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Full Length Article

Over-expression of Cytochrome P450s in *Helicoverpa armigera* in Response to Bio-insecticide, Cantharidin

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Abstract

Cantharidin, a well known natural compound produced by beetles of family Meloidae and Oedemeridae, developed as bio-insecticide in China, was investigated for its effect of cytochrome P450 *O*-demethylase activity using sub-lethal concentration. Results showed that cytochrome P450 *O*-demethylase activity remained significantly high at 12 to 48 h after treatment. To study the gene expression of *CYP6B7* at molecular level, gene specific primers were designed according to gene sequence of *CYP6B7*, whereas *β-actin* gene from *Helicoverpa armigera* was used as internal control. Semi qRT-PCR results showed that fluorescent intensity ratio of *CYP6B7* and control mRNA transcript level remained at 1.01, 1.23, 1.68 and 1.62 folds after 12, 24, 36 and 48 h, respectively after treatment by sub-lethal dose of cantharidin in artificial diet. In light of our experimental results we may conclude that over-expression of P450s in general and *CYP6B7* in specific may be involved in resistance towards cantharidin in *H. armigera*. Moreover, there is a potential risk of insecticide resistance against cantharidin in areas, where P450s associated resistance has already developed in lepidopteran. © 2013 Friends Science Publishers

Keywords: Cantharidin; *Helicoverpa armigera*; Cytochrome P450; *CYP6B7*; mRNA

Introduction

Cytochrome P450 monooxygenases belong to protein super family of diverse and widely distributed enzymes, which are mainly involved in metabolism of endogenous and exogenous compounds of structurally diverse nature (Hudgson, 1985; Nelson, 1987). They have been found to have the capability of oxidizing broad range of exogenous compounds and are therefore important in defining interaction of animals and plants (Gonzalez and Nebert, 1990; Schuler, 1996). Herbivores usually use P450s to metabolize allelochemicals that allow utilization of host plants which are toxic to other species (Berenbaum, 1990).

It is widely believed that the exposure of herbivores to secondary plant metabolites such as alkaloids, terpenoids, flavonoids and quinines, which are used as defense mechanism, may have forced the evolution of P450s (Ma *et al*., 1994; Prapaipong *et al*., 1994).

Monooxygenases metabolize large number of compounds due to abundance of P450 isoforms and substrate specificity of some isoform. Generally, metabolism by monooxygenases commonly results in detoxification of substrate however activation in some cases is possible. For instance organophosphates insecticides commonly used for pest control are activated by monooxygenases (Agosin, 1985).

The activity level of monooxygenase show significant increase when exposed to certain type of natural or synthetic compounds acting as substrate known as induction of P450 (Qiu *et al*., 2003). P450 monooxygenases in insects may be induced by insecticides (Huang and Leng 1992), plant allelochemicals (Tan and Guo 1996; Amichot *et al*., 1998) and herbicides (Miota *et al*., 2000) etc.

Helicoverpa armigera commonly known as cotton bollworm is an important pest of many agricultural crops of economic importance all over the world. The polyphagous nature of this pest is attributed to its extensive damage to wide variety of crop species. In previous studies resistance has been mentioned as main reason behind its outbreak (Forrester *et al*., 1993). Cytochrome dependent detoxification has been implicated as one of the main reason of its insecticidal resistance (Forrester *et al*., 1993; Scott *et al*., 1998). In China, cantharidin insecticides are under investigation for the control of lepidopteran insects. One commercial bioinsecticide, cantharidin AS has already been registered. The insecticidal and anti-feedant activities of cantharidin are well established fact as elucidated by (Zhang *et al*., 2003) on armyworm and diamondback moth. However, no data is available on its interaction with cytochrome P450 monooxygenases.

In our present studies possible role of P450 monooxygenases in resistance mechanism of insect toxin, cantharidin was investigated *in vivo* by biochemical and molecular methods.

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Materials and Methods

Reagents and Chemicals

P-Nitroanisol and NADPH were purchased from BODI and Wolsen, respectively, whereas Cantharidin, Diethylpyrocarbonate (DEPC), phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT) and ethylenediaminetetraacetate acid (EDTA) were procured from Sigma-Aldrich. Molecular weight marker DL2000 and *rTaq* polymerase enzyme were from Takara. Other chemicals used in the experiments were of commercial grade.

Rearing of Insect

H. armigera larvae were procured from Henan Jiyuan Baiyun Industry Co., Ltd. China and reared until F1 for use in bioassay. Groups of 24 larvae were placed into 24 chamber plastic boxes. The boxes were placed in an incubator at 27+1°C and 40 to 50% RH with 12 h photoperiod on artificial diet (Ahmed and McCaffery, 1991).

Statistical Analysis

SPSS 17.0 software was used for analysis of photometric data (SPSS Inc., Chicago, IL). Significance of the effect of cantharidin on enzymes specific activity was determined by independent *t*-test. Means were considered significantly different at $P \leq 0.05$.

Insect Treatment for Enzyme Assay

Laboratory prepared artificial diet as mentioned above was mixed with experimentally determined sub-lethal dose of 0.01 mg/g of cantharidin, dissolved in acetone. Acetone was added to the control artificial diet. Acetone was allowed to evaporate for one h before introduction of larvae into it. Larvae of early third instar, starved for eight h, were introduced to the cantharidin-treated artificial diet and control. Ten larvae per replication were collected from cantharidin-treatment and control groups at 12, 24, 36 and 48 h for determination of enzyme activity. The experiment was replicated thrice. Collected larval samples were flash frozen in liquid nitrogen just after collection and stored at -80°C.

Protein Determination

Protein contents within homogenates were determined using bovine serum albumin as standard (Bradford, 1976).

Specific Activity of Cytochrome P450 *O*-demethylase

The activity of *p*-nitroanisole *O*-demethylase was determined followed by Hansen and Hodgson (1971). Midguts from larvae both in treatment and control groups were homogenized on ice by glass homogenizer in pre-cooled 0.1M PBS of pH 7.2 having 1mM EDTA, 1 mM PMSF, 1 mM DTT and 10% glycerol. Homogenates were subjected to centrifugation at 10,000×g at 4°C for 15 min. The Supernatants obtained were used as enzyme extract solutions. Enzyme extract solutions of 0.5 mL containing 0.1M sodium phosphate (pH 7.8) and 0.005M NADPH was prepared. Reaction was initiated by the addition of 10 µL 0.002M nitroanisole and placed for incubated in water bath at 25°C with shaking for 30 min. The reaction was terminated by the addition of 0.5 mL of IM HCl. The product *p*-nitrophenol was extracted with $CHCl_3$ and then centrifuged to get two fractions. The $CHCl_3$ fraction was back extracted with 0.5M NaOH. The optical density of NaOH solution was determined at 400nm and the product was quantified using the experimentally determined curve.

RNA Extraction and Reverse Transcription Reaction

Frozen insects subjected to cantharidin treatment and stored at -80°C were used for total RNA extraction. The midguts were dissected from a total of 30 larvae per treatment and homogenized using liquid nitrogen before addition of RNAiso Plus (TaKaRa). Three biological replicates were used for each treatment and control groups. The total RNAs were extracted individually from treatment and control following manufacture's instructions. The quality of RNA samples were examined by running on agarose gel. DNase-I (Fermentas) was used to remove DNA contamination. The cDNAs were synthesized individually for treatment and control by reverse transcription using RevertAid™ Reverse Transcriptase (Fermentas) in 20 uL reaction following the recommended protocol provided by the manufacturer.

Gene Cloning and Sequencing

Helicoverpa armigera cytochrome P450 (*CYP6B7*) was amplified from cDNA by polymerase chain reaction (PCR) by upstream primer (5' GCA**GGATCC**ATGTGGGTCTTATATCTAC3') and downstream primer (5' GAC**GTCGAC**TTAAGATACAATCTTCCTAGG3'), respectively. Bam Hl restriction site was incorporated to sense primer, whereas Sal 1 restriction site was incorporated to antisense primer for double restriction digestion. Amplification reaction was performed by PCR program: First step denaturation for 3 min at 94ºC followed by 30 cycles of 94ºC for 30 s, 55ºC 45 s, 72ºC for 2 min and final extension of 5 min at 72ºC. Target gene amplified product was gel purified by gel extraction kit (Biomiga). Gel purified PCR product were then ligated to pMD-19T vector (TaKaRa) and transformed into *Escherichia coli* DH5α. The transformants were selected on LB agar plates containing 100 µg/mL ampicillin after overnight incubation at 37°C. At first the presence of target gene was identified by double restriction digestion of plasmid extracted from positive clones. Resultants clones were then sequenced by Shanghai Sunny Biotech Co., Ltd.

Sequence Analysis and Phylogenetic Analysis

Sequences were analyzed by DNAman software package (Lynnon, Quebec, Canada). Amino acid sequence was deduced by http://web.expasy.org/translate/NCBI was used to BLAST amino acid sequence to see its conformity with target sequences.

Semi qRT-PCR Analysis for Gene Expression

Semi qRT-PCR was performed to compare the expression level of *CYP6B7* mRNA in midgut of treatment and control groups after induction by sub lethal dose of cantharidin. The expression level of *CYP6B7* gene transcript was examined by Semi qRT-PCR analysis of cDNA synthesized from RNA isolated from midgets of insects treated with cantharidin. Two gene specific primers, sense (5'AATATCTTGATGGAGTAACA3') and anti-sense (5'GATTAAGTGAGAGTTGGTA3') were designed to amplify the cDNA fragment of *CYP6B7* of 135bp using Beacon Designer 7 (Premier Biosoft). To normalize gene expression *H. armigera β-actin* (GenBank EU527017) was used as endogenous control. A pair of primers was used to amplify Haβ-actin, sense (5'GTATTGCTGACCGTATGC3') and antisense (5'ATCTGTTGGAAGGTGGAG3'). Electrophoresis and visualization of amplified products were performed as mentioned above.

Results

CYP6B7 of *H. armigera* was amplified using gene specific primers. A product of 1515bp was obtained after PCR reaction (Fig. 1). The gene ligated to pMD-19T sequencing vector was confirmed by double restriction digestion and sequencing (Fig. 1).

Effect of Cantharidin on Activity of Cytochrome P450 *O*-demethylase

Higher cytochrome P450 *O*-demethylase specific activity was induced by sub-lethal dose of cantharidin-treated artificial diet. The specific activity of P450 *O*-demethylase was induced at 12 h after treatment and remained significantly high at ($P \leq 0.05$) compared to untreated control. Specific activities of *O*-demethylase were seen increasing at 24, 36 and 48 h and remained highly significant at ($P \leq 0.01$) compared to untreated control (Fig. 2).

Semi qRT-PCR Analysis for Gene Expression

The expression level of *CYP6B7* mRNA was investigated after treatment by cantharidin using semi qRT-PCR. *CYP6B7* mRNA expression level obviously changed in treatment compared to control (Fig. 3). No bigger change was seen at 12 h after treatment. Expression level however changed after 12 h. Fold change in fluorescent intensity of gene *CYP6B7* mRNA expression in midgut of *H. armigera* induced by cantharidin remained at 1.23, 1.68 and 1.62 after 24, 36 and 48 h, respectively (Fig. 4).

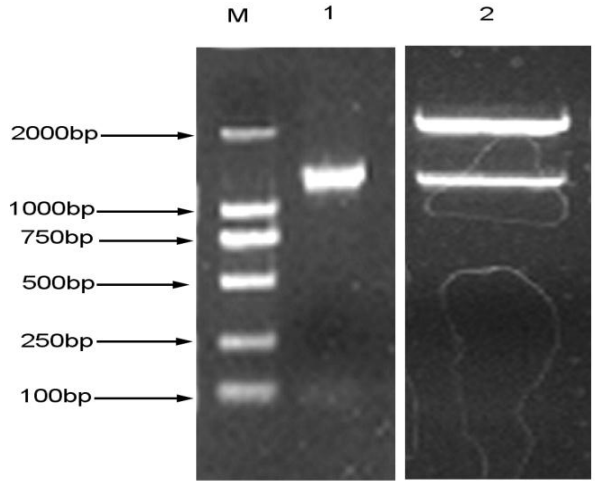

Fig. 1: PCR amplification of *CYP6B7*. Lane M, DNA marker; Lane 1, Target amplification of *CYP6B7*; Lane 2, Double restriction digestion of *CYP6B7*

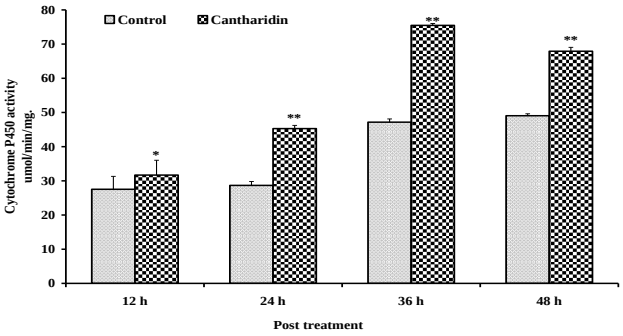

Fig. 2: Specific activity of cytochrome P450s at different intervals after treatment. Asterisks show significant difference between control and treatment by independent *t*-test at α 0.05 level

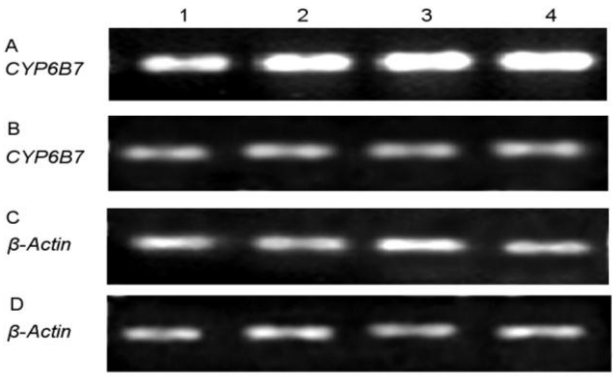

Fig. 3: Expression level of *CYP6B7* compared to control and β-actin. (A) Lane 1-4, expression level of mRNA transcript of *CYP6B7* in treatment; (B) Expression level of mRNA transcript of *CYP6B7* in control; (C) Expression level of mRNA transcript in *β-actin* in treatment; (D) Expression level of mRNA transcript in *β-actin* in control

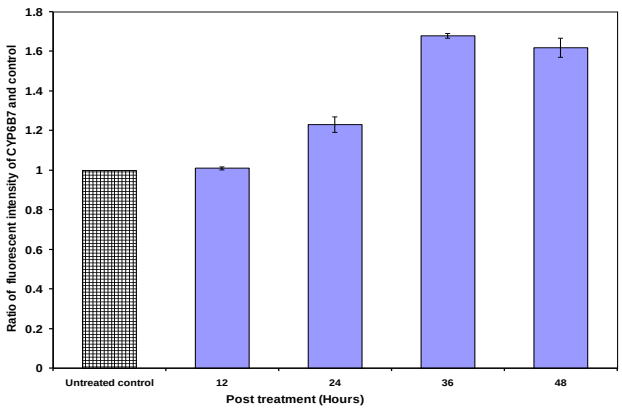

Fig. 4: Ratio of fluorescent intensity of *CYP6B7* mRNA transcript level and control, normalized by *β-actin* as internal control. Imagej software package was used to calculate fluorescent intensity of the bands. Error bars represent the mean ± SD

Discussion

In our present study cantharidin treatment of sub-lethal dose increased activity of P450s in general and midgut-specific *CYP6B7* in particular. Higher level of P450s in insect midgut is responsible for metabolism of substances. Lepidopteran larvae are the most ferocious feeder of crops and higher P450 monooxygenases activity has been reported in their midgut tissue (Thongsinthusak and Krieger, 1976; Qiu *et al*., 2003).

Activity of cytochrome P450s monooxygenases in present study remained largely high, showing its possible involvement in resistance towards cantharidin. Similarly, insecticide metabolism is catalyzed by cytochrome P450s, an important enzyme family. Enhanced levels of P450s have been reported in resistant insects such as cotton bollworm, diamondback moth and are regarded as major resistance mechanism to pyrethroids (Martin *et al*., 2002; Sonoda 2010). In our study the increased level of P450s showed similar mechanism of resistance by *H. armigera* with the above mentioned studies. Semi qRT-PCR results showed higher mRNA transcript of *CYP6B7* in cantharidin-treatment, compared to control. These results suggest that the mechanism of resistance towards cantharidin detoxification may be similar to pyrethroids detoxification by *CYP6B7*. Likewise, pyrethroids resistant field population of *H. armigera* from Australia was reported with over-expressed levels of *CYP6B7* (Ranasinghe *et al.*, 1998). In China, main resistance mechanism of resistance towards pyrethroids has been documented as enhanced oxidative metabolism (Shen and Wu 1995; Yang *et al.*, 2005). A higher activity of P450s in the present experiment showed that the Chinese strain of *H. armigera* showed similar mechanism of resistance towards cantharidin. In short, the use of cantharidin insecticide in fields with especially high

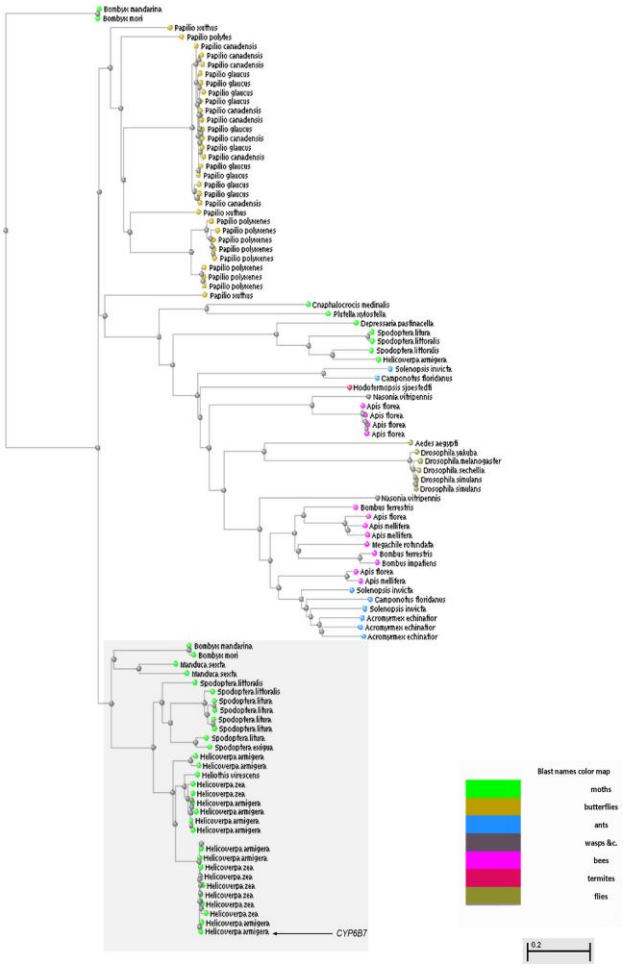

Fig. 5: Neighbor-joining phylogenetic tree of *CYP6B7* and its closest relatives from the NCBI database. Phylogenetic tree was generated by subjecting *CYP6B7* amino acid sequence to BLASTP in NCBI

CYP6B7 associated resistance in *H. armigera* and phylogenetically related insect species (Fig. 5) may be counterproductive.

Higher activity of P450s in general and *CYP6B7* in specific were found to be induced by cantharidin treatment showing their possible involvement in cantharidin resistance and detoxification. In the light of our experiment there is a potential risk of P450s associated insecticidal resistance if it is not used judiciously for control of lepidopteran pests.

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