosphorothioate, purity 99.5%), chlorpyrifos oxon (O,O-diethyl-O-(3,5,6-trichlorl-2-pyridinyl) phosphate, purity 99.5%), paraoxon (p-nitrophenyl phosphate, purity 99%), malafoxon (O,O-dimethyl-S-1,2-di (carboethoxy) ethyl phosphorothiolate, purity 99%) and demeton-S-methyl (S-2-ethylthioethyl O,O-dimethyl phosphorothioate, purity 99%) were purchased from Chem Service (West Chester, PA, USA), whereas bovine serum albumin (BSA) was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.3. Insecticide bioassay

The susceptibility of the locust to malathion was evaluated using topical application. Six different concentrations of malathion were prepared in acetone as a solvent. Sixteen to 22 fifth-instar locust nymphs, as a replicate, were individually treated with 5 μl of each malathion dose or acetone (control) in the abdomen between the second and third sterna. Each dose or control was repeated three times. Mortality was assessed after the treated locusts were maintained at rearing room (room temperature: 24 °C, room humidity: 60%) for 24 h. Mortality data were analyzed by probit analysis using the SPSS program (SPSS Inc., Chicago, USA). Resistance ratio and its 95% confidence intervals were calculated by the method of Robertson and Preisler [14].

2.4. Preparation of AChE

AChE was prepared by homogenizing each of 32 locust heads from each population in 1.0 ml of ice-cold 0.1 M phosphate buffer (pH 7.5) containing 0.3% (v/v) Triton X-100. The homogenates were centrifuged at 15,000g for 20 min at 4 °C. After supernatants were transferred to new tubes, enzyme preparations were divided into four groups. The enzyme preparations within each group which represented 8 locusts were combined as a replicate. The enzyme preparations were used to compare AChE kinetics and sensitivity to each of four selected OP compounds between the two populations. All procedures were carried out on ice to minimize losses of enzyme activity.

2.5. Assay of AChE activity

AChE activities were determined based on the method of Ellman et al. [15] as modified by Zhu and Clark [16] using ATC as a substrate. Briefly, 20 μl of enzyme preparation was added each well of a microplate and mixed by 180 μl of the ATC-DTNB mixture. The final concentration in the reaction is 0.25 mM for ATC and 0.4 mM for DTNB. The AChE activity was determined immediately for 2 min at 405 nm in 24 °C with a SpectraMax 190 microplate reader and SOFTmax computer software (Molecular Devices, Menlo Park, CA, USA). All assays were corrected for non-enzymatic activity using the same mixtures except for using 20 μl of 0.1 M phosphate buffer (pH 7.5) containing 0.3% (v/v) Triton X-100 instead of the enzyme preparation.

2.6. Kinetic analysis of AChE

To determine the kinetic parameters of AChE, 50 μl of enzyme preparation and 50 μl of 1.2 mM DTNB was mixed with 50 μl ATC (final reaction concentration: 3.9 μM–8 mM). The initial velocity was determined based on the reaction for 30 s for ATC at room temperature (ca. 24 °C) and pH 7.5 using a SpectraMax 190 microplate...
2.7. In vitro inhibition of AChE

Because the oxidative analogs of OP insecticides were effective inhibitors of AChE, four OP oxon analogs (paraoxon, malaoxon, chlorpyrifos oxon and demeton-S-methyl) were used to compare the sensitivity levels of AChE between the two populations.

Inhibition of AChE by each of four OP compounds was performed as previously described [18]. Briefly, 10 μl of each of five to six concentrations of an OP compound was mixed with 10 μl of properly diluted enzyme using a multichannel pipette and incubated for 2 min at room temperature. The remaining activity of AChE was measured immediately after 180 μl of ATC and DTNB solution was added to the inhibition mixture as the same as assay of AChE activity. The final concentrations of ATC and DTNB were 0.25 and 0.4 mM, respectively. The bimolecular rate constant (k_i) was determined by calculating the slope of each linear regression of the log percentage of residual AChE activity against the OP concentration according to the method of Aldridge and Davison [19].

2.8. Non-denaturing polyacrylamide gel electrophoretic analysis of AChE

Non-denaturing polyacrylamide gel electrophoresis (PAGE) of AChE was carried out based on the method as previously described by Zhu and Clark [16] using a DYCZ-24D Electrophoresis Cell (Liu-Yi, Beijing, China). Thirty-two locust heads were divided into two groups, and each group (representing 16 locusts as a replicate) was homogenized in 0.1 M phosphate buffer (pH 7.5) containing 0.3% (v/v) Triton X-100. After centrifugation at 15,000 g for 20 min at 4°C, supernatants were transferred to fresh tubes and used as enzyme sources for AChE electrophoretic analysis. The gel (4% and 7% polyacrylamide in stacking and separation gels, respectively) was run at a constant voltage of 150 V for 1 h on an ice-bath. The AChE bands were visualized by incubating the gels in staining mixture overnight at room temperature [20].

2.9. Protein assay

Protein content of each enzyme preparation was determined according to Smith et al. [21] using BSA as a standard. Measurements were performed with the microplate reader at 560 nm [16].

2.10. Data analysis

All comparisons were subjected to Student’s t-test using SPSS program (SPSS Inc., Chicago, USA).

3. Results

3.1. Malathion susceptibility bioassay

The comparisons of malathion susceptibility of the locust between the two populations were presented in Table 1. The LD_{50} values for malathion susceptibility of the locust were 4.13 and 34.97 μg/g body weight in Wudi and Huangliu populations, respectively. Results indicated that Huangliu population was 8.5-fold less susceptible to malathion than Wudi population.

3.2. Assay of frequency distributions of locusts with respect to specific activity of AChE

Fig. 1 shows the frequency distributions of locusts with respect to the AChE specific activity in Huangliu and Wudi populations. The Huangliu population displayed a high frequency of individuals with higher percentage of specific activity of AChE than that of Wudi population. The mean of AChE activity in Huangliu population was 4.8-fold higher than that of Wudi population when ATC was used as a substrate (Table 2). Further, AChE activity of Huangliu population was less homogeneous than that of Wudi population.

3.3. Kinetic and electrophoretic analysis of AChE

Fig. 2 shows the effect of substrate (ATC) concentration on AChE activity and Hanes plots (inserted) for AChE kinetics in Wudi and Huangliu populations. The overall patterns of the enzyme responses to the change of substrate concentration were different between the two populations, suggesting that AChE in Huangliu population was kinetically different from that in Wudi population. The kinetic study indicated that AChE from Huangliu population had 2.6-fold lower affinity (i.e. higher K_m values) to the substrate than that in Wudi population (Table 2). In contrast, the catalytic activity of AChE toward ATC in Huangliu population was 5.0-fold higher (i.e., higher V_max values) than that in Wudi population. Significantly increased AChE activity in Huangliu population was also confirmed by non-denaturing PAGE, showing remarkably increased intensity when gels were stained for the AChE activity (Fig. 3).

3.4. In vitro inhibition studies of AChE

Four OP oxon analogs (paraoxon, malaoxon, chlorpyrifos oxon and demeton-S-methyl) were used to compare the sensitivity levels of AChE between the two populations (Fig. 4). Table 3 summarized various inhibition constants determined from the inhibition reactions of four OP compounds on AChE from the two populations. Significant differences were observed in k_i values between Huangliu and Wudi populations for all the four OP inhibitors. Specifically, AChE from Huangliu popula-
tion were 9.8-, 2.4-, 8.0- and 7.7-fold less sensitive to inhibition by paraoxon, malaoxon, chlorpyrifos oxon, and demeton-S-methyl, respectively, than that from Wudi population based on the differences in the $k_i$ values between the two populations.

### Table 1

Comparison of malathion susceptibility of the fifth-instar nymphs of *L. migratoria manilensis* collected from Wudi and Huangliu counties

<table>
<thead>
<tr>
<th>Population</th>
<th>n$^a$</th>
<th>$\chi^2$</th>
<th>$P^b$</th>
<th>Slope ± SE</th>
<th>LD$_{50}$ (μg/g body weight) (95% CL)</th>
<th>LD$_{50}$ ratio$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wudi</td>
<td>346</td>
<td>0.86</td>
<td>0.98</td>
<td>1.370.19</td>
<td>4.13 (2.98–6.37)</td>
<td>—</td>
</tr>
<tr>
<td>Huangliu</td>
<td>378</td>
<td>1.13</td>
<td>0.93</td>
<td>2.14 ± 0.33</td>
<td>34.97 (28.91–47.67)</td>
<td>8.5 (4.8–11.3) **</td>
</tr>
</tbody>
</table>

$^a$ Number of the locust nymphs used in each bioassay.  
$^b$ $P > 0.05$ indicates a significant fit between the observed and expected regression lines in a probit analysis.  
$^c$ LD$_{50}$ ratio and 95% CL were calculated by LD$_{50}$ of Huangliu/LD$_{50}$ of Wudi according to the method of Robertson and Preisler (1992). *, a significant difference between the LD$_{50}$ of the two populations was based on the non-overlapping 95% CLs of LD$_{50}$ values.

### Table 2

Activities and kinetic parameters of AChE extracted from two populations of *L. migratoria manilensis* in hydrolyzing the substrate acetylthiocholine (ATC)$^a$

<table>
<thead>
<tr>
<th>Population</th>
<th>AChE activities (μmol/min/mg)</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (μmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wudi</td>
<td>7.22 ± 1.31</td>
<td>40.77 ± 5.79</td>
<td>8.40 ± 0.29</td>
</tr>
<tr>
<td>Huangliu</td>
<td>34.58 ± 3.18*</td>
<td>104.12 ± 10.38*</td>
<td>42.13 ± 8.39*</td>
</tr>
</tbody>
</table>

$^a$ Results on AChE activities are presented as the mean ± SD of 32 individual heads ($n = 32$), and results on AChE kinetics are presented as the mean ± SD of four replicates ($n = 4$); each with triplicate analyses.  
$^*$ There is significant difference between the two populations ($P < 0.05$, Student’s $t$-test).

### 4. Discussion

#### 4.1. Decreased malathion susceptibility in Huangliu locust population

Huangliu and Wudi populations breed both in the sea-coast locust area, and the oceanic climate of appropriate temperature and humidity is the primary factor leading to locust outbreaks. In Huangliu of Hainan Province, the droughty savannah had been formed and the ecological environment was deteriorated due to the magnitude of...
forest plants being seriously destroyed for more than 60 years. Consequently, infestations of *L. migratoria manilensis* became extremely destructive [22]. Insecticides, mainly OPs, have been applied frequently for nearly 20 years to control the locust. The local growers have complained about control failures probably due to reduced susceptibility to insecticides. In contrast, the use of OPs has been discontinued for locust control in Wudi County of Shandong Province due to the reduced breeding frequencies of the locust since 1995. Thus, Huangliu population had much higher insecticide selection pressures than Wudi population. Indeed, our bioassay results revealed 8.5-fold decreased susceptibility to malathion in the Huangliu population compared with Wudi population (Table 1). Although detailed information on the usage of insecticides for locust control in these two regions are currently not available, our results support the notion that Huangliu population was less susceptible than Wudi population to malathion, which agrees with the notion of different insecticide selection pressures between the two populations.

![Fig. 3. Non-denaturing PAGE for AChE extracted from Huangliu (HL) and Wudi (WD) populations of *L. migratoria manilensis*. Each lane was loaded with 400 μg of AChE preparations. Enzyme preparations of 32 locust heads from each population were divided into two groups, and each group represented 16 locusts as a replicate. Gel (7% separating gel and 4% stacking gel) was run at a constant voltage of 150 V for 1 h on an ice-bath and AChE was stained for its activity using ATC as substrate.](image1)

![Fig. 4. Inhibition of AChE from Huangliu (HL) and Wudi (WD) populations of *L. migratoria manilensis* by selected organophosphate inhibitors (paraoxon, malaoxon, chlorpyrifos-oxon, and demeton-S-methyl) at room temperature. Each point represents the mean of four determinations (n = 4). The correlation coefficients (r) of all linear regression lines are >0.97 except for Huangliu population with paraoxon which is 0.94 (P < 0.01).](image2)
4.2. Increased activity and reduced sensitivity of AChE associated with malathion resistance

The present study indicated that Huangliu population possessed an altered AChE with decreased sensitivity to inhibition by paraoxon, malaoxon, chlorpyrifos oxon and demeton-S-methyl, and decreased affinity and increased catalytic activity toward the model substrate ATC. Our results agreed well with a previous report by Zhu et al. [13] with respect to the decreased sensitivity and increased activity of AChE in conferring OP resistance in greenbugs. Specifically, AChE from Huangliu population showed 2.6- to 9.8-fold reduced affinity to ATC (i.e., increased K_m values) (Table 2, Fig. 2) and 2.4- to 9.8-fold reduced sensitivity to inhibition by OP compounds (i.e., decreased k_i values) (Fig. 4, Table 3) compared with AChE from Wudi population. Thus, it was clear that Huangliu population possessed a qualitatively altered AChE. Furthermore, AChE from Huangliu population also showed 4.8- and 5.0-fold higher specific activity and V_max in the enzyme kinetics, respectively (Table 2, Fig. 2). Both significantly decreased sensitivity to OP inhibition and affinity to ATC, and significantly increased catalytic activity in Huangliu population strongly suggest that AChE in Huangliu population may have undergone both qualitative and quantitative alterations although we can not completely rule out the possibility of only qualitative changes leading to a more catalytically active enzyme. In addition, the sensitivity to paraoxon and the activity of AChE from Huangliu population were more heterogeneous than that from Wudi population (Figs. 1 and 4). Apparently, such a heterogeneous sensitivity of AChE to paraoxon in Huangliu population was likely caused by mixed AChE with different sensitivity levels to the OP as previously reported in OP-resistant greenbugs [23].

Nevertheless, our bioassay revealed only 8.5-fold resistance to malathion in Huangliu population compared with Wudi population. Apparently, Huangliu population was only mildly resistant to malathion compared with Wudi population. Such levels of insecticide resistance are usually not considered to be high for many field crop insects, but can be considered to be substantial for migratory insects which are less documented for their resistance to insecticides, particularly in locusts.

Although the decreased malathion susceptibility in Huangliu population is likely due to reduced sensitivity and increased activity of AChE, we can not be sure as to whether or not Wudi population can be considered as a “true” malathion-susceptible population. However, several important evidences, including discontinuous use of OP insecticides for more than 10 years in the region of Wudi County where the locusts were collected, high sensitivity of AChE to all four OP compounds examined, and high homogeneities of AChE in responses to OP inhibition, suggested that Wudi population was likely to be a proximal insecticide-susceptible population found in the field.

Nevertheless, further studies would be necessary to compare the susceptibility spectra of the two populations to various insecticides. It is possible that Huangliu population may show higher resistance levels to other OPs than to malathion as suggested by differential sensitivity levels of AChE from Huangliu population (Table 3). This notion is supported by only 2.4-fold reduction of AChE’s sensitivity to malaoxon but with an 8.5-fold resistance to malathion in Huangliu population. It is possible that higher resistance levels may be observed with other OPs, such as parathion, chlorpyrifos and demeton-S-methyl, because AChE from Huangliu population showed 7.7- to 9.8-fold reduced sensitivities to their corresponding oxon analogs (Table 3). In addition, it would also be very important to understand molecular mechanisms of the altered AChE associated with reduced sensitivity and increased activity in L. migratoria manilensis. Such knowledge may help us develop molecular tools that can be used to detect OP resistance in the field populations [24,25].

Acknowledgment

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References


