

Laboratory Evaluation of Chlorpyrifos Against Resistant and Susceptible Strains of a Moth Pest of Crop Plants, *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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Abstract The cotton leafworm, *Spodoptera littoralis* Boisadual (Lepidoptera: Noctuidae), is an invasive species causing serious damage in crop production and is commonly controlled for seven decades with the organophosphorus (OP) compounds. Despite their low effectiveness, about 90 of these compounds are still in use and play a major role in pest control. The sensitivity of acetylcholinesterase (AChE) to chlorpyrifos was compared in susceptible strain (LS) and resistant strain (FR) of *S. littoralis*. The FR was significantly resistant to chlorpyrifos (70.64-fold) compared to LS strain. Inhibition studies revealed apparent differences in sensitivity of AChE enzyme from resistant and susceptible larvae to chlorpyrifos. In comparison to AChE from LS, the enzyme of insect's resistant to chlorpyrifos has Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) values 1.98- and 2.54-times higher. Overall, the current study supports the importance of AChE-altered in created resistance to OP compounds.

Keywords Acetylcholinesterase · Enzyme inhibition · Enzyme kinetics · Organophosphate resistance · *Spodoptera littoralis*

Abbreviations

AChE Acetylcholinesterase
FR Resistant strain
LS Susceptible strain

OP Organophosphorus compounds
RR Resistance ratio

Introduction

The cotton leafworm, *Spodoptera littoralis* Boisadual (Lepidoptera: Noctuidae), is a worldwide economically important key pest. The immature stages of *S. littoralis* are commonly controlled with the application of synthetic insecticides. However, the indiscriminate use of these insecticides can cause the development of resistant populations (Ismail et al. 2020; Hilliou et al. 2021). Since the mid-1950s, widespread use of OP compounds as insecticides around the world is one of the main reasons for the outbreaks of pests (Fournier 2005). Despite OP compounds have become largely ineffective, they still play a major role in pest control. So urgent action is required to manage the resistance and expand the lifespan of these compounds (Hilliou et al. 2021). Carbamate and OP compounds are potent AChE (EC3.1.1.7), inhibitors which blocks the enzyme activity via a reversible complex formation followed by phosphorylation or carbamylation of AChE (Dawkar et al. 2013; Umar and Aisami 2020). AChE hydrolyzes the neurotransmitter acetylcholine (ACh) to terminate neuronal excitement at the postsynaptic membrane (Mwila et al. 2013). Decrease in sensitivity of the target enzyme, AChE, to OPs has as well been reported in more than 30 insecticide resistant pest species (Mutero et al. 1994; Afzal et al. 2015; Moradi et al. 2019). Knowledge of AChE kinetics is a prerequisite for understanding the effects of mutation in resistant insects. However, studies concerning the biochemical basis of pest resistance to insecticides are scarce. The present study focused on the possible involvement of AChE in conferring resistance in OP-resistant insect populations. Therefore, the sensitivity

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of AchE to chlorpyrifos was compared through the kinetic parameters of AchE in the two strains of *S. littoralis*. This study will help in resistance management by understanding and overcoming resistance problems.

Materials and Methods

Insects

The originally FR strain of the cotton leafworm, *Spodoptera littoralis* Boisadual (Lepidoptera: Noctuidae) was collected from the cotton fields of Biala district, Kafr El-Sheikh Governorate, Egypt. These populations have been exposed to frequent use of various insecticides including OP insecticides in these fields. The collected *S. littoralis* egg mass was carefully transferred to the laboratory. A reference susceptible strain LS of *S. littoralis* was obtained from Department of Insect Population Toxicology, Central Agricultural Pesticides Laboratory, Agriculture Research Center, Giza, Egypt, and a laboratory population has been maintained in the rearing chamber without exposure to any insecticide for over 15 years. Two strains were reared on castor bean leaves (*Ricinus communis* L.) and maintained in the rearing chamber under controlled conditions of 25 ± 1 °C, $65 \pm 5\%$ relative humidity, and a photoperiod of 16:8 (h light: dark).

Insecticide and Chemicals

The OP insecticide chlorpyrifos (*O,O*-diethyl-*O*-3,5,6-trichloro-2-pyridyl phosphorothioate, purity 99.5%) was purchased from Chem. Service (West Chester, PA). Bovine serum albumin (BSA) protein assay standard was purchased from Bio-Rad Laboratories (Hercules, CA). 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and Pyridostigmine bromide were purchased from Sigma Chemical (St. Louis, MO).

Toxicity Bioassay

The toxicity of the chlorpyrifos to 3rd instar larvae (< 12 h) of *S. littoralis* was evaluated in two strains through topical application. Larvae were treated on the dorsum with 2 µL of each concentration of insecticide solution or acetone (control). Three replicates ($n = 10$ larvae per replicate) for each concentration as well as the control. Larvae were moved to a glass jar for 24 h, then mortality was noted. If larvae were not able to move in a coordinated way when gently stimulated with a fine brush, the larvae were considered as dead. The jars were placed in the rearing chamber at 25 ± 1 °C, $65 \pm 5\%$ relative humidity and 16:8 L:D. The dose of insecticide that was lethal to 50% of tested larvae (LD_{50}) was calculated using probit analysis using the SPSS program (SPSS Inc., Chicago, IL).

Sample Preparation and Enzyme Assays

The larvae alive after treatment with chlorpyrifos of two strains were homogenized in 200 µL phosphate buffer pH (7.0). The homogenates were centrifuged at 4 °C, 10,000 rpm for 10 min. The supernatants were used as the enzyme source. AchE activity was determined according to colorimetric method of Ellman et al. (1961) using ATChI as substrate. Fifty µL of supernatant was added to a tube containing 140 µL of phosphate buffer (0.1 M, pH 7), 50 µL substrate (ATChI, 75 mM), and 20 µL DTNB (0.1 M). After 15 min of incubating the reaction mixture at room temperature, 0.5 mL of pyridostigmine bromide (1 mM) was added to stop the reaction. Level of enzyme activity was measured at 412 nm using a spectrophotometer (Shimadzu UV-1600®) at 412 nm. AchE activity was expressed as units per mg of protein after measurement of protein by Bradford (1976) method, using BSA as a standard.

Assays of Activity, Kinetics, and Sensitivity of AchE

Kinetic parameters of AchE were determined based on the AchE activity at two substrate concentrations (1 and 10 mM) as previously described in the assay of AchE activity. The enzyme kinetic parameters, Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were estimated using Lineweaver–Burk plot (Lineweaver and Burk 1934). Estimation of I_{50} value (the inhibitor concentration required to inhibit 50% of the enzyme activity) was done by pre-incubating the AchE solution in different concentrations (0.01–2 µM) of chlorpyrifos for 10 min. Afterward, the substrate was added to the reaction mixture containing phosphate buffer (pH 7.0), and DTNB. Also, the inhibitory constant (K_i) was determined according to the method of Dixon and Webb (1964).

Statistical Analysis

The data obtained from the toxicity test were corrected using Abbott's formula (1925) before analysis, and data were subjected to probit. $RR = LD_{50} FR / LD_{50} LS$. All tests and control (without chlorpyrifos) assays were corrected by blanks for non-enzymic hydrolysis. Assays were performed in triplicate. According to the Lineweaver–Burk plot and Dixon-plot, it was estimated how chlorpyrifos inhibited AchE. The mean values of the recorded data were subjected to analysis of variance (ANOVA), followed by Tukey's test at $P < 0.05$ significant level using SAS program (SAS Institute, 2004).

Results

Resistance Level

The LS and the FR strains of *Spodoptera littoralis* showed significant difference in toxicity to chlorpyrifos; LD₅₀ value of LS strain = 4.07 µg/larva, $\chi^2 = 0.39$, $df = 2$, $P = 0.96$; LD₅₀ value of FR strain = 287.49 µg/larva, $\chi^2 = 6.54$, $df = 2$, $P = 0.62$.

In Vitro AChE Inhibition

The in vitro inhibitory effect of chlorpyrifos on AChE in both LS and FR strains at 0.1 and 0.05 µM showed high inhibitory activity in LS strain and there was significant difference between the two concentrations (91.67% and 76.81% at 0.1 and 0.05 µM, respectively). In contrast, the effect of chlorpyrifos was low on AChE in the FR strain at two concentrations (29.34% and 14.66% at 0.1 and 0.05 µM, respectively) (Fig. 1).

Sensitivity of AChE

Table 1 provides inhibition constants determined from the inhibition reaction of chlorpyrifos on AChE from the two strains. Significant differences were observed in I_{50} and ki values between the two strains. The I_{50} and ki values of AChE from FR strain were 5.22- and 1.24-fold, respectively, less sensitive to inhibition by chlorpyrifos than LS strain.

Kinetics of AChE

A comparison of the AChE sensitivity and resistance in the two strains showed the V_{max} and K_m values for AChE in

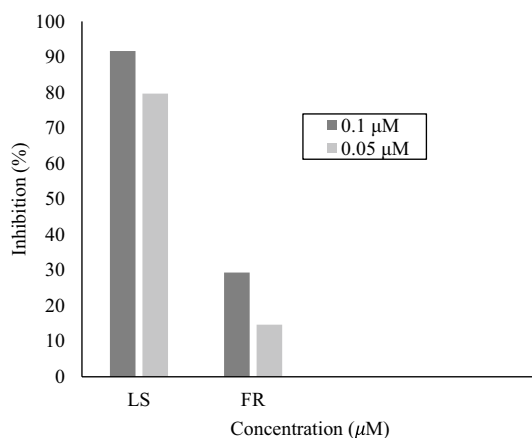


Fig. 1 In vitro inhibition of *Spodoptera littoralis* larvae AChE by chlorpyrifos in LS and FR strains

Table 1 I_{50} and K_i values of chlorpyrifos on AChE in the LS and FR strains of *Spodoptera littoralis*

Insecticide	Strain	I_{50} (µM) ^a	K_i (µM) ^b
Chlorpyrifos	LS	0.069 ± 0.037a	3.86 ± 0.13a
	FR	0.36 ± 0.015b	4.80 ± 0.38b

Data are means ± SE of three replicates. Means of I_{50} and ki values followed by the same letters in each column are not significantly different (Tukey's test, $P < 0.05$).

^a($df = 2$; $F = 128.628$)

^b($df = 2$; $F = 774.334$)

the FR strain decreased by 2.54- and 1.98-fold, respectively (Table 2).

Discussion

Based on the results, FR strain showed a high rate of resistance to chlorpyrifos 70.64-fold compared to the LS strain. Previous studies demonstrated that resistance to OP insecticides has developed in many insects such as *Bemisia tabaci* (Gennadius) (Zhang et al. 2012), *Phenacoccus solenopsis* (Tinsley) (Afzal et al. 2015), *Drosophila melanogaster* (Meigen) (Daisley et al. 2018) and *Spodoptera littoralis* (Boisadual) (Ismail 2020).

In this study, the results indicated that the I_{50} and ki values of AChE in the FR strain are about 5.22- and 1.24-fold, respectively, higher than in the LS strain and this resistance is attributable to the modification of AChE enzyme to an insensitive form (Houndété et al. 2010; Afzal et al. 2015; Moradi et al. 2019; Ismail 2021). All findings in other studies have shown that AChE insensitivity to OP insecticides plays an important role in the differences of susceptibility in several insects, including *Cydia pomonella* (Linnaeus) (Reyes et al. 2007), *Helicoverpa armigera* (Hübner) (Farnsworth et al. 2010), *Laodelphax striatellus* (Fallén) (Xu et al. 2014) and *Spodoptera littoralis* (Boisadual) (Ismail 2020).

Kinetic analyses clearly showed that the K_m and V_{max} values of AChE in the FR strain are about twofold less

Table 2 Kinetic parameters of AChE in the LS and FR strains of *Spodoptera littoralis*

Insecticide	Strain	V_{max} (mmol/min/mg) ^a	K_m (µM) ^b
Chlorpyrifos	LS	61.7 ± 0.88a	17.5 ± 5.69a
	FR	156.7 ± 4.15b	34.7 ± 8.43b

Data are means ± SE of three replicates. Means of V_{max} and K_m values followed by the same letters in each column are not significantly different (Tukey's test, $P < 0.05$).

^a($df = 2$; $F = 1421.223$)

^b($df = 2$; $F = 160.912$)

compared to those from the LS strain. Alteration in K_m and V_{max} values between FR and LS strains may affect AchE sensitivity for various organophosphorus based insecticides. Various studies show that higher K_m and V_{max} activities are indicators of resistance (Gao et al. 1998; Ismail 2008; Lang et al. 2010; Hwang et al. 2014). In general, all the results showed that the decreased sensitivity to AchE, played an important role in increasing the OP resistance.

Conclusion

Overall, these results support a direct causal relationship between the insensitivity of AchE and resistance to OP insecticides. Therefore, rotation of OP insecticides with insecticides of other classes in application schedule against insect pests may slow the development of resistance from OP insecticides.

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Availability of Data and Material All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no conflict of interests.

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

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