CONTRIBUTIONS OF MONOOXYGENASES AND ESTERASES TO PYRETHROID RESISTANCE IN THE TOBACCO BUDWORM, HELIOTHIS VIRESCENS

Guomin Shan and Jim Ottea
LA Agric. Expt. Station, LSU Agric. Center
Baton Rouge, LA

Abstract

An in vitro assay was used to measure the metabolism of cypermethrin in homogenates of pyrethroid-susceptible and -resistant tobacco budworms. Total metabolism was greater in field-collected and laboratory-resistant larvae compared to that in a laboratory-susceptible strain. In addition, metabolsim of cypermethrin was greater in a late- than early-season collection. These results confirm that enhanced metabolism is associated with pyrethroid resistance in this insect. Three metabolites were identified from homogenates of resistant larvae: phenoxybenzoic acid, phenoxybenzyl alcohol and 4'-HO-cypermethrin. Phenoxybenzoic acid was the predominant metabolite produced in crude homogenates from all strains suggesting that hydrolysis of cypermethrin by esterases plays a key role in resistance in these strains.

Introduction

The tobacco budworm, Heliothis virescens (F.), is one of the most important insects attacking cotton and has caused significant economic damage in the United States (Sparks 1981). In 1996, this pest, together with the corn earworm, Helicoverpa zea, infested more than 80% of all U. S. cotton and caused more than $100 million in total cotton losses and costs of control (Williams, 1997). Insecticide resistance is a major factor responsible for our inability to manage populations of H. virescens on cotton (Sparks, 1981; Sparks et al., 1993). Because of heavy use of insecticides on cotton, H. virescens has developed high levels of resistance to organophosphate, carbamate and pyrethroid insecticides (Sparks et al., 1993).

The major mechanisms of resistance to pyrethroids in H. virescens are enhanced metabolic detoxication and reduced target site sensitivity (Nicholson and Miller, 1985; Payne et al., 1988; McCaffery et al., 1989; Gladwell et al., 1990; Ottea et al., 1995). Metabolism of pyrethroids in H. virescens occurs by both oxidative and hydrolytic attack (Nicholson and Miller, 1985; Dowd et al., 1987). Studies by Little et al. (1989), Ottea et al. (1995) and more recently by Shan et al. (1997) have shown the importance of P450-monooxygenases, which are associated with metabolic resistance to pyrethroids in laboratory and field-collected strains of H. virescens. In studies with H. virescens, Lee et al. (1989) and Little et al. (1989) suggested that the 4' and 2' positions on the phenoxybenzyl moiety of cypermethrin are the main sites of metabolism, whereas oxidation at geminal dimethyl groups is less important. Oxidative metabolism in a number of other insect species has been shown to occur at these sites as well as the geminal dimethyl groups of the cyclopropane ring (Shono et al., 1978, 1979; Casida and Ruzo 1980; Leahey, 1985). In addition, in studies with the related cotton pest, Helicoverpa armigera, Gunning et al. (1995, 1996) found that ester hydrolysis is a predominant pathway of metabolism of esfenvalerate. Based on the synergism of cypermethrin toxicity by DEF, Shan et al. (1997) also suggested that esterases might play an important role in H. virescens. To more clearly establish and define the role of P450-monooxygenases and esterases in H. virescens in pyrethroid resistance, the fate of cypermethrin in vitro was studied in pyrethroid-susceptible and –resistant strains. In addition, the efficacy of enzyme inhibitors was examined.

Materials and Methods

Chemicals

Radiolabeled ^{14}C-cypermethrin (53 mCi/mmol, benzyl labeled) was obtained from ICI Chemical Corporation. Unlabeled cypermethrin (cyper) was kindly provided by Zeneca Agrochemicals (Richmond, CA). Imidazole, 3-bromo-benzaldehyde, 4-methoxyphenol, *tert*-butyl dimethylsilyl (TBDMS) chloride, copper(I) chloride, 3-phenoxybenzoic acid, 3-phenoxybenzaldehyde, 3-phenoxybenzaldehyde cyanohydrin, and 3-phenoxybenzyl alcohol were purchased from Aldrich Chemical Company (Milwaukee, WI). Piperonyl butoxide (PBO) and paraoxon were purchased from ChemService Inc. (West Chester, PA). NADPH, NADP+, and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). Permethric acid (3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid; PA) was prepared from its methyl ester (Shan et al. 1997). All other chemicals were of analytical quality and purchased from commercial suppliers.

Insects

Pyrethroid-susceptible and -resistant laboratory strains of *H. virescens* were studied. The susceptible strain (LSU) was established in 1977 (Leonard et al., 1988) and has been reared in the laboratory since that time without exposure to insecticides. The resistant Pyr-R strain was derived from a field collection made in August, 1995 from the Red River Research Station (Bossier City, LA). Insects from this collection were reared for one generation then selected as fifth stadium larvae with cypermethrin (1.75 μg/larva) for three generations. Based on comparisons between LD_{50}s, Pyr-R insects are ca. 58-fold resistant relative to LSU-S insects. Preliminary results from neurophysiological and molecular genetic assays suggest that reduced neuronal sensitivity is not a major resistance mechanism in this strain (Park and Taylor, unpublished).

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Two strains of insects were collected from the Macon Ridge (MRS) location of LSU Agricultural Center's Northeast Research Station and studied. Collections of neonates were made at this site in 1997 from velvet leaf (Abutilon threoplasti) during June (MRS-June) and from cotton during August (MRS-August). Leaves containing insects were transported to the laboratory in styrofoam coolers and reared on a pinto bean-based diet. Insects from these collections were studied during the field-collected generation.

Preparation of 4'-HO-Cypermethrin

Methoxyphenol, 3-bromobenzaldehyde, and PA were used to synthesize 4'-HO-cypermethrin . The method of Sheldon et al. (1976) was used to prepare 4'-methoxyphenoxybenzaldehyde. Yield: 6.2 g, 56%. 1H NMR (CDCl3) d3.8 (s, 3H, CH3), 6.9-7.5 (m, 8H, Aromatics), 9.9 (s, 1H, CHO). MS (*m/z*): M+=228. Methoxyphenoxybenzaldehyde (11.2 g, 0.05 mol) and pyridine-hydrochloride (24 g, 0.25 mol) were added to a flask, then the mixture was stirred and heated with increasing temperature. A homogenous liquid was formed at 150 °C, which darkened to a deep purple color at 190 °C. After stirring at 190 °C for 5 h, the mixture was allowed to cool to 120 °C, then poured into water (250 mL). More water (100 mL) and EtOAc (150 mL) were added, stirred for 10 min and separated. The aqueous portion was extracted with EtOAc (2x 100 mL). Combined organics were washed with brine (3x 150mL), dried and concentrated to a dark viscous oil. The crude product was purified using silica column chromatography with EtOAc/hexane (30:70; v/v) as eluting solvent. The solvent was removed to give a brown colored gum (4.2 g, 39%).

Imidazole (1.7 g, 0.025 mol) and tert-butyldimethyl silyl chloride (TBDMS; 3.8 g, 0.02 mol) were dissolved in dimethylformamide (20 mL) then stirred at 15°C under nitrogen. Then, 4'-hydroxyphenoxybenzaldehyde (4.2 g, 0.02 mol) was dissolved in dimethylformamide (10 mL) and added dropwise over 20 min to form a red solution. The mixture was stirred at 20°C for 3 h, and then poured into ice water (200 mL). The aqueous phase was extracted with EtOAc (2 x 30 mL) and combined organics were washed with water, sodium bicarbonate (1%, 30 mL), water, and dried over MgSO4. The solvent was removed to give dark red oil. After purification by silica column chromatography (hexane/EtOAc = 9:1; v/v), the solvent was removed to yield product (5.2 g, 93%).

The 4'-TBDMS-O-cypermethrin was prepared by using a general procedure of esterification described by Hu et al. (1985) and modified by Shan et al. (1997). The 4'-HO-cypermethrin was made by the method of Corey et al. (1978) with removal of the silyl protecting group. 4'-TBDMSO-cypermethrin (1.4 g, 2.5 mmol) was dissolved in tetrahydrofuran (10 mL), then water (10 mL) and acetic acid (30 mL) were added quickly. The mixture was stirred at 55°C for 24 h, cooled to room temperature, then water (20 mL) and EtOAc (20 mL) were added. After separation, the aqueous phase was extracted with EtOAc (2 x 10 mL). Combined organics were washed with water (20 mL) and saturated NaCl solution (3x 20 mL), dried with anhydrous sodium sulfate and then concentrated to a dark yellow oil (0.85 g, 80%). This crude product was purified by column chromatography with hexane/EtOAc (8:2; v/v) as eluent. The solvent was removed to give dark green, viscous oil, which was submitted for prolonged concentration under high vacuum at 45°C to obtain a thick, viscous brown gum (0.45 g). 1H NMR (CDCl3) d1.18-1.3 (q, 6H, CH3), 5.3 (b, 1H, OH), 5.6 (m, 1H, aH), 6.3 (d, 1H, vinyl H), 6.8-7.3 (m, 8H, Aromatics). FAB-MS (*m/z*): (M+H)+=432.

Identification and Analysis of ^{14}C-Cypermethrin and Metabolites

The positions of cypermethrin and metabolites were measured using thin-layer chromatography (TLC) and precoated silica gel plates (G-60, F-254, 20 x 20 cm, 0.25 mm layer thickness; Whatman Inc., New Jersey) developed in two dimensions (Gaughan et al., 1977). The solvent systems and Rf values for cypermethrin and standards are shown in Table 1. Each plate was spotted with 1 ml of ^{14}C-cypermethrin (1520dpm) and 3-phenoxybenzoic acid (PBA), HO-cypermethrin (HO-cyper), 3-phenoxybenzaldehyde, 3-phenoxybenzyl alcohol (PBalc) and unlabelled cypermethrin as standards. Plates were developed in solvent system A, dried then developed in the second direction using either B (A/B) or developed twice using solvent system C (A/Cx2).

Identity of metabolites was verified using reverse phase HPLC and/or GC-MS. For HPLC studies, a Supelcosil column (LC-18-s, 25 cm x 4.6 mm, 5 μm ODS stationary phase; Supelco) was used, and eluted with water-acetonitrile containing 0.5% (v/v) acetic acid with a linear gradient from 30% to 100% acetonitrile over 25 min at a flow rate of 1 mL/min (Waters E600 solvent delivery system). The eluent was monitored for UV density at a wavelength of 254 nm with a Waters 486 Tunable Absorbance Detector. The retention times measured for reference compounds are listed in Table 1. The identity of the relatively less-polar compounds (i.e., cypermethrin and 4í-HO-cypermethrin) were confirmed by GC/MS. After separation by one-dimensional (A) or two-dimensional (A/C x 2) TLC, individual compounds was excised from the plate and extracted into diethyl ether, which was concentrated to 50 μL and injected into the GC/MS. A capillary column (DB-5, 20 m x 0.18 mm) was used with the following temperature programming: Tinit 408C for 3 min then 208C/min up to Tfinal 2808C, and compounds were detected by a Hewlett-Packard 5971A mass selective detector.

For analysis of cypermethrin metabolism, cypermethrin was separated from metabolites on pre-channeled TLC plates (linear-K, Whatman Inc., New Jersey) developed in solvent system A. One lane of each plate was spotted with a

solution containing ^{14}C-cypermethrin and unlabelled standards. After development, plates were dried, and peaks of radioactivity were localized using a BioScan System 200 imaging scanner (Washington DC). Peaks were assigned to regions of radioactivity that constituted greater than 0.25% of total radioactivity for each sample. Percent metabolism was defined as radioactivity in assigned peak/total radioactivity from all assigned peaks. Regions of UV density corresponding to unlabeled reference standards were visualized under UV light @ 254 nm.

Enzyme Preparation

After removal of heads and gut contents, carcasses were rinsed with 1.15% KCl. The insect tissue was placed into homogenization buffer (0.1 M pH 7.6 sodium phosphate containing EDTA (1 mM), dithiothreitol (1 mM), phenylthiourea (1 mM), and phenylmethylsulfonyl fluoride (4 mM; Rose et al., 1995), and homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 15,000g for 15 min, and the supernatant was used as enzyme resources (crude homogenate).

***in vitro* Assays**

Aliquots from crude homogenates (1 mg/mL protein) were incubated in a reaction mixture containing (final concentration): 50 μM cypermethrin, 0.25 μM ^{14}C-cypermethrin (3050 dpm), 1 mM NADPH and 20 μL of an NADPH regenerating system (0.25 mM NADP+, 2.5 mM glucose-6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase; Rose et al. 1995) in a total volume of 200 μL. Homogenates were incubated in the absence or presence of NADPH to establish the contribution of P450 monooxygenases to cypermethrin metabolism. For inhibition experiments, various concentrations of the monooxygenase inhibitor, PBO, or the esterase inhibitor, paraoxon, were added in 10 μL ethanol. Reaction mixtures were incubated for 45 min at 30^{o}C, then terminated by adding 20 μL of concentrated HCl. Cypermethrin and its metabolites were extracted into 850 μL of diethyl ether (3x), which was evaporated to about 100 μL under a stream of nitrogen and spotted onto pre-channeled TLC plates (Whatman Inc., New Jersey).

Spectrophotometric Esterase Assay

Esterase activity in the cytosol was measured with alpha-naphthyl acetate (ANA), using the method of Gomori (1953) as modified by van Asperen (1962), Grant et al. (1989), and Ibrahim and Ottea (1995). Substrate solution was prepared by dissolving 18 mg of Fast Blue B salt in 30 mL of 0.1 M phosphate buffer (pH 7.0) followed by addition of 600 μL of 0.113 M ANA (in buffer containing 50% acetone). Following filtration, 230 μL of substrate solution (2.03 mM final concentration) was added to an individual well of a microplate with 10 μL of enzyme homogenate. For inhibition studies, PBO or paraoxon (0.0001-10 mM in 10 μL of ethanol) was added prior to addition of substrate. Reactions with 10 μL ethanol or without protein were used as controls for measurements of inhibition or activity, respectively. Microplates containing reaction mixtures were incubated at 30 ^{o}C and changes in optical density (OD) during the initial 10 min of reactions were measured at 595 nm using a Thermomax (Molecular Devices) microplate reader. Results were corrected for non-enzymatic activity and the percentage inhibition (%I) was calculated as %I = 100 x (ODcontrol-OD inhibitor)/ODcontrol.

Results

Three metabolites were measured following incubation of crude homogenates (15,000*g* supernatant) with benzyl-labeled ^{14}C-cypermethrin. Based on Rf values and HPLC retention times for standards (Table 1), these products were identified as PBacid, PBalc and 4'-HO-cyper. In incubations with Pyr-R homogenates, the predominant products were PBacid and PBalc, which represented 7.6 and 7.5% of the total radiactivity detected by radiometric scanning, whereas HO-cyper was a minor metabolite (1.1% of total radioactivity). Additional, polar products with unknown identities also were detected by radiometric scanning (Rf<0.15; 2.9% of total radioactivity).

Levels of ^{14}C-cypermethrin metabolism were similar between Pyr-R and the field-collected strains, although metabolite profiles differed (Table 2). In tests with MRS-June, PBacid was the predominant metabolite detected. Moderate amounts of PBalc (3.1%) also were detected as well as a low level of HO-cyper (0.7%). Total metabolism and levels of all three metabolites were higher in MRS-August compared with -June larvae with the greatest increase seen in amounts of HO-cyper. LSU strain larvae produced very low levels (1.6%) of PBalc and no HO-cyper was detected, whereas a low level of PBacid was measured.

In incubations with inhibitors, both paraoxon and PBO were potent inhibitors of cypermethrin hydrolysis (Figure 1). Paraoxon was the more potent inhibitor with an I_{50} that was over 700 times lower that that for PBO (0.005 mM vs. 3.65 mM). Paraoxon was less potent as an inhibitor of ANA hydrolysis (I_{50}= 0.35 mM) and was only 10-fold more potent than PBO (I50= 24.7 mM).

Discussion

The results of this study suggest that metabolism of cypermethrin in *H. virescens* larvae is associated with resistance, and occurs by both oxidative and hydrolytic pathways. A metabolite in the Pyr-R strain of H. virescens was identified as 4'-HO-cypermethrin, confirming that the phenoxybenzyl moiety of conventional pyrethroids is a site for detoxication by P450 monooxygenases in this strain. These results are consistent with those from previous studies of metabolism of pyrethroids in H. virescens (Lee et al. 1989, Little et al. 1989) and the related pest, H. armigera (Lee et al. 1989).

Hydrolysis of cypermethrin appears to be a major metabolic resistance mechanism in this strain. A hydrolytic product of cypermethrin, PBacid, was the predominant metabolite in both LSU and Pyr-R larvae, and was detected at high levels in homogenates from field-collected strains. The suggestion that esterases are important for cypermethrin metabolism and resistance in H. virescens is consistent with evidence of synergism of cypermethrin toxicity by DEF (Graves et al., 1991; Shan et al., 1997), and previous studies in H. virescens (Dowd et al., 1987) and the related pest, H. armigera (Gunning et al. 1996).

A second product, PBalc, was detected as a major metabolite of in incubations with NADPH. THis finding is consistent with the study of esfenvalerate metabolism in H. armigera reported by Gunning et al. (1995). PBacid and PBalc are products of ester cleavage. The metabolite (phenoxybenzaldehyde cyanohydrin) is then converted into either PBacid or possibly PBalc (Shono et al., 1979). However, the production of PBalc is NADPH-dependent in mammals (Shono et al., 1979) and insects (this study), which suggests that oxidases may enhance ester cleavage of cypermethrin. Characterization of enzymes catalyzing this reaction require further study.

Inhibition experiments with PBO and paraoxon suggest that the monooxygenase inhibitor, PBO, also inhibits esterases. This finding suggests that the utility of these synergists as indicators of oxidative and hydrolytic resistance mechanisms should be reevaluated.

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Table 1. Chromatographic properties of cypermethrin and potential metabolites.

	Rf value with indicated solvent system*			HPLC tR
Compound	A	A/B	A/CX2	(min)
Cypermethrin	0.74	0.84	0.90	22.9
4'-HO-cyper	0.53	0.84	0.89	21.6
PBacid	0.27	0.81	0.65	9.90
PBalc	0.39	0.81	0.59	13.8
3-phenoxy-benzaldehyde	0.68	0.84	0.90	13.3
3-phenoxy-benzaldehyde cyanohydrin	0.44	----	-----	10.6

* TLC solvent systems used: A = toluene/ethyl acetate/acetic acid (75:25:1, v/v/v); B = butanol/acetic acid/water (15:5:1, v/v/v); C = benzene saturated with formic acid/ethyl ether (10:3, v/v).

Table 2. Metabolism of ^{14}C-cypermethrin by crude homogenates of laboratory-reared and field-collected H. virescens larvae*.

Strain	Cyper	PBacid	PBalc	HO-cyper	Other
Pyr-R	82 (11)	7.6 (1.0)	7.5 (0.9)	1.1 (0.4)	2.9 (0.4)
MRS-June	86 (8.6)	6.8 (0.7)	3.1 (0.4)	0.7 (0.3)	4.0 (0.4)
MRS-Aug	82 (11)	9.1 (1.2)	3.8 (0.7)	3.9 (0.7)	2.1 (0.3)
LSU-S	93 (23)	3.7 (0.8)	1.6 (0.4)	< 0.25	2.1 (0.5)

* Reaction contained 1.0 mg/mL of 15,000g supernatants and NADPH. Results are expressed as percent of total radioactivity (SD), which was defined as radioactivity in assigned peak/total radioactivity from all assigned peaks. More than 97% counts of radioactivity was extracted from reaction mixtures.

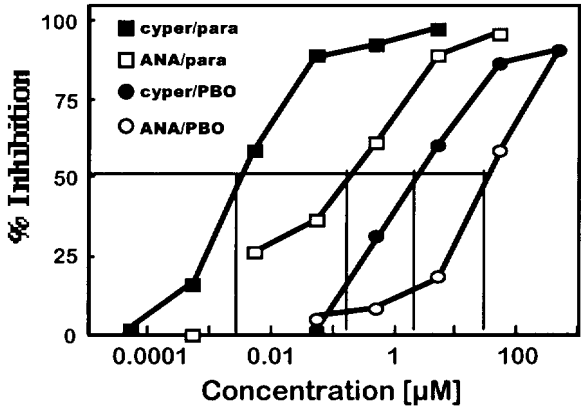

Figure 1. Inhibition of hydrolysis of cypermethrin (cyper) and alpha-naphthyl acetate (ANA) by paraoxon (para) and piperonyl butoxide (PBO).