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Full Length Research Paper

Resistance comparison of domesticated silkworm (*Bombyx mori* L.) and wild silkworm (*Bombyx mandarina* M.) to phoxim insecticide

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In this study, the resistance difference to phoxim between *Bombyx mori* L. and *Bombyx mandarina* M was investigated. For the both silkworm species, the whole body of each larval were collected, and on the third day of the 5th instar, the brain, midgut, fat bodies, and silk gland were collected for enzymatic activity assay of acetylcholinesterase (AChE). Our results showed that in the early larval stages, the resistance difference to phoxim was not significant between the two species. However, in the 4th and 5th instar, the resistance differences showed significant increase. When compared to *B. mori* L, the LC_{50} of *B. mandarina* was 4.43 and 4.02-fold higher in the 4th and 5th instar, respectively. From the 1st to 5th instar, the enzymatic activities of AChE of *B. mandarina* were 1.60, 1.65, 1.81, 1.93 and 2.28-fold higher than that of *B. mori*, respectively. For the brain, midgut, fat body, and silk gland on the third day of the 5th instar, the enzymatic activity ratios of *B. mandarina* to *B. mori* were 1.90, 2.23, 2.76, and 2.78, respectively. The AChE-I_{50} values of *B. mori* and *B. mandarina* detected by eserine method were 5.02×10^{-7} and 5.23×10^{-7} mol/L, respectively. Thus, our results indicate that the higher enzymatic activities of AChE and the insensitivity to specific inhibitor of the enzyme might be the underlying mechanisms for higher phoxim resistance in *B. mandarina*.

Key words: *Bombyx mori* L., *Bombyx mandarina* M., phoxim, acetylcholinesterase activity, resistance.

INTRODUCTION

The domesticated silkworm *Bombyx mori* L., a member of the family Bombycidae, is a well-studied lepidopteran model system with rich repertoire of genetic information on mutations affecting morphology, development, and behavior (Arunkumar et al., 2006). This species has been used as a source of silk, and has lost some characteristics due to long-term breeding under artificial conditions.

The wild silkmoth, *Bombyx mandarina* M., is believed to be the ancestor of *B. mori* (Banno et al., 2004), as these two species can cross-breed and yield fertile hybrid offspring. *B. mandarina* includes significant variation within species (Yukuhiro et al., 2002). From the aspect of morphological and physiological characteristics, *B. mandarina* was very similar to *B. mori* (Astaurov et al., 1959; Yoshitake, 1968). Due to long-term natural selection, there was a difference of resistance to insecticides between the two species (Shen et al., 2003). *B. mori* had a weak resistance to insecticide, and its production was reduced by more than 30% annually because of insecticide poisoning in China. On the other hand, being one of the major pests in mulberry fields, *B. mandarina* showed increasing resistance to insecticide owing to its wide use.

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Abbreviations: **AChE,** Acetylcholinesterase; **OP,** organophosphate; **CB,** carbamate; **ATC-DTNB,** acetylthiocholine iodide-5, 5'-dithio-bis (2-nitrobenzoic acid).

Table 1. Comparison of resistance of *B. mori* to phoxim at different instars.

Instars	Regression equation	LC_{50} (ng/mL^{-1})	(95% FL)	Resistance ratio
1st	Y = - 43.1671 + 20.0179x	254.8	247.4 ~ 262.4	1.00
2nd	Y = - 16.3687 + 8.5586x	313.9	294.1 ~ 334.9	1.23
3rd	Y = - 21.7041 + 8.84129x	1048.1	985.0 ~ 1115.2	4.11
4th	Y = - 19.781 + 7.7398x	1591.4	1484.0 ~ 1706.5	6.25
5th	Y = - 2.6411 + 2.2099x	2868.8	2185.6 ~ 3765.6	11.26

The organophosphorus insecticides have been of interest for years because of their toxicological activities in a wide variety of organisms, including the overall changes observed in acetylcholinestease activity (Nath et al., 1999). Phoxim, a widely-used broad-spectrum organophosphorus insecticide, is well-known for its potential insect knockdown capacity. Although beneficial in protecting the crop against insect pests, phoxim has posed a grave environmental problem due to its indiscriminate use in the fields.

Acetylcholinesterase (AChE, 2 EC 3.1.1.7), encoded by the *ace* gene, catalyzes the hydrolysis of the neurotransmitter acetylcholine to terminate nerve impulses at the postsynaptic membrane. AChE is one of the targets of organophosphate (OP) and carbamate (CB) insecticides. Structural alteration of AChE, resulting in insensitive enzyme, is one of the major mechanisms of the OP and CB resistance in more than 25 arthropod species (Fournier et al., 1994).

In 1972, Kattera reported that *B. mori* larvae were resistant to organphosphorus insecticides (Kattera, 1972). However, recent studies have demonstrated the toxic impact of organophosphorus insecticides on *B. mori*. AChE activity (Nath et al., 1999) and two AChE cDNAs have been recently cloned from *B. mori* (Seino et al., 2007). Here, we explored the difference in the activity of AChE and the resistance to phoxim between *B. mori* and *B. mandarina.* The present results are significant to the study of resistance evolution of Lepidorptera as well as the understanding of the mechanism of pesticide resistance of insects.

MATERIALS AND METHODS

Insect

The larvae of *B. mori* (Dazao strain), and *B. mandarina* (Suzhou strain), maintained in our laboratory, were reared on mulberry leaves under a 12-h light/12-h dark-photo period.

Measure of resistance

Twenty grams of fresh mulberry leaves were soaked in a solution containing each working concentration of phoxim for 1 min. After being dried in the air, the leaves were used to rear the newly molted larvae of *B. mori* and *B. mandarina* in each instar. Three independent experimental tests were done, with three repeats in each test, and 30 larvae in each test. The mortality number of the larvae was counted after 24 h.

Samples for detection of AChE enzymic activity

The crude enzyme was prepared and studied according to Shang et al. (2007). The whole body of each newly molted *B. mori* and *B. mandarina*, the brain, midgut, fat body and silk gland of the third day 5th instar from *B. mori* and *B. mandarina* were selected for AChE activity assay. All the samples were homogenized in phosphate buffer (25 mmol/L, pH 8.0) containing 0.5% Triton X-100. The homogenates were centrifuged at 15 000 g for 30 min at 4℃ using a refrigerated centrifuge (KUBOTA 3700, KuBoTa Corporation, Tokyo, JAP). The supernatant was filtered through glass wool to remove lipids. The filtrates were used to detect the enzyme activity.

AChE activity and inhibition assays

AChE activity was assayed at 412 nm using acetylthiocholine iodide (Sigma-Aldrich, St. Louis, USA) as the substrate (Ellman et al., 1961). Inhibitor specificity of the enzyme was tested according to Zhu et al. (1991). In brief, the samples were pre-incubated with several different concentrations of the inhibitor (eserine, Sigma) at 37℃ for 5 min. Residual activity was determined with a microplate reader at 405 nm for 2 min after ATC-DTNB (acetylthiocholine iodide-5,5'-dithio-bis (2-nitrobenzoic acid) Sigma solution was added to the reaction mixture. The median inhibition concentration (I_{50}) of each inhibitor was determined based on log-concentration versus probit (% inhibition) regression analysis. Protein concentration was determined by the protein-dye binding method with a standard protein of bovine serum albumin (BSA) (Bradford et al., 1976).

RESULTS

Comparison of phoxim resistance in the two species

A definite increase of resistance to phoxim is shown when the larvae of *B. mori* grew (Table 1). The resistance ratios of the 2nd, 3rd, 4th and 5th instar to the 1st instar were 1.23, 4.11, 6.25 and 11.26 fold, respectively.

The trend of resistance increase of *B. mandarina* was similar to that of *B. mori* larvae. The result is as presented in Table 2. The resistance ratio of 2nd, 3rd, 4th and 5th instar to 1st instar were 1.13, 4.42, 18.81 and 30.82-fold, respectively.

Detection of AChE activity

From the 1st to 5th instar, the AChE activity of *B.*

Table 2. Comparison of resistance of *B. mandarina* to phoxime at different instars.

Instars	Regression equation	LC_{50} (ng/mL^{-1})	(95% FL)	Resistance ratio
1st	Y = - 16.0684 + 8.1869x	374.5	348.4 ~ 402.6	1.00
2nd	Y = - 41.2076 + 17.5843x	424.4	409.5 ~ 439.9	1.13
3rd	Y = - 24.6709 + 9.2177x	1655.3	1556.9 ~ 1760.04	4.42
4th	Y = - 25.7289 + 7.9858x	7045.7	6565.3 ~ 7561.1	18.81
5th	Y = - 27.7556 + 8.06320x	11543.9	10777.1 ~ 12365.3	30.82

Table 3. Comparison of AChE activity in each instar of *B. mori* and *B. mandarina*.

Instars	AChE activity (nmol/min/mg.pro)		Bmm/Bm* ratio
	B. mori	*B. mandarina*	
1st	11.42 ± 1.05	18.24 ± 1.62	1.60
2nd	13.86 ± 2.67	22.85 ± 1.43	1.65
3rd	15.11 ± 0.84	27.33 ± 1.61	1.81
4th	17.31 ± 1.26	33.46 ± 2.34	1.93
5th	18.35 ± 2.11	42.01 ± 1.04	2.28

Bmm/Bm, *B. mandarina*/ *B. mori*.

Table 4. Comparison of AChE activity in different tissues of *B. mori* and *B. mandarina*.

Tissues	AChE activity (nmol/min/mg.pro)		Bmm/Bm ratio
	B. mori	*B. mandarina.*	
Brain	30.82±0.10	58.51±2.34	1.90
Midgut	13.54±0.30	30.14±0.61	2.23
Fat body	13.86±0.55	38.11±0.97	2.75
Silk gland	14.73±0.10	40.82±0.65	2.77
Blood	6.02±0.13	15.31±0.12	2.54

mandarina was higher than that of *B. mori*, the ratio range (Bmm/Bm) were 1.6 to 2.28. There was a definite increase in AChE activity during the larvae growing period. The result is as shown in Table 3.

The AChE activity in the tissues of *B. mori* and *B. mandarina* is presented in Table 4. The result showed that the AChE activity in *B. mandarina* was higher than that of *B. mori*. The Bmm/Bm ratios were 1.90, 2.23, 2.75, 2.77 and 2.54 in brain, midgut, fat body, silk gland and blood, respectively.

The filtrates of the 2nd instar newly molted *B. mori* and *B. mandarina* were used as a source of enzyme to test for the AChE I_{50}. The I_{50} of *B. mori* and *B. mandarina* stimulated by the reagent of eserine were 5.02×10^{-7}, and 5.23×10^{-7} mol/L, respectively. Both enzymes were sensitive to the inhibition by eserine. However, the *B. mori* enzyme was more sensitive than *B. mandarina* enzyme.

DISCUSSION

B. mori has been used for over 5, 000 years and is an important economical insect. The use of insecticides, especially organophosphates, on the mulberry has deleterious effects on the silkworm (Radhakrishna et al., 1992). The resistance of silkworm to organophosphate and carbomate insecticides is not well-studied, and no replacement of amino acids is found in the cloned *ace*1 sequence (Shang et al., 2007). Recently, completed genome sequence of *B. mori* (Mita et al., 2004; Xia et al., 2004) provides molecular genetic resources for resolving a broad range of biological problems (Nagaraju et al., 2002). The functions of some important genes have been studied in *B. mori* (Ling et al., 2008; Zhao et al., 2007; Xu et al., 2008). It is not inconceivable that domesticated silkworms nowadays are all descended from an initial stock of *B. mandarina* (Yoshitake, 1968). Hence, the *B. mandarina* is a good model for evolution analysis of *B. mori*. (Arunkumar et al., 2006; Banno et al., 2004; Yukuhiro et al., 2002; Lu et al., 2002; Sakaguchi et al., 1998). Furthermore, the *B. mandarina* is a pest in fields and it will be informative for the pest control when the resistance to insecticides of *B. mandarina* is well studied.

The difference of resistance to insecticides has been

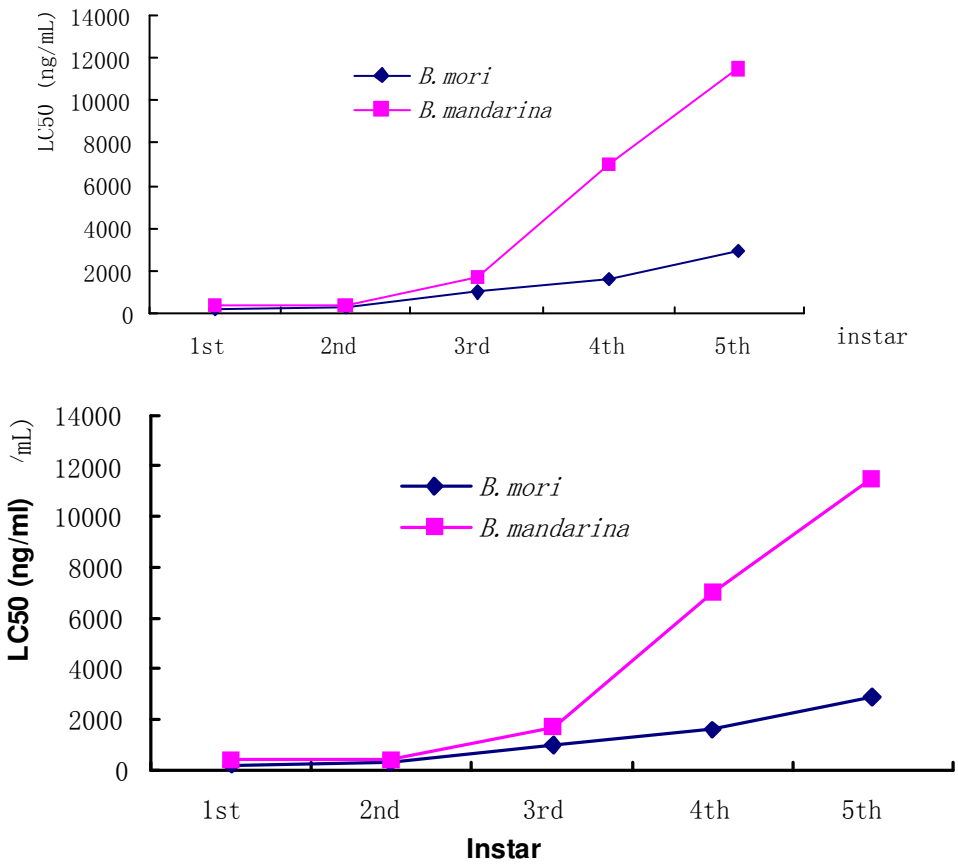

Figure 1. Comparison of resistance of *B. mandarina* and *B. mori* to phoxime in each instar.

found in the development period in a certain instar of *B. mori* (Ma et al., 2005). There is no linear relationship between the difference of resistance to OP and body height of *B. mori*. It may be due to the increase in the detoxification enzymes, generally attributed to be found in the microsomes (Kattera, 1972). In the natural environment, the development proceeding of *B. mandarina* is quite different (Shen et al., 2003). The insects can avoid the harm from insecticides when they are in the molting stage. Hence, directly investigating the insecticide resistance of the insects in field is not suitable. In this study, the larvae of *B. mandarina* were cultured to keep them consistent in development, avoiding the error of analyzing the data obtained from larvae grew in the field. The results indicate that the insecticide resistance of *B. mandarina* from each larval stage is significantly stronger than that of *B. mori*, particularly in the older larvae (Figure 1). This might be a reason why insecticide poisoning of *B. mori* often occur while *B. mandarina* remains unaffected.

Alteration of AChE has been considered as one of the several resistance mechanisms to organophosphate and carbamate insecticides in many insect species (Soderlund et al., 1990). In resistant strains with altered AChE, the reduced sensitivity of AChE to active inhibitors appears to have resulted from the reduced affinity of the enzyme to the inhibitor molecule rather than from the lowered rate constant for acylation (Oppenoorth, 1985). It means that the inhibition of altered AChE could be analyzed *in vitro* through the conventional biochemical experiments with suitable AChE inhibitors. In this study, eserine is chosen as an AChEs inhibitor, and the sensitivity of other AChEs inhibitors remains to be further studied.

Two acetylcholinesterase genes (*ace*1 and *ace*2) of *B. mandarina* (Suzhou strain) and *B. mori* (Dazao strain) have been cloned in our laboratory. The sequence analysis showed that *ace*1 has two mutations (G664S and S307P) and *ace*2 has four mutations (M18I, N233S, I310V and G621S) (unpublished data). This observation reinforces the previous findings in the *ace* gene between *B. mori* and *B. mandarina*.

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