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ARTICLE

HPLC Assay for Characterizing α-Cyano-3-phenoxybenzyl Pyrethroids Hydrolytic Metabolism by *Helicoverpa armigera* (Hübner) Based on the Quantitative Analysis of 3-Phenoxybenzoic Acid

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A new HPLC assay for characterizing the hydrolytic metabolism of α-cyano-3-phenoxybenzyl pyrethroids in *Helicoverpa armigera* (Hübner) through quantitative analysis of the common metabolite 3-phenoxybenzoic acid (3-PBA), converted from 3-phenoxybenzaldehyde (3-PBAld) by aldehyde-oxidizing enzymes, has been established. The method was validated by measuring β-cypermethrin metabolism in *H. armigera*. The hydrolytic activity based on 3-PBA toward β-cypermethrin was consistent with that based on (*trans*+*cis*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA). The analysis system is based on using liquid−liquid extraction and HPLC with a C18 column, gradient elution, and UV detection at 230 nm. The analytical methodology was validated by its repeatability, linearity, accuracy, and limits of detection. The developed and validated HPLC assay method was applied to examine the inhibition effect of *S,S,S*-tributyl phosophorotrithioate (DEF) and piperonyl butoxide (PBO) on β-cypermethrin hydrolytic metabolism, and to evaluate the inducibility of six α-cyano-3-phenoxybenzyl pyrethroids hydrolytic metabolism by 2-tridecanone, a generalized "inducer" of detoxifying enzyme activities.

KEYWORDS: HPLC; pyrethroid; hydrolytic metabolism; *Helicoverpa armigera* (Hübner); 3-phenoxybenzoic acid; 2-tridecanone

INTRODUCTION

Synthetic pyrethroid insecticides are used extensively in agriculture, household and public health to control pests (*1*, *2*). Pyrethroids are a class of important insecticides because of their selective toxicity against pests rather than mammals, and their good spectrum of activity against crop damaging pests (*2*−*4*). Traditionally, pyrethroids can be grouped into two subclasses (Type I and II) based on their chemical structure and toxicological actions (*5*). Type I pyrethroids (e.g., permethrin) are esters of primary alcohols, while type II pyrethroids are esters of secondary alcohols that contain a cyano group at the α-carbon of the alcohol moiety. α-Cyano-3-phenoxybenzyl pyrethroids, such as cypermethrin, β-cypermethrin, α-cypermethrin, deltamethrin, λ-cyhalothrin, fenvalerate, and fenpropathrin, are the dominant pyrethroids against agricultural pests in China.

Ester hydrolysis by carboxylesterases (CarEs) and oxidation by cytochrome P450s are the main detoxification of pyrethroids in both animals (*3*, *6*, *7*) and insects (*6*, *8*−*10*), and the hydrolysis by CarEs is more important for most pyrethroids (*11*). Enhanced CarEs activity in insects is thought to contribute to the insects' resistance to ester-containing insecticides (*12*−*15*). Therefore, it is very important to identify the role of CarEs involved in insect resistance to ester-containing insecticides by characterizing CarEs activities.

To characterize CarEs hydrolytic activity, many standard (model) substrates for a general CarEs assay have been developed, such as fluorescent substrates (*16*−*18*) based on the fluorescent estimation of products on the hydrolysis of 4-methylumbelliferylesters, and colorimetric substrates based on the colorimetric estimation of α/β-naphthol (*15*, *16*, *19*, *20*) or *p*-nitrophenol (*4*) that are produced by hydrolysis of naphthylesters or *p*-nitrophenylesters, respectively. In addition to general esterase substrates, pyrethroid-like compounds have been synthesized and used for determining pyrethroid-specific CarEs activity, such as pyrethroid colorimetric (*21*−*23*) and fluorescent surrogates (*17*, *24*, *25*). However, in insecticide-resistant insects, the increases in CarEs metabolism toward model substrates and pyrethroid-like surrogates are indirect, and an increase in CarEs metabolism toward the insecticide itself is more conclusive.

In direct characterization of CarEs hydrolytic metabolism toward pyrethroid insecticides, hydrolysis rates were based on the production of pyrethroid acid moieties (*4*) or pyrethroid alcohol moieties such as 3-phenoxybenzylalcohol (*4*, *17*, *26*, *27*) for Type I pyrethroids, and 3-phenoxybenzaldehyde (3-PBAld) (*4*, *17*, *26*, *28*) for type II pyrethroids by HPLC(/MS) or GC(/MS)

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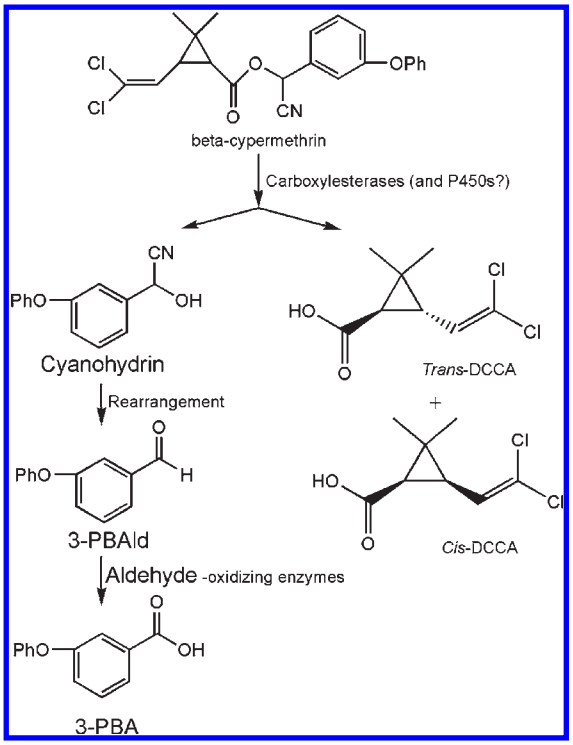

Figure 1. The major metabolic pathway for α-cyano-3-phenoxybenzyl pyrethroids in *H. armigera*: β-cypermethrin as an example.

in animal CarEs assay, or by thin-layer chromatography (TLC) using radiometric methods (*29*) in insects CarEs assay, while almost no HPLC or GC assays for CarEs in insects have been reported.

It was reported that 3-phenoxybenzoic acid (3-PBA) was the predominant metabolite of cypermethrin produced by crude homogenates of *H. virescens* (*30*). In our research, 3-PBA was also found as one of the major metabolites of type II pyrethroids instead of 3-PBAld. Therefore, 3-PBA turned out as the common metabolite of α-cyano-3-phenoxybenzyl pyrethroids in the midgut of *H. armigera*. The major enzymatic pathway of type II pyrethroids metabolism in *H. armigera* was proposed in **Figure 1**.

2-Tridecanone, a naturally occurring toxin in tomato leaves, can cause both qualitative and quantitative changes in P450 spectral properties in *H. virescens* larvae (*31*), induce specific P450 genes in the gut of *M. sexta* larvae (*32*, *33*), elevate P450 activities in *H. virescens* (*34*) and *H. armigera* larvae (*35*), enhance glutathione *S*-transferase (GSTs) activities (*36*) as well as CarEs activities (*37*) in *H. armigera* larvae, and also result in increased tolerance to diazinon and increased in vitro degradation of diazinon in *H. virescens* larvae (*38*). However, there is relatively little knowledge of 2-tridecanone induction of pyrethroids hydrolytic metabolism.

In the present study, in vitro metabolism of type II pyrethroids to 3-PBA, and the oxidation of 3-PBALD to 3-PBA in *H. armigera* were examined. A new HPLC assay for determining pyrethroids metabolic activities in *H. armigera* midgut by quantitative analysis of 3-PBA was developed and validated. The established HPLC assay was successfully applied to examine the inhibition effect of DEF and PBO on β-cypermethrin hydrolytic metabolism, and to evaluate the induction effect of 2-tridecanone on hydrolytic metabolism of type II pyrethroids including β-cypermethrin, α-cypermethrin, deltamethrin, λ-cyhalothrin, fenpropathrin, and fenvalerate in midgut of the sixth-instar larvae.

MATERIALS AND METHODS

Reagents and Materials. Pyrethroids, β-cypermethrin ((*S*)-α-cyano-3-phenoxy-benzyl (1*R*)-*cis*-,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate and (*R*)-α-cyano-3-phenoxybenzyl (1*S*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethy-lcyclopropanecarboxylate, 99.0% purity), deltamethrin ((*S*)-α-cyano-3-phenoxybenzyl (*R*)-cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate, 99.0% purity), λ-cyhalothrin ((*S*)-α-cyano-3-phenoxybenzyl (*Z*)-(1*R*)-*cis*-3-(2-chloro-3,3,3-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylate and (*R*)-α-cyano-3-phenox-ybenzyl(Z)-(1*S*)-*cis*-3-(2-chloro-3,3,3-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylate, 99.2% purity), fenpropathrin ((*RS*)-α-cyano-3-phenoxybenzyl 2,2,3,3-tetramethylcyclopropanecarboxylate, 99.2% purity), fenvalerate ((*RS*)-α-cyano-3-phenoxybenzyl (*RS*)-2-(4-chlorophenyl)-3-methylbutyrate, 98.0% purity), were obtained from Institute for the Control of Agrochemicals, Ministry of Agriculture, Beijing City, China (ICAMA). α-Cypermethrin (*cis*, β-cypermethrin) ((*S*)-α-cyano-3-phenoxybenzyl (1*R*)-*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate and (*R*)- α-cyano-3-phenoxybenzyl (1*S*)-*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (94.4% purity) was obtained from Suzhou Fumeishi Chemical Co., Ltd. *cis*-,*trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (*cis*-,*trans*-DCCA, 99.0% purity) was provided by Jiangsu Yangnong Chemical Group Co., Ltd. The chemical structures of pyrethroids examined and their common metabolite 3-PBA, and β-cypermethrin metabolites (*cis*- and *trans*-DCCA) are shown in **Figure 2**. Phenylmethylsulfonyl fluoride (PMSF, 99.0% purity), 1-phenyl-2-thiourea (PTU, 98.0% purity), NADPH Na_4 (98.0% purity), and 3-phenoxybenzaldehyde (98.0% purity) were purchased from Sigma-Aldrich Chemical (St. Louis, MO). HPLC grade acetonitrile, methanol, and 2-propanol were purchased from Fisher (Fisherchemicals, USA). Ultrapure water was prepared by using a Milli-Q academic water purification system (Milford, MA, USA). Piperonyl butoxide (PBO, 98% purity) and *S*,*S*,*S*-tributyl phosophorotrithioate (DEF, 98.0% purity) were purchased from Chem Service (West Chester, PA). Tris base and DL-dithiothreitol (DTT) were obtained from Promega Corporation (Madison, WI). Ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin (BSA) were purchased from Beijing Tongzheng Biological Company. 2-Tridecanone (96.0% purity) and 3-phenoxybenzoic acid (99.0% purity) were purchased from Alfa Aesar (Ward Hill, MA, USA). Other chemical reagents of analytical grade were purchased from Beijing Chemical Reagents Company.

Insects. Cotton bollworm, *H. armigera* (Hübner), population was collected from Handan of Hebei Province, China, in 1998 and reared on an artificial diet in a conditioned room maintained at 26 ± 1 °C, 70−80% relative humidity, with a 16:8 (L:D) photoperiod. Adults were held under the same conditions and supplied with a 10% sugar solution. For exploring 2-tridecanone induction of type II pyrethroids hydrolytic metabolism, larvae were fed the regular artificial diet without 2-tridecanone (control larvae) or were shifted to an artificial diet into which 0.2% 2-tridecanone (m/m) was incorporated and fed to sixth instar larvae within 2 h after ecdysis, exposed to 0.2% 2-tridecanone (induced larvae) for 48 h.

Enzyme Preparation. Crude homogenates of *H. armigera* midgut were prepared as described previously (*35*, *39*) with some modification. Briefly, the midgut of two-day-old final instar larvae was obtained by dissection on ice. The midgut was gently shaken to free of its contents and rinsed in an ice-cold 1.15% (m/v) potassium chloride aqueous solution. Sixty midguts were homogenized, on ice, in 5 mL of homogenization buffer (0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM PTU, and 15% (v/v) glycerol), and the homogenizer was rinsed with a further 10 mL of homogenization buffer. Both rinses were pooled (15 mL). The homogenate was centrifuged at 4 °C, 10000*g* for 20 min. The supernatant was filtered through glass wool and collected into a clean ice-cold eppendorf tube, and used immediately for in vitro pyrethroid metabolism assay.

Determination of protein concentration was carried out by an improved method (*40*) described previously using bovine serum albumin as the standard.

In Vitro Pyrethroids Metabolism and Inhibition by DEF and PBO. The in vitro metabolism reactions of type II pyrethroids catalyzed by the crude homogenates of *H. armigera* midguts were performed in a total volume of 2 mL at 30 °C for 30 min in a water bath with occasional shaking. The incubation mixture consisted of 0.1 M pH 8.0 Tris-HCl buffer, 100 μM type II pyrethroid substrates, 7.5 mM $MgCl_2$, 0.75 mg/mL BSA, 1 mM NADPH or 1 mM NAD^+ and 10 μL of 2-propanol (solvent for DEF and PBO). The reaction mixtures were preincubated for 5 min, and reactions were initiated by adding 0.5 mL of enzyme sample (about

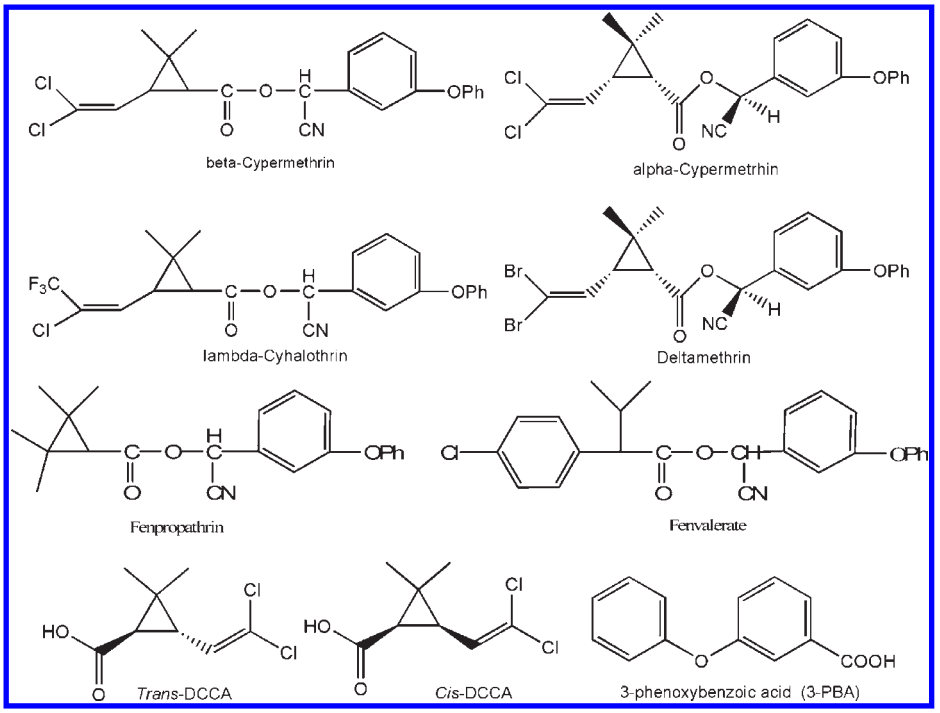

Figure 2. Chemical structures of α-cyano-3-phenoxybenzyl pyrethroids, common metabolite 3-PBA, and β-cypermethrin metabolites *cis*- and *trans*-DCCA.

2 mg of proteins). After a 30 min incubation, metabolic reactions were terminated by extracting with 2.5 mL of ice-cold ethyl acetate/*n*-hexane (2:1, v/v) containing 0.3% phosphoric acid. Another 1.5 and 1 mL of ice-cold ethyl acetate/*n*-hexane (2:1) was added to extract the remaining metabolites. The organic fraction of three extracts were combined together and evaporated to dryness under a gentle nitrogen stream. The residue was redissolved in 200 μL of acetonitrile and a 20-μL filtered solution was injected for HPLC analysis. Metabolic reactions at each substrate were performed in triplicate. Additionally, control incubations (without enzyme samples) and blank incubations (without substrates) were prepared in order to differentiate between metabolites originating from the enzyme samples and possible metabolites from the incubation procedure (*41*). In order to eliminate the influence of organic solvents and pH values on the metabolic enzyme activity, 500 μL 0.1 M pH 7.4 phosphate buffer were added instead of enzyme samples in the control incubations, and all the other components added were replaced by corresponding solvents as control.

For the inhibition study, serial concentrations of DEF (10 μL) and PBO (10 μL) were added to the reaction mixtures.

For examining the influence of pH values on metabolic product and enzymatic activity, 0.1 M pH 6.8 phosphate buffer was added instead of 0.1 M pH 8.0 Tris-HCl buffer.

In Vitro 3-PBAld Metabolism to 3-PBA. In order to evaluate the catalytic conversion of 3-PBAld to 3-PBA by aldehyde-oxidizing enzymes in *H. armigera*, 100 μM 3-PBAld was added to the incubation mixture as substrate.

Identification of the Metabolites of Beta-cypermethrin. UPLC−ESI−MS/MS determination was performed using Waters Acquity UPLC system (Waters, Milford, MA, USA) coupled to a Micromass Quattro Premier triple quadrupole mass spectrometer (Waters) using an electrospray source. UPLC separation was achieved using a Waters Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm) with a mobile phase flow rate of 0.3 mL/min. The mobile phase consisted of mixtures of acetonitrile/0.1% HCOOH in water (45/55, v/v). The eluents were monitored by UV detection of wavelength of 214 nm at 25 °C. Determination was performed using a Waters Micromass Quattro Premier tandem quadrupole mass spectrometer (Waters, Manchester, UK). Drying gas as well as nebulizing gas was the nitrogen generator. For operation in the MS/MS mode, collision gas was argon 99.995% with a pressure of 3.5×10^{-3} mbar in the collision cell. Capillary voltage of 3.0 kV was used in negative ionization mode. The interface temperature was set to 400 °C and the source temperature was set to 110 °C, with the cone voltage set at 20 V. Collision energy of 20 eV and dwell times of 0.2 s/scan were chosen. Instrument control and data processing were done using Masslynx 4.1 software. The characteristics of the metabolites were compared with those of authentic samples.

HPLC Analysis System. HPLC-DAD analysis of pyrethroids and pyrethroid metabolites was performed on an Agilent 1100 HPLC system (Agilent Company, USA) combined with a quaternary pump, online degasser, diode array detector (DAD), 7725i injection valve equipped with a 20-μL loop, and column thermostat, using a ZORBAX SB-C18 column (250 mm × 4.6 mm i.d., 5 μm, Agilent, USA). The mobile phases used were solvent A (acetonitrile), B (methanol), and C (H_2O, adjusted to pH 2.1 with 85% phosphoric acid). The analytes were eluted with the following gradient program (linear increase): 0 min (0% A, 5% B, 95% C), 15 min (37% A, 5% B, 58% C), 25 min (60% A, 5% B, 35% C), 50 min (85% A, 5% B, 10% C), 51 min (95% A, 5% B, 0% C), 56 min (95% A, 5% B, 0% C), 61 min (0% A, 5% B, 95% C) and 69 min (0% A, 5% B, 95% C), at a flow rate of 0.8 mL/min. Products were detected at 230 nm. Data collection and analysis were conducted using ChemStation software (Agilent Technologies, Wilmington, DE). The mixtures of three standards, *trans*-DCCA (0.2666 μg), *cis*-DCCA (0.2812 μg), and 3-PBA (0.2944 μg), were injected to the HPLC analysis system six times to determine the precision of the developed HPLC method. Under these chromatographic conditions, *trans*-DCCA, 3-PBA, and *cis*-DCCA eluted at approximately 26.7, 27.1, and 27.9 min, respectively.

Calibration Procedures. The mixed standards of *trans*-, *cis*-DCCA, 3-PBA, and β-cypermethrin (*trans*/*cis* isomer ratio, 0.9328 by NHPLC and 0.9367 by RHPLC in this study) were prepared in acetonitrile. Calibration standards for 3-PBA, *trans*- and *cis*-DCCA were run using an external standards and peak areas were plotted against the quantities (ng) of the analytes injected. For β-cypermethrin, quantification of hydrolysis was based on the production of the total acid products *trans*-DCCA plus *cis*-DCCA, and the alcohol product 3-PBA as well. For the other pyrethroids examined, the hydrolysis rates were based on production of 3-PBA. The standard curves were used to determine recovery of 3-PBA, *trans*- and *cis*-DCCA.

Recovery Studies. The mixtures of *trans*-DCCA, *cis*-DCCA, and 3-PBA over three concentration levels were added to the incubation mixtures instead of pyrethroid substrates, and 0.5 mL of enzyme sample was added immediately after the metabolic reaction was terminated.

Limits of Detection. Limits of detection were determined at the lowest concentration to be detected, taking into consideration a 1:3 baseline noise/signal ratio.

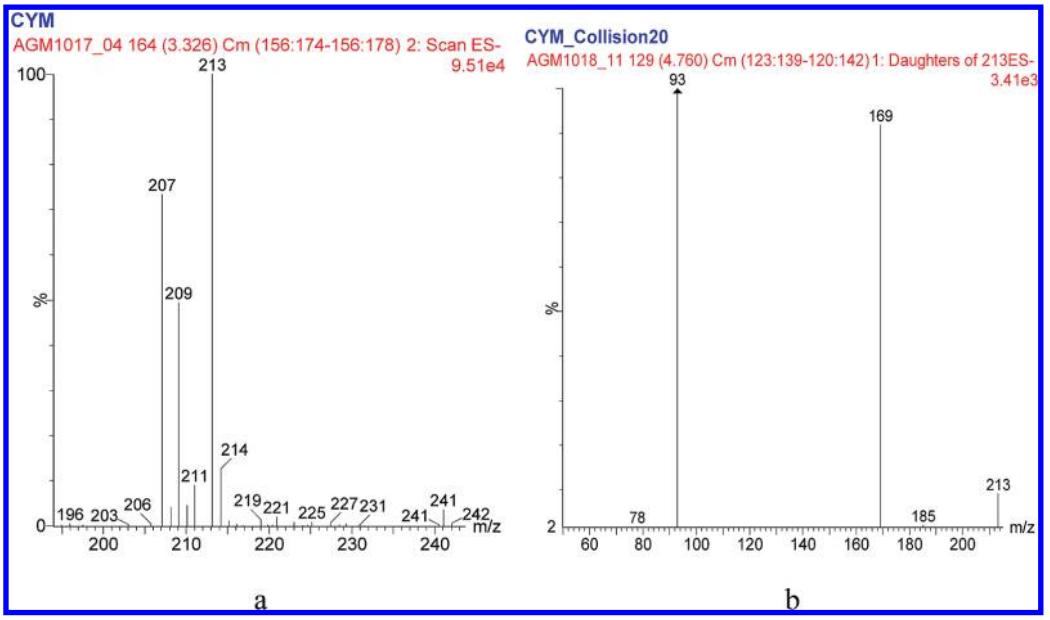

Figure 3. Negative ion electrospray mass spectra of metabolite. (**a**) Full scan. (**b**) Product ion spectra of *m/z* 213 at 20 eV.

Application to Evaluate the Induction Effect of 2-Tridecanone on type II Pyrethroids Hydrolytic Metabolism. The HPLC method based on the quantitative analysis of a common metabolite (3-PBA) was applied to investigate whether or not hydrolytic metabolism of six type II pyrethroids can be induced as influenced by 2-tridecanone induction for 48 h in the midgut of the sixth-instar *H. armigera* larvae.

Data Analyses and Statistics. The enzymatic activity data are presented as means (±S.E.) of three replicates expressed as pmol of 3-PBA or (*trans*+*cis*)-DCCA/mg protein/min. Statistical analysis was performed by using paired *t* tests, or using analysis of variance (ANOVA) with the Duncan test when comparing more than two means, using SPSS 11.5 software. A value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Metabolites Identification. *β*-Cypermethrin metabolites were unambiguously identified based on their abundant $(M - H)^-$ ions, UV spectra, retention time, and tandem mass spectrometric analysis compared with authentic standards. Products with characteristic isotopic ion spectra of *m/z* 207, 209, 211 and corresponding isotopic ratio of 9:6:1 (**Figure 3a**), showing the presence of double chlorine atoms, and a maximal UV absorption wavelength of 214 nm, were identified as DCCA. Products with characteristic ion spectra of *m/z* 213 (**Figure 3a**), fragment ion spectra of *m/z* 93, 169, 185 (**Figure 3b**), and maximal UV absorption wavelength of 292 nm, were identified as 3-PBA. The collision-induced dissociation reactions for 3-PBA were proposed (**Figure 4**). Under the chromatographic conditions of UPLC−ESI−MS/MS determination, metabolites DCCA and 3-PBA were simultaneously eluted. After in vitro *β*-cypermethrin metabolism in the *H. armigera* midgut, there were no other detectable products in this assay besides 3-PBA, *trans*-DCCA, and *cis*-DCCA .

A common metabolite of other type II pyrethroids except *β*-cypermethrin was identified as 3-PBA based on UV spectra, retention time compared with an authentic standard under the chromatographic conditions described in the HPLC analysis system.

HPLC Analysis System for Mixed Standards of Metabolites and Beta-cypermethrin. On the basis of the three metabolites identified, we develop a HPLC analysis system suitable for the separation of *trans*-, *cis*-DCCA, 3-PBA, *trans*-, and *cis*-*β*-cypermethrin.

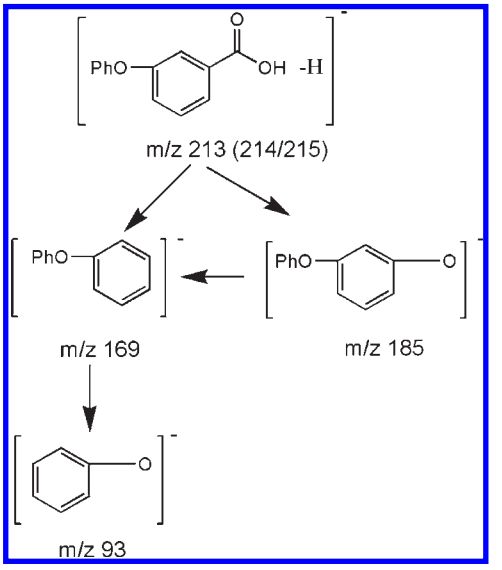

Figure 4. Proposed collision-induced dissociation reactions for 3-phenoxybenzoic acid.

Through the HPLC analysis of the mixed standard solution of *trans*-, *cis*-DCCA, 3-PBA and *β*-cypermethrin, satisfactory separation was obtained with perfect resolutions between *trans*-DCCA and 3-PBA (1.64), and between *trans*-*β*-cypermethrin and *cis*-*β*-cypermethrin (1.93) (**Figure 5**).

To assess the precision of the method, we injected the same standard solution six times. The RSD of the peak area response and retention time showed satisfactory reproducibility of the system (< 1%) (**Table 1**) for all three compounds.

Calibration curves were developed in the range 7.2−724.4 ng for *trans*-DCCA, 1.5−219.2 ng for *cis*-DCCA, and 16.0−799.9 ng for 3-PBA. The regression equations of the curves and their coefficients of determination (R^2) were calculated as follows: *trans*-DCCA, *y* (area) = 2.4799*X* (ng) − 2.0342, 0.99997; *cis*-DCCA, *y* (area) = 2.5183*X* (ng) + 0.1706, 0.9999; 3-PBA, *y* (area) = 6.0260*X* (ng) − 9.1190, 0.99996. The method showed a linear relationship between peak areas and concentrations over this range for all three compounds. A signal three times higher

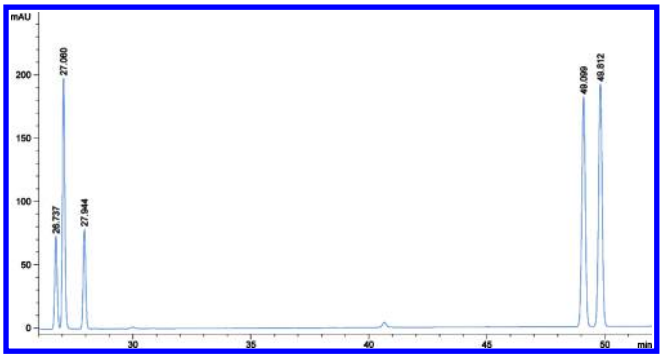

Figure 5. HPLC-DAD chromatograms of mixed standard solution of metabolites and parent β-cypermethrin. The compounds (containing 239.976 ng of 3-PBA, 217.308 ng of *trans*-DCCA, 229.248 ng of *cis*-DCCA; 586.077 ng of *trans*, β-cypermethrin and 628.299 ng of *cis*, β-cypermethrin) were detected at 230 nm. Peak at retention time (t_R) of 26.737 min = *trans*-DCCA; peak at t_R of 27.060 min = 3-PBA; peak at t_R of 27.944 min = *cis*-DCCA, peak at t_R of 49.099 min = *trans*, β-cypermethrin, peak at t_R of 49.812 min = *cis*, β-cypermethrin.

Table 1. Precision for Determination of 3-PBA, *trans*- and *cis*-DCCA

standard	injected (μg)	peak area	RSD (n = 6) (%)
trans-DCCA	0.2666	661.9	0.88
cis-DCCA	0.2812	714.7	0.88
3-PBA	0.2944	1774.9	0.93

Table 2. Recovery of *trans*-DCCA, *cis*-DCCA, and 3-PBA

		recovery (n = 3)	
standard	amount added (μg)	mean (%)	RSD (%)
trans-DCCA	2.1731	92.11	4.73
	1.0866	94.58	4.24
	0.2173	94.12	3.17
cis-DCCA	2.2925	89.03	5.12
	1.1463	91.81	4.97
	0.2293	93.01	3.49
3-PBA	2.3998	91.86	2.57
	1.1999	94.83	4.32
	0.2400	91.10	3.46

than the noise was regarded as the detection limit. Accordingly, the detection limits of the three constituents were all 0.15 ng.

The accuracy of the HPLC method was evaluated by the recovery studies at three concentration levels. The recoveries of *trans*-DCCA, *cis*-DCCA, and 3-PBA added to samples ranged from 89.03% to 94.83%. The relative standard deviations (RSD) of recoveries of three constituents ranged between 2.57 and 5.12% (**Table 2**) and are considered satisfactory for trace analyses.

Analysis of Metabolites and Unmetabolized Parent Pyrethroids by HPLC. The established HPLC analysis system developed on the basis of β-cypermethrin metabolic assay was suited for assay of all the metabolic reactions of the examined pyrethroids, and was successfully used to characterize type II pyrethroids hydrolytic metabolism. **Figure 6** shows a typical metabolite profile of in vitro metabolism of six type II pyrethroids by *H. armigera* midgut (**a**) and unmetabolized parent pyrethroid compounds (**b**). All the metabolites and unmetabolized parent pyrethroids eluted during 26−30 min and 47−52 min, respectively. In this study, a 69-min-consumed gradient program was used, for obtaining a perfect separation for *trans*- and *cis*-isomers of β-cypermethrin. However, if only the monitoring of metabolites is necessary, in some

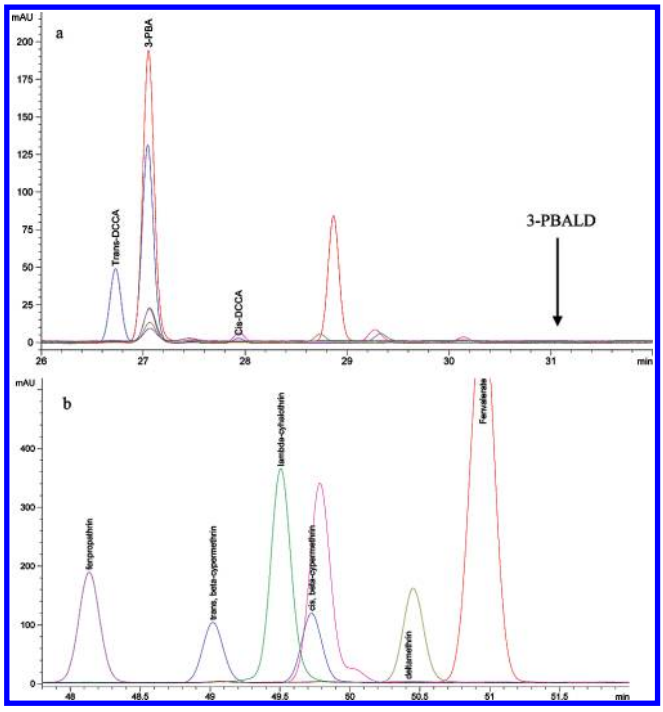

Figure 6. HPLC-DAD chromatograms of metabolites of 100 μM pyrethroids by *H. armigera* larvae induced by 0.2% 2-tridecanone (**a**) and unmetabolized parent pyrethroid compounds (**b**) on a ZORBAX SB-C18 (4.6 mm × 250 mm, 5 μm, made by Agilent in USA) analytical column. The unmetabolized parent pyrethroid in (**b**) is expressed with the same color line with its corresponding metabolites in (**a**). The compounds were detected at 230 nm. Peak at t_R of 26.729 min = *trans*-DCCA; peak at t_R of 27.053 min = 3-PBA; peak at t_R of 27.936 min = *cis*-DCCA, peak at t_R of 48.135 min = fenpropathrin, peak at t_R of 49.020 min = *trans*, β-cypermethrin, peak at t_R of 49.056 min = λ-cyhalothrin, peak at t_R of 49.786 min = α-cypermethrin (*cis*, β-cypermethrin), peak at t_R of 50.417 min = deltamethrin, peak at t_R of 50.952 min = fenvalerate.

cases about 20 min can be saved through rapidly increasing the elution concentration of organic solvent acetonitrile after 31-min of gradient elution.

Comparison of Hydrolytic Activity toward Beta-cypermethrin Based on the Formation of 3-PBA with that on (*trans*+*cis*)-DCCA. In order to examine the accuracy of this developed HPLC assay for characterizing pyrethroids hydrolytic metabolism, hydrolytic activity of *H. armigera* toward β-cypermethrin based on the formation of 3-PBA was compared with that of on (*trans*+*cis*)-DCCA. **Figure 7a** shows no significant difference between 3-PBA-presented and (*trans*+*cis*)-DCCA-presented hydrolytic activity under pH 6.8−8.0 reaction buffer in control larvae, and no significant difference under pH 8.0 reaction buffer in induced larvae as well. Hydrolytic product of β-cypermethrin under 0.1 M pH 8.0 Tris-HCl reaction buffer in control larvae and induced larvae, and under 0.1 M pH 6.8 phosphate reaction buffer in control larvae were all the same, suggesting that β-cypermethrin undergoes the same enzymatic pathway described in **Figure 1** under pH 6.8−8.0 reaction buffer (final pH 7.0−7.8), and that induction of *H. armigera* larvae by 0.2% 2-tridecanone does not result in new metabolic products. These facts imply that we can accurately characterize hydrolytic metabolism of β-cypermethrin and other α-cyano-3-phenoxybenzyl pyrethroids by *H. armigera* based on the production of 3-PBA in control and induced larvae.

Aldehyde-Oxidizing Enzymes Activity in *H. armigera*. The oxidative activity of crude homogenates of *H. armigera* midgut toward 3-PBAld was examined. 3-PBA was formed from

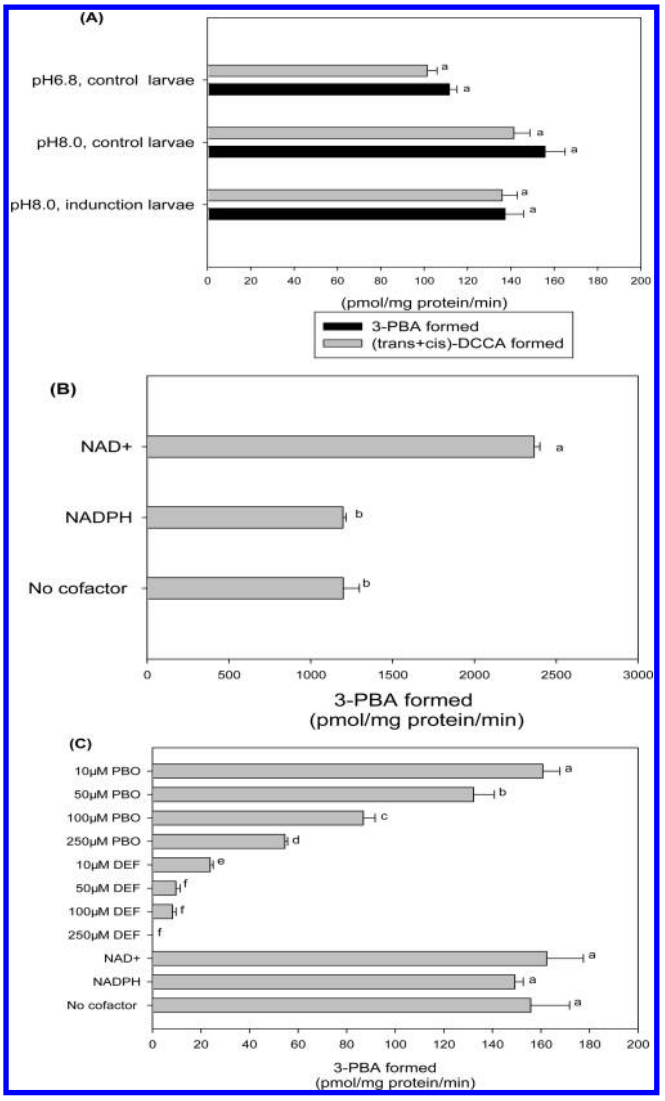

Figure 7. Hydrolytic activity toward 100 μM β-cypermethrin (**A**, **C**) and oxidative activity toward 100 μM 3-PBAld (**B**) of the midgut of *H. armigera*. (A) Hydrolytic activity based on the formation of 3-PBA and (*trans*+*cis*)-DCCA under 0.1 M pH 8.0 Tris-HCl (control larvae and induced larvae by 0.2% 2-tridecanone) and 0.1 M pH 6.8 phosphate reaction buffer (control larvae). (**B**) Oxidative activity of *H. armigera* midgut for conversion of 3-PBAld to 3-PBA. (**C**) Discussion of detoxifying enzymes in *H. armigera* midgut responsible for the metabolism of β-cypermethrin with cofactors (1 mM NADPH and 1 mM NAD^+) and detoxifying enzyme inhibitors (10−250 μM DEF for hydrolase and 10−250 μM PBO for cytochrome p450s) based on the production of 3-PBA. Each bar represents the mean ± SE (n = 3). Incubation was performed at 30 °C with 0.5 mL of enzyme sample (about 2 mg of protein). Both DEF and PBO were added at a concentration of 1×10^{-5}−2.5×10^{-4} M. Metabolites formed were determined by HPLC at 230 nm. The same letter indicates no significant difference between 3-PBA-presented and (*trans*+*cis*)-DCCA-presented hydrolytic activity using Paired t tests (**A**). A different letter indicates a significant difference ($p < 0.05$) among treatments in ANOVA multiple comparison analysis (**B** and **C**).

3-PBAld by crude homogenates, and the oxidative activity toward 3-PBAld was doubled in the presence of NAD^+ (**Figure 7B**).

Three observations from our present study support our hypothesis that the 3-PBAld, resulting from cyanohydrin after hydrolysis of type II pyrethroids, was completely oxidized to 3-PBA during incubation. First, the results in **Figure 7C** show no difference in hydrolytic activity of *H. armigera* midgut toward β-cypermethrin between "with" and "without" cofactor NAD^+. Second, the results in **Figure 7A** show no significant difference between 3-PBA-presented and (*trans*+*cis*)-DCCA-presented hydrolytic activity of *H. armigera* midgut toward β-cypermethrin. Lastly, it is found in **Figure 7B** that, when 3-PBAld was used as substrate, the aldehyde-oxidizing enzymes present in the crude homogenates could convert as much as 7.7-fold and 15.2-fold 3-PBAld to 3-PBA than that produced by β-cypermethrin metabolism (**Figure 7C**) in *H. armigera* midgut "with" and "without" cofactor NAD^+, respectively.

There are many reports to characterize type II pyrethroids hydrolytic metabolism based on the formation of 3-PBAld in animals (*4*, *17*, *26*, *28*). In our study, owing to the complete conversion of 3-PBAld to 3-PBA by aldehyde-oxidizing enzymes, development of a new HPLC assay method, using only one standard, for characterizing hydrolytic metabolism of all the α-cyano-3-phenoxybenzyl pyrethroids in *H. armigera* based on the quantitative analysis of 3-PBA is feasible and accurate. Concerning the high activity responsible for the conversion of 3-PBALD to 3-PBA by *H. armigera*, we explain that crude homogenates contain appropriate enzymes and endogenous cofactors (NAD^+ or/and NADPH) to accomplish the relevant oxidations (*6*).

Basic Enzymatic Pathway of Beta-cypermethrin Metabolism in *H. armigera* and Inhibition by DEF and PBO. In our research, three metabolites resulting from ester cleavage of β-cypermethrin were identified, and no metabolites generated by nonester cleavage were detectable supplemented with and without NADPH (intrinsic NADPH may be in the crude homogenates). 3-PBA was formed when 3-PBAld was added to the incubation mixtures as substrate, and the aldehyde-oxidizing enzymes activity responsible for 3-PBAld conversion to 3-PBA was fortified by the addition of NAD^+, and almost not affected by NADPH (**Figure 7C**). **Figure 1** shows the major enzymatic pathway of type II pyrethroids metabolism in *H. armigera* .

3-PBA is the major metabolite of the aromatic portion of the alcohol moiety of α-cyano-3-phenoxybenzyl pyrethroids in rats (*42*, *43*), in tea leaves (*44*), and in *H. virescens* (*30*) by way of 3-PBAld. In *H. virescens*, both aldehyde dehydrogenase (ALDH) and aldehyde oxidase activity were observed (*45*). In rats, both ALDH in the presence of NAD^+ (*46*) and cytochrome P450 isoforms in the presence of NADPH (*27*) have been identified and reported to contribute to the conversion of 3-PBAld to 3-PBA. To date, no specific insect P450 enzyme responsible for oxidizing aldehyde has been identified, although some substrates (e.g., C-26 hydroxyecdysteroids) indicate aldehyde oxidation metabolism by insect P450s (*47*). The P450 isoforms identified in rats (*27*) responsible for 3-PBALD oxidation have not been identified in insects (through search in "NCBI genebank"). Presently, we can just conclude that the aldehyde-oxidizing enzymes present in *H. armigera* midgut can be fortified by NAD^+, and much has to be done to characterize and identify the enzymes through biochemical and molecular biological methods. We would focus on the aldehyde-oxidizing enzymes as the subject of subsequent studies.

DEF, as an inhibitor for hydrolases, at 10 μM showed an 84.7% (a significant effect) inhibition of the metabolic enzyme activities, while PBO, as an inhibitor for cytochrome P450, at 10 μM had no inhibition effect on the metabolic enzyme activities at all. In addition, DEF at 50−250 μM had 93.8−100% inhibition of the metabolic enzyme activities, while PBO at 50 μM had only slight inhibition (15.1%) effect on the metabolic enzyme activities. These data indicate that CarEs, inhibited by DEF, were likely mainly responsible for β-cypermethrin hydrolysis.

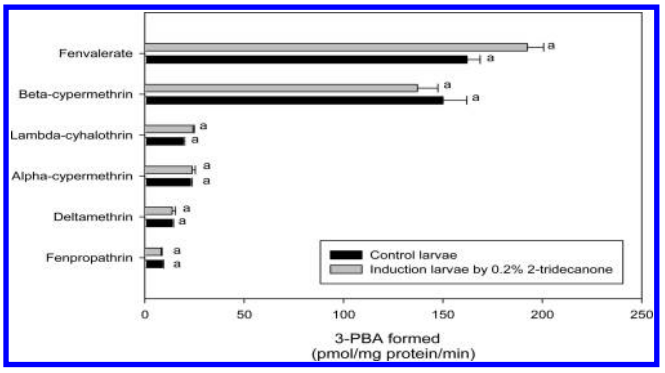

Figure 8. Hydrolytic activity toward six α-cyano-3-phenoxybenzyl pyrethroids (final concentration 100 μM) based on the production of 3-PBA of *H. armigera* larvae reared on a regular artificial diet or shifted to an artificial diet into which 0.2% 2-tridecanone (m/m) was incorporated and fed to sixth-instar larvae within 2 h after ecdysis. The sixth-instar newly molted larvae were exposed to 2-tridecanone (induced larvae) or continually exposed to an artificial diet (control larvae) for 48 h prior to dissection. Metabolites formed were determined by HPLC at 230 nm. The same letter indicates no significant difference in hydrolytic activity between control larvae and induced larvae using paired *t* tests.

It has been proposed that cyanohydrin may be generated by hydrolytic or/and oxidative metabolism of α-cyanophenoxybenzyl pyrethroids, at least for *cis* pyrethroids (*6*). In addition, it has been proposed that, a high concentration of DEF and PBO could inhibit cytochrome P450 monooxygenases (*48*) and hydrolases (*49*), respectively. Considering the important reports above, we conduct two separate inhibitor experiments using PBO and DEF with a moderate concentration for discussing whether CarEs or/and P450s are involved in the metabolic reaction. Several detoxifying enzymes could present together in crude homogenates which are responsible for metabolic reactions. In addition, DEF is not completely specific for esterases and may also be inhibiting oxidases, and intrinsic NADPH may be in crude homogenates with adequate concentrations to serve as a cofactor for P450s in metabolic reactions, so we cannot exclude the possibility of oxidative ester cleavage by P450s in β-cypermethrin hydrolysis.

2-Tridecanone Induction of type II Pyrethroids Hydrolytic Metabolism. The in vitro hydrolytic metabolism activity of six α-cyano-3-phenoxybenzyl pyrethroids between control larvae and induced larvae by 2-tridecanone was compared. The results in **Figure 8** show no difference in hydrolytic activity of *H. armigera* midgut toward all six type II pyrethroids comparing "with" and "without" induction by 2-tridecanone, which indicated that pyrethroids hydrolytic metabolism were not induced or elevated by 2-tridecanone. All six type II pyrethroids yielded 3-PBA (**Figure 6a**) and shared the same enzymatic pathway described in **Figure 1**. In addition, both control larvae and induced larvae by 2-tridecanone shared the same enzymatic pathway, and similar inhibition effect by DEF and PBO. In our other experiments, we find elevated cytochrome P450 monooxygenases activities in crude homogenates of 2-tridecanone induction *H. armigera* midgut responsible for the metabolism of other insecticide substrates.

The results in **Figure 8** also show that the pyrethroid hydrolytic rate appears to be dependent largely upon the compounds' structure. The rate of pyrethroids hydrolysis ranked in following order: fenvalerate > β-cypermethrin > α-cypermethrin, λ-cyhalothrin > deltamethrin > fenpropathrin, which indicated that the incorporation of cyclopropane group reduced the rate of pyrethroids hydrolysis, and that the tetramethylcyclopropanecarboxylate and solely *cis*-substituted pyrethroids including α-cypermethrin, λ-cyhalothrin and deltamethrin, were metabolically stable.

ABBREVIATIONS USED

CarEs, carboxylesterases; *trans*-DCCA, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid; *cis*-DCCA, *cis*-3-(2, 2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid; 3-PBA, 3-phenoxybenzoic acid; 3-PBAld, 3-phenoxybenzaldehyde; DEF, *S*,*S*,*S*-tributyl phosophorotrithioate; PBO, piperonyl butoxide; ALDH, aldehyde dehydrogenases.

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