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CYP6B6 is involved in esfenvalerate detoxification in the polyphagous lepidopteran pest, *Helicoverpa armigera*

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ABSTRACT

The cotton bollworm, *Helicoverpa armigera*, is a polyphagous pest that has a strong capacity to evolve resistance against various classes of insecticides. Cytochrome P450 enzymes have been suspected involved in pyrethroid metabolism and resistance in this pest. However, how many and which P450s are involved in pyrethroid metabolism is largely unknown. In this study, CYP6B6 and NADPH-cytochrome P450 reductase (HaCPR) from *H. armigera* were successfully co-expressed in *Escherichia coli*. Incubation of esfenvalerate with the recombinant CYP6B6-HaCPR monooxygenase complex revealed that CYP6B6 was able to transform esfenvalerate into 4′-hydroxy fenvalerate. *Kcat* and *Km* values for the formation of 4′-hydroxyfenvalerate by the *E. coli*-produced CYP6B6 were determined to be 1.65 ± 0.11 min^{-1} and 4.10 ± 0.84 μM respectively. Our results demonstrate that CYP6B6 has the ability to hydroxylate esfenvalerate, thus plays a role in fenvalerate detoxification.

1. Introduction

The cotton bollworm is a polyphagous pest that has caused serious yield losses in many economically important crops [1]. Resistance of *Helicoverpa armigera* has been documented to almost all kinds of insecticides including pyrethroidss over the last 50 years [2,3]. Although the increased adoption of Bt cotton worldwide has reduced the use of chemical insecticides for *H. armigera* control, insecticide resistance remains or re-emerges to be problematic in China and some other countries [3–5]. For example, a recent extensive survey revealed that *H. armigera* populations collected from northern China showed 43- to 830-fold resistance to fenvalerate [3]. Therefore, there is an outstanding need to investigate the molecular mechanisms of resistance to conventional insecticides in *H. armigera*.

Cytochrome P450s (CYPs) are a superfamily of enzymes ubiquitous in nature. Insect cytochrome P450s have been long recognized as very important enzymes involved in xenobiotics metabolism and insecticide resistance [6]. Several studies indicate that multiple *CYP* genes may contribute to insecticide resistance in either population-specific or more general ways [4,5,7–9]. For example, constitutive overexpression of CYP9A12 and CYP9A14 was observed in a laboratory-selected pyrethroid resistance strain (YGF) of *H. armigera*, and both CYP9A12 and CYP9A14 exhibited clearance activity towards esfenvalerate [10]. Likewise, overexpression of CYP6B7 was observed in the fenvalerate-resistant strains of *H. armigera* derived from both China [11] and Australia [12], and a further study showed that silencing of CYP6B7 increased the susceptibility of *H. armigera* to fenvalerate [13]. More recently, the presence of CYP337B3, a cytochrome P450 capable of metabolizing fenvalerate into the nontoxic 4′-hydroxyfenvalerate, was reported to be responsible for 40–50 fold resistance to fenvalerate in an Australian population [14]. Similar mechanism is present in a Pakistani population of *H. armigera* [15]. However, a study on Chinese populations of *H. armigera* with ~250–1200 fold resistance to fenvalerate revealed that although *CYP337B3v2* was in high frequency in these populations, the difference in the P450 mediated resistance levels among different populations could not be explained by *CYP337B3* [16], suggesting that other P450(s) may contribute to resistance.

Thus far, implicating CYP in pyrethroids metabolism or resistance in the cotton bollworm is largely based on synergism of the insecticides by P450 inhibitors, higher levels of mRNA or/and activities towards model substrates in resistant strains [5,9]. Few studies on the capacity of individual P450s in insecticide metabolism in the cotton bollworm have been published [8,13–15], thus knowledge about how many and which P450(s) are responsible for insecticide metabolism and resistance in *H. armigera* is very limited. We attempted to address this knowledge gap.

CYP6B6 was identified in the cotton bollworm in 1998 [12]. It exists in cluster with CYP6B2 and CYP6B7 [17] and is expressed in all the developmental stages of the cotton bollworm [18]. CYP6B6 transcription is highly inducible by deltamethrin in both midgut and fat body of the larvae of *H. armigera* [19], and CYP6B6 silencing could reduce its

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tolerance to insecticides (bifenthrin, 3-cyfluthrin, chlorpyrifos) [20]. These observations strongly suggest that CYP6B6 may play roles in the metabolism of insecticides including pyrethroids. However, no substrate for CYP6B6 is biochemically identified until today.

In this study, we firstly established procedures for recombinant functional expression of both CYP6B6 and NADPH-cytochrome P450 reductase (HaCPR) from *H. armigera* in *Escherichia coli* (*E. coli*). Furthermore, we performed metabolism studies to confirm that esfenvalerate is a substrate of CYP6B6. Our results demonstrated that CYP6B6 could metabolize esfenvalerate via 4′-hydroxylation.

2. Materials and methods

2.1. Isolation of CYP6B6 cDNAs

Ten 2nd instar larvae of *H. armigera* from a laboratory colony derived from a field collection from Anyang, Henan Province of China in 2005 were used to isolate RNA. Total RNA was extracted using TRIzol according to the manufacture's protocol (Invitrogen, CA, USA). cDNA was synthesized by using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Gene-specific primers (Table 1) were designed to obtain the intact open reading frame (ORF) of CYP6B6 by using the PrimeSTAR® HS DNA Polymerase (TaKaRa) and cDNA as the template. The PCR product was purified on gel, cloned into pEASY-T1 vector, and transformed into *E. coli*. At least three clones were sequenced from both directions to confirm the nucleotide sequence of the PCR product (Invitrogen, Beijing). We identified three amino acid variations (M62L, A360V and I379T) in our CYP6B6 compared with a CYP6B6 allele (AY950636) cloned from the midgut of the cotton bollworm (Fig. S-1-1).

2.2. Construction of recombinant pCW-CYP6B6 and pACYC-HaCPR plasmids

To construct recombinant plasmid of CYP6B6 for functional expression, the first ten amino acid residues (MWILYFPAVI) in the N-terminus of CYP6B6 were replaced by the eight residues (MALLLAVF) of bovine 17-α hydroxylase (17-α strategy [21]). The N-terminal-modified CYP sequence was inserted into pCWori + vector (pB54) at the *Nde*I and *Kpn*I digestion sites in order to optimize mRNA translation. The primers used for plasmid construction were listed in Table 1. The resultant right recombinant plasmid (pCW-CYP6B6) was used for functional expression.

The pACYC-HaCPR plasmid was constructed through the following steps. A DNA product containing the *Ptactac* promoter, the pelB leader peptide, and the ORF of HaCPR plus two digestion sites (*Sal*I and *Bgl*II), was amplified by PCR using the plasmid pB508-HaCPR [22] as DNA template and the Ptac-HaCPR-F and Ter-HaCPR-R primers (Table 1). Then the PCR product was ligated into the vector pACYC-184 [23] after cutting with *Sal*I and *Bgl*II enzymes. The ligation was transformed in *E. coli* DH5α. The clone with the right sequence was screened.

Table 1
Primers used in this study.

Primer name	Sequence (5′-3′)	Purpose
CYP6B6-cF	ATGTGGATCTTGTATTTTCCGGC	Cloning the ORF of CYP6B6
CYP6B6-cR	TTAAGATACGAGCTTCCTAGGG	
17α-6B6-F	GGTCATATGGCTCTGTTATTAGCAG TTTTTTCAGTGCTAATCGTCACTCT	N-terminal modification of CYP6B6
17α-6B6-R	CTCGGTACCTTAAGATACGAGCTTCCTAGGG	
Ptac-HaCPR-F	AGCGTCGACATTCGATGGTGTCCTGGCA	Constructing the pACYC-HaCPR plasmid
Ter-HaCPR-R	GCGTACTATGGTTGCTTTGACG	

2.3. Functional co-expression of CYP6B6 and HaCPR in E. coli

Both pCW-CYP6B6 and pACYC-HaCPR were co-transformed into *E. coli* BL21(DE3) for dual plasmid coexpression of CYP6B6 and HaCPR. The starter culture was prepared by inoculating 5 mL of Luria Broth (LB) (containing 100 μg mL^{-1} of both ampicillin and chloromycetin) with a single colony carrying both recombinant pCW-CYP6B6 and pACYC-HaCPR plasmids and allowed growth overnight at 37 °C and 200 rpm. 1 mL of the overnight cultures was transferred into 100 mL TB media. When the OD_{600} of the culture reached 0.7–1.0, 1 mM IPTG and 0.5 mM ALA were added. After a further 40 h growth at 23 °C with 180 rpm shaking, cells were pelleted by spinning at 3000*g* for 25 min at 4 °C. The harvested cells were resuspended in 1 × TSE buffer (50 mM Tris-acetate (pH 7.6), 250 mM sucrose, and 0.25 mM EDTA) and 0.25 mg mL^{-1} lysozyme on ice for 1 h. The resultant spheroplast was re-suspended in ice cold buffer (100 mM potassium phosphate, pH 7.6, 20% (*v*/v) glycerol, 1 mM PMSF and 0.1 mM DTT), then sonicated (Scienta-IID, China) and centrifuged at 12,000*g* for 30 min at 4 °C. The 12,000*g* supernatant was further centrifuged at 180,000*g* for 1 h. The membrane fraction was prepared by re-suspending the 180,000*g* pellets in 1 × TSE buffer, and used for the further assays.

2.4. Determination of CYP6B6 content and CPR activity

Protein concentration was determined according to Bradford method [24]. The content of expressed CYP6B6 protein was estimated by the method established by Omura and Sato [25]. The recombinant CPR activity was measured at 30 °C by recording the change of absorbance at 550 nm according to the method of Pritchard et al. [26].

2.5. Esfenvalerate metabolism

Esfenvalerate (99.5%, Fluka) (the most toxic (2S,αS) isomer of racemic fenvalerate) was dissolved in dimethyl sulfoxide (DSMO) as a stock solution of 2 mM. The reaction mix containing 2 μL(4 nmol)of esfenvalerate, 30 pmol CYP6B6, 0.2 M Tris-HCl buffer (pH = 7.4), 0.25 mM $MgCl_2$ and the NADPH-generation system (1 mM NADPH, 1 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase per mL in a total volume of 200 μL). Controls were set up in parallel by omitting the NADPH. Piperonyl butoxide (PBO) was used in the inhibition assay at a final concentration of 50 μM. Reactions were conducted at 30 °C, 250 rpm for 1 h and stopped by the addition of acetonitrile at a final concentration of 84%, and votexing for 10 min. The reaction mix was centrifuged at 14,000*g* for 5 min at 25 °C after an additional 10 min of shaking, and the supernatants were filtrated through a 0.22 μm PTFE membrane. Aliquots of the filtrated supernatants were used for HPLC and mass spectrometric analyses.

Kinetic analysis was performed by measuring the product formation under linear conditions with time and amount of enzyme. The reactions were conducted at 30 °C, 250 rpm for 10 min, containing 15 pmol CYP6B6 in membrane and varying substrate concentrations between 0 and 40 μM. Three independent experiments were carried out with each in duplicate. Kinetic parameters were calculated from the plots of substrate concentrations against the initial velocities by fitting to the nonlinear Michaelis–Menten equation using GraphPad Prism 5 (San Diego, CA, USA).

2.6. High-pressure liquid chromatography (HPLC) analysis

HPLC analysis was performed on a Agilent HPLC 1260 Series equipped with a HC-C18 column (250 mm × 4.6 mm, 5 microcon, Agilent) by using solvents water (A) and acetonitrile (B) in a gradient program(0–5 min, 5% B; 5–45 min, 100% B; 45–50 min, 100% B; 50–55 min, 5% B; 55–60 min, 5% B). The substrate esfenvalerate and its metabolite(s) were detected at 230 nm. Column temperature and injection volume were set at 28 °C and 100 μL respectively.

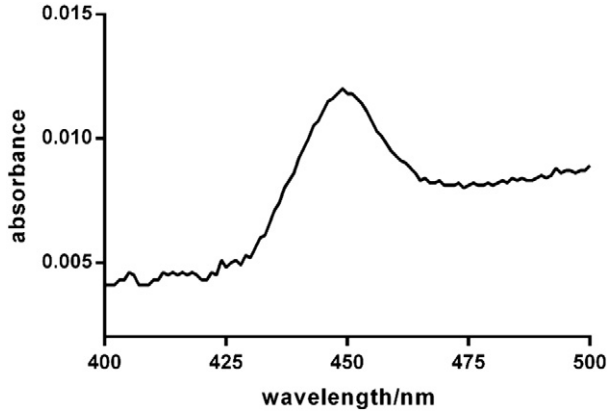

Fig. 1. Reduced CO-difference spectrum of *E. coli* membrane containing expressed *H. armigera* CYP6B6. A typical absorbance at 450 nm was observed.

2.7. Mass spectrometric analysis for fenvalerate metabolism

Mass spectrometric analysis was performed to confirm the occurrence of CTP6B6-mediated reaction and the identity of potential metabolite(s). Agilent 1200-6520 HPLC-Q-TOF/MS was employed for the structural analysis of the metabolite(s). Substances were separated by a 2.1x150mm SB-C18 column (5 microcon, Agilent, USA) at 30 °C with following conditions: 0–5 min, 20% B; 5–45 min, 95% B; 45–50 min, 95% B; 50–55 min, 20% B; 55–60 min, 20% B (A: 0.15% fromic acid and 0.15% ammonium formate in milli-Q water, B: HPLC grade acetonitrile). The instrument was run in the positive ionization mode at capillary voltage of 3500 V and drying gas temperature of 300 °C. For MS/MS analysis, the voltages of fragmentor and collision were set at 135 V and 25 V respectively.

3. Results

3.1. Heterologous co-expression of CYP6B6 and HaCPR

The strategies of 17α N-terminal modification of CYP6B6 and pelB addition to HaCPR were adopted to construct the recombinant plasmids pCW-CYP6B6 and pACYC-HaCPR respectively. Both plasmids were co-expressed in *E. coli*. Under the conditions described in this study, approximate 0.7 ± 0.14 nmol CYP6B6 per mg protein and 35.46 ± 3.71 μmol cytochrome c reduced per min per mg protein (~10 nmol per mg protein) were detected in the membrane fraction. The heterologously expressed CYP6B6 exhibited a CO-difference spectrum with a peak at ~450 nm (Fig. 1).

3.2. Esfenvalerate metabolism by H. armigera CYP6B6

To assess the ability of CYP6B6 in metabolizing esfenvalerate, *in vitro* metabolism assays were conducted using the *E. coli* produced CYP6B6 and HaCPR enzymes. The catalytic activity was inspected by measuring substrate depletion and appearance of potential metabolite(s). HPLC analysis revealed the appearance of an additional peak with a higher hydrophilicity in the reactions in the presence of NADPH (Fig. 2A). This metabolite was undetectable in the reactions without NADPH and in the PBO inhibition assay (Fig. 2A). No other metabolite was observed by HPLC analysis under conditions described in this work. Paralleled NADPH-dependent depletion of esfenvalerate (29.2 ± 3.1% in 1 h,

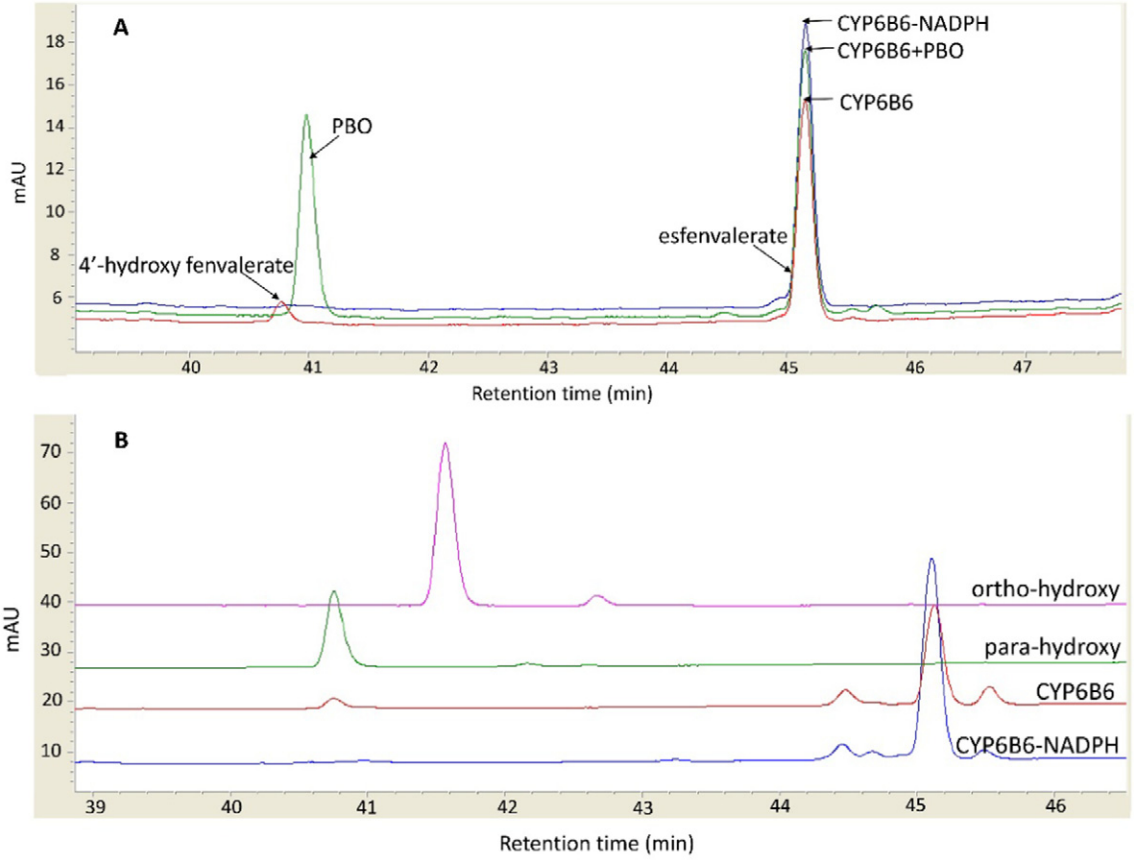

Fig. 2. HPLC chromatogram of metabolite(s) of esfenvalerate and the authentic substances detected at 230 nm. A: *In vitro* metabolism studies (with NADPH, without NADPH and with 10 nmol PBO) were performed by incubating 4 nmol esfenvalerate with 30 pmol CYP6B6 at 30 °C for 1 h; B: Co-chromatography of the esfenvalerate-CYP6B6 reactions with authentic references (*para*- and *ortho*-hydroxylfenvalerate).

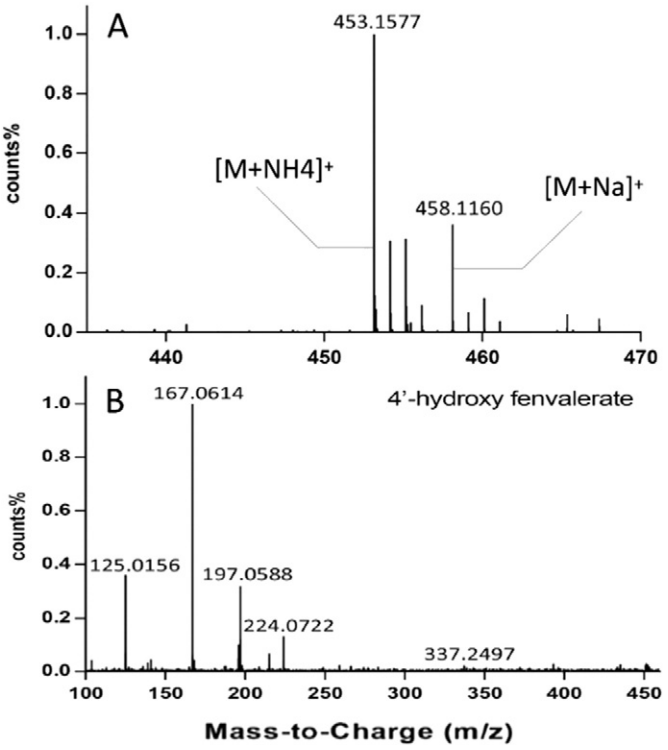

Fig. 3. HPLC-Q-TOF/MS analysis of the metabolite of esfenvalerate metabolism by CYP6B6 and the authentic 4′-hydroxylfenvalerate. A: The mass spectrum of the metabolite (M); B: The MS/MS of the authentic 4′-hydroxyfenvalerate.

with the retention time of 45.2 min) was observed (Fig. 2A). The retention time (40.7 min) of the metabolite (M) was correlated well with that of 4′-hydroxyfenvalerate by co-chromatography with authentic references (Fig. 2B).

The identity of the metabolite was confirmed by mass spectrometric (MS) analysis. The HPLC-Q-TOF/MS analysis detected a compound with ion mass of 453.1581 and 458.1172, indicating the presence of $[M + NH_4]^+$ ($\Delta m = 0.5$ mDa) and $[M + Na]^+$ ($\Delta m = 0.8$ mDa) (Fig. 3A). The major fragment ions at m/z 224, 197, 167 and 125 were found in the MS/MS spectrum of both the metabolite and the authentic 4′-hydroxyfenvalerate (Fig. 3B), indicating that the metabolite was 4′-hydroxyfenvalerate.

3.3. Enzyme kinetics

The enzyme kinetics study showed that the *Kcat* and *Km* values for the formation of 4′-OH fenvalerate by CYP6B6 were 1.65 ± 0.11 min^{-1} and 4.10 ± 0.84 μM respectively (Fig. 5).

4. Discussion

The widespread use of pyrethroids has resulted in many *H. armigera* populations with varying levels of resistance worldwide [3–5,14,15]. Several P450s have been suggested to be responsible for pyrethroid resistance in *H. armigera* [4,7–16,28], however, critical metabolism evidence has been reported in only a few studies [8,14,15]. This situation is largely caused by the diversity of CYP genes in the pest and the technical difficulty in identifying the biochemical function of a specific P450 [6,27]. In the current study, we successfully co-produced both CYP6B6 and its redox partner HaCPR simultaneously in *E. coli* (Fig. 1). The stable expression of functional monooxygenase complex (CYP6B6-HaCPR) in sufficient quantity allows us to perform substrate screening.

Our results of metabolism studies have definitively demonstrated that esfenvalerate is a substrate of CYP6B6. Incubation of esfenvalerate with the CYP6B6-HaCPR complex generated a metabolite along with depletion of the substrate in the presence of NADPH (Fig. 2). Subsequent HPLC-Q-TOF/MS analysis identified that the metabolite was 4′-hydroxylfenvalerate (Figs. 3 & 4). Previous studies have shown that 4′-C of pyrethroids is the most preferred site of hydroxylation by most insect P450s [29]. The metabolic fate of esfenvalerate by CYP6B6 revealed in this work (hydroxylation in the phenoxybenzyl 4′-position) is in keeping with pyrethroids with the phenoxybenzyl alcohol moiety in *H. armigera* [14,15] and in other insects [30–34]. To our knowledge, only four P450s (CYP9A12, CYP9A14, CYP9A17 and CYP337B3) are known capable of metabolizing pyrethroids in *H. armigera*, we add CYP6B6 in the list. Moreover, CYP6B6 represents the second pyrethroid-metabolizing P450 identified with known metabolite (after CYP337B3 [14–15]) in *H. armigera*.

The hydroxylation of esfenvalerate by recombinant CYP6B6 follows the Michaelis–Menten kinetics, with calculated *Kcat* and *Km* values being 1.65 ± 0.11 min^{-1} and 4.10 ± 0.84 μM respectively (Fig. 5). The catalytic efficiency (*Kcat*/*Km* = 0.4 min^{-1} μM^{-1}) of *H. armigera* CYP6B6 in esfenvalerate metabolism is comparable to those established for several other insect CYPs (e.g. CYP6M2, CYP6P4 and CYP6Z1) involved in pyrethroid hydroxylation [31,33–35]. To our knowledge, there is no other published kinetic data of pyrethroid metabolism for

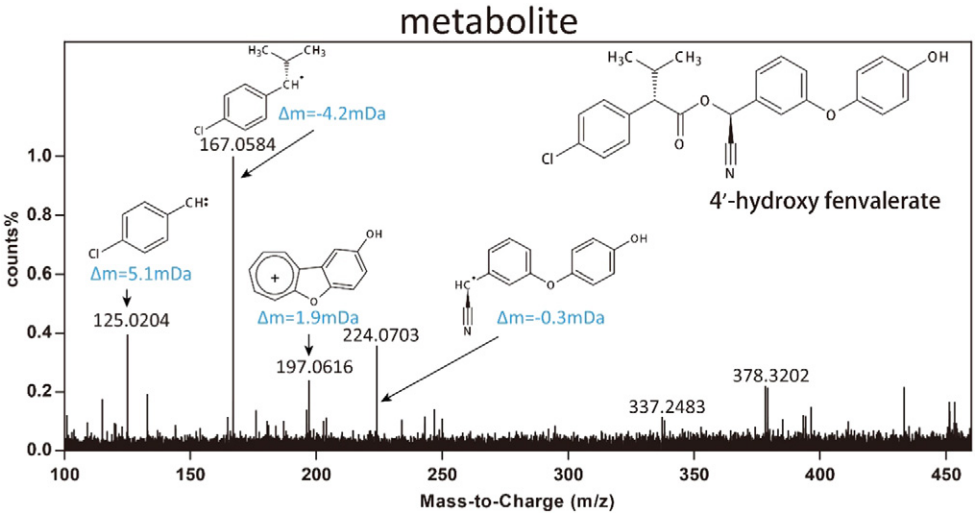

Fig. 4. Structures of the major fragment ions detected on the MS/MS of the metabolite deduced from their formula calculated accurate mass value.

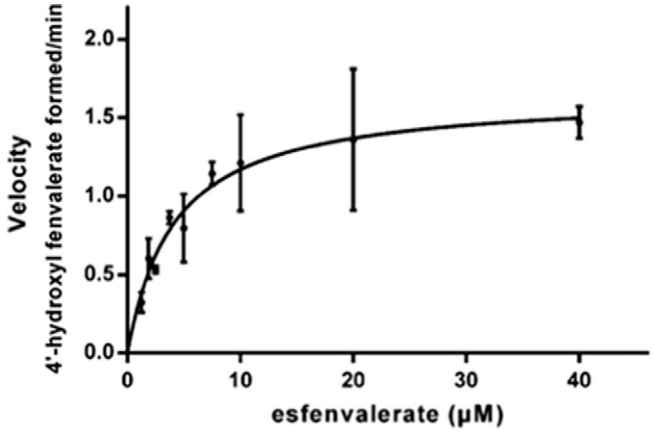

Fig. 5. Kinetics analysis of *E. coli* produced *H. armigera* CYP6B6 for esfenvalerate 4′-hydroxylation. The substrate concentrations used in this assay were 0, 1.25, 1.875, 2.5, 3.75, 5, 7.5, 10, 20, 40 μM. Data are mean ± SD (n = 3). The *Kcat* (1.65 ± 0.11 min^{-1}) and *Km* (4.10 ± 0.84 μM) were calculated from the plot of substrate concentrations against the initial velocities by fitting to the nonlinear Michaelis–Menten equation ($R^2 = 0.7984$) using GraphPad Prism.

individual CYPs from *H. armigera*, a comparison of catalytic efficiency among CYPs in the cotton bollworm is not currently possible. Compared with the formation of hydroxyl fenvalerate catalyzed by CYP6B6, the CYP6B8 from the generalist *H. zea* (coexpressed with the house fly P450 reductase in Sf9 Cells) has a greater *Vmax* for of 12.7 min^{-1} and a much greater *Km* of 85 μM for α-cypermethrin clearance, resulting in a lower metabolic efficiency (*Vmax*/*Km* = 0.15 min^{-1} μM^{-1}) [36].

A previous bioassay has demonstrated that hydroxylation of fenvalerate in 4′-position is a detoxification mechanism *in vivo* in the cotton bollworm, suggesting that 4′-hydroxyfenvalerate is intrinsically nontoxic, or/and it can be metabolized to a nontoxic compound or excreted more easily than fenvalerate [14]. The finding that CYP6B6 is able to transform esfenvalerate into 4′-hydroxyfenvalerate indicates that CYP6B6 plays a role in esfenvalerate detoxification in the cotton bollworm. Therefore, it is logical to expect that inhibiting the activity or reducing the level of CYP6B6 can enhance the insecticidal efficacy of esfenvalerate against the cotton bollworm.

5. Conclusion

In this work, we realized the co-production of both CYP6B6 and HaCPR from *H. armigera* in *E. coli*. The availability of recombinant CYP6B6-HaCPR complex makes it possible to conduct substrate screening and further studies of the biochemistry of CYP6B6. Our metabolism assays have demonstrated that CYP6B6 has the ability to hydroxylate esfenvalerate with a catalytic efficiency of 0.4 min^{-1} μM^{-1}. Based on the findings obtained in this investigation, together with the results of earlier studies, it is strongly indicated that CYP6B6 plays a role in the detoxification of esfenvalerate. We suggest that CYP6B6-inhibition/interference based strategies could be used to the control of the cotton bollworm.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.pestbp.2017.02.006.

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