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Changes in the activity and the expression of detoxification enzymes in silkworms (*Bombyx mori*) after phoxim feeding

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ABSTRACT

Silkworm (*Bombyx mori*) is an economically important insect. However, non-cocoon caused by chemical insecticide poisoning has largely hindered the development of sericulture. To explore the roles of detoxification enzymes in *B. mori* after insecticide poisoning, we monitored the activity changes of cytochrome P450 monooxygenase, glutathione-*S*-transferase, and carboxylesterase in *B. mori* midgut and fatbody after phoxim feeding. At the same time, the expression levels of detoxification enzyme-related genes were also determined by real-time quantitative PCR. Compare to the control levels, the activity of P450 in the midgut and fatbody was increased to 1.72 and 6.72 folds; the activity of GST was no change in midgut, and in fatbody increased to 1.11 folds; the activity of carboxylesterase in the midgut was decreased to 0.69 folds, and in fatbody increased to 1.13 folds. Correspondingly, the expression levels of detoxifying enzyme genes *CYP6ae22*, *CYP9a21*, *GSTo1* and *Bmcce* were increased to 15.99, 3.32, 1.86 and 2.30 folds in the midgut and to 3.58, 1.84, 2.14 and 4.21 folds in the fatbody after phoxim treatment. These results demonstrated the important roles of detoxification enzymes in phoxim metabolism. In addition, the detected activities of such enzymes were generally lower than those in cotton bollworms (*Helicoverpa armigera*), which may contribute to the high susceptibility of *B. mori* to insecticides. Our findings laid the foundation for further investigations of the molecular mechanisms of organophosphorus pesticide metabolism in *B. mori*.

1. Introduction

Organophosphorus pesticides are widely used in agriculture because of their broad spectrum of activities [1]. However, the resistance to such pesticides is gradually increasing with continued application of them. The insects obtain resistance by two major mechanisms: reduction in the susceptibility of acetylcholinesterase (AChE) to organophosphorus insecticides [1,2]; and increases in the activities of enzymes, including esterase (EST), cytochrome P450 monooxygenases, and glutathione-*S*-transferases, to hydrolyze and isolate the organophosphorus pesticides [3].

Phoxim [*O*,*O*-diethyl *O*-(alpha-cyanobenzylideneamino) phosphorothioate] is a highly effective organophosphorus insecticide widely used in agriculture and forestry. Tang et al. [4] reported that cytochrome P450 oxidase and GST play important roles in bollworms' phoxim-resistance, with much higher activities of these two enzymes being observed in the resistant strains. Rodríguez et al. [5] revealed that the activity of detoxification enzyme was significantly elevated in the Spain strain *Adoxophyes orana* treated with chlorpyrifos-ethyl, azinphos-methyl, or phosalone; in that study, a mechanism with EST as the major player for the pesticide resistance was also proposed. Therefore, it is critical to understand the important roles of detoxification enzyme family in organophosphorus pesticide metabolism and resistance, which has been largely hindered by the lack of genomic information of related insects.

Changes in expression levels or in gene structures of detoxification enzymes directly generate corresponding changes in their detoxification capacities. Specifically, many studies had indicated that induced overexpression of detoxification genes is tightly correlated with insects' pesticide resistance. For example, Wang et al. [6] found that the expression of *Cyp6ae22* was significantly increased in the midgut (1.5 folds) and fatbody (2.5 folds) of *Bombyx mandarina* by deltamethrin pesticide. And some GSTs and carboxylesterases also participated in detoxification in insects. Ma et al. [7] found that *GSTo1* may play some roles in the resistance to organophosphorus pesticides, and the K_m and V_{max} for the CDNB as the substrate were high than that of the control. Wang et al. [8] used the gene chip found that after phoxim treatment, the expression of *Bmcce* (probe ID: sw12117) was 2-fold than that of the control. In this paper, we tested the activities of three different enzymes

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(cytochrome P450, CYP450; glutathione-*S*-transferase, GST; carboxylesterase, CCE) in the midgut and fatbody and explored their roles in the metabolism and detoxification of phoxim insecticide. We then chose four genes to study their expression at the transcriptional level. We found that phoxim-treatment enhanced the expression of detoxification genes, suggesting that these genes may participate in the metabolism and detoxification of phoxim insecticide.

The *Bombyx mori* belongs to *Saturniidae* of *Lepidoptera* and was derived from the Chinese *B. mandarina* [9]. After about 5700 years of domestication [10], *B. mori* is highly sensitive to adverse environments, as a result of being constantly kept isolate [11]. Furthermore, the *B. mori* is also the first one in *Lepidoptera* whose genome has been completely sequenced. Therefore, it has become the model organism to study the mechanisms of pesticide resistance in *Lepidoptera*. Wang et al. [8] reported the gene expression profiles in *B. mori* after phoxim treatment using gene chip. Peng et al. [12] revealed the expression patterns of *ace* genes in different developmental stages after phoxim treatment. However, the activity changes and gene expression of detoxification enzymes after phoxim treatment have remained unknown.

In this study, the characteristics of activity changes of three major detoxification enzyme families in phoxim-fed 3-day-old fifth instar *B. mori* were determined using the method of enzyme activity dynamics. At the same time, the expression levels of related detoxification enzyme genes were also investigated. The findings promote more clear understanding of the role of metabolism in pesticide resistance, which may facilitate the development of new methods for effective control of *Lepidoptera* insects.

2. Materials and methods

2.1. Insects

The *B. mori* strain Dazao was maintained in our laboratory. This strain originated in Guangdong province, China. And it was sensitive to insecticides.

The status of insecticide exposure: gastric juice spitting, body distortion, body shrinking, and head and chest protrusion (Fig. 1).

2.2. Chemicals and reagents

p-Nitroanisole, Reduced coenzyme II NADPH, 1-chloro-2, 4-dinitrobenzene, CDNB, and phoxim were purchased from Sigma. Fast Blue RR salt and α-naphthyl acetate were purchased from Fluka. Phenylmethylsulfonyl fluoride (PMSF), ethylene diamine tetra acetic acid (EDTA), and Dithiothreitol (DTT) were purchased from Promega. Phenylthiourea (PTU) and reduced Glutathione were purchased from Sinopharm Chemical Reagent Co., Ltd. phoxim were purchased from sigma company.

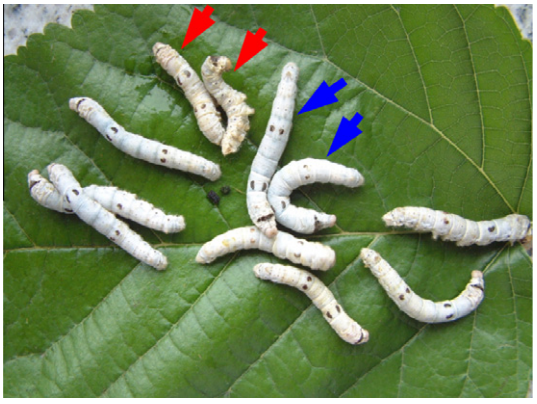

Fig. 1. Symptoms of phoxim poisoning of *B. mori*.

2.3. Phoxim treatment

One hundred milligrams phoxim was dissolved in 1 mL of acetone as stock solution, which was further diluted with double distilled water (4.0 μg/mL) as working solution. Mulberry leaves were dipped in 200 mL of working solution for 1 min, before being air-dried.

Mulberry leaves treated with water as control, 3-day-old fifth instar larvae (weight 1 ± 0.19 g) were fed with phoxim-treated leaves for 24 h.

2.4. Enzyme activity determination

2.4.1. Enzyme preparation

B. mori both phoxim treatment group and control group were dissected on ice for the isolation of midgut and fatbody tissues (0.2 g/tissue/insect). The tissues were then homogenized in 1 mL 0.1 M pH 7.8 buffer (1 mM EDTA, l mM DTT, 1 mM PTU, 1 mM PMSF, and 20% glycerol, before being centrifuged at 12,000g at 4 °C. The supernatant was used for enzyme activity determination.

2.4.2. Determination of enzyme activities and total protein levels

The activity of cytochrome P450 was determined with *p*-nitroanisole (PNOD) as substrate, following previous methods of Rose et al. [13] and Yang et al. [14]. The activities of glutathione-*S*-transferase were determined by its binding to CDNB following the GST–CDNB method described previously [15]. The Esterase-a-NA activities were determined according to the Villi-Asperen method [16]. The total protein levels were determined by the Bradford method [17].

2.5. Total RNA extraction and real time quantitative RT-PCR

2.5.1. Total RNA extraction

After 24 h of feeding with phoxim, the midgut and fatbody tissues were isolated and stored at −80 °C. The total RNA was extracted from the tissues by using the TRIzol kit, and the M-MLV RT kit was used for reverse transcription.

2.5.2. Primer design and real time quantitative PCR

The sequences of these genes were from NCBI. The primers for real-time PCR were designed by using the Primer premier 5.0 software (Table 1).

A total of 20 μL reaction was set up for qPCR: 10 μL of SYBR Pre-Mix Ex Taq™, 1 μL of each primer, 2 μL of cDNA template, and 6 μL of water. The PCR conditions were: denaturation at 95 °C for 1 min, followed by 40 cycles of 5 s at 95 °C, 10 s at 55 °C, and 10 s at 72 °C. The PCR was repeated 3 folds for each sample, and *actin3* was used as the internal reference gene.

2.6. Statistical analysis

The statistical analysis was carried out by using the SPSS software with the paired value *t*-test (two-tailed) and significant difference analysis (analysis of variance, ANOVA). Differences with $p \leqslant 0.05$ were considered as statistically significant.