MECHANISMS OF RESISTANCE TO ORGANOPHOSPHATE INSECTICIDES IN THE TOBACCO BUDWORM, HELIOTHIS VIRESCENS

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Abstract

The utility of microplate and electrophoretic assays for detection of metabolic mechanisms of resistance to profenofos in the tobacco budworm, Heliothis virescens (F.) was assessed. Mean esterase (EST) activities were significantly higher in profenofos-resistant than -susceptible larvae and activities were correlated with resistance to profenofos. In addition, qualitative and quantitative variation was observed in electrophoretic gels stained with_α-_and β- naphthyl acetates. Staining of ESTs was more intense with resistant larvae than those from a susceptible strain, and an esterase was detected in 100% of individuals from a profenofos-selected strain but was absent in the susceptible individuals examined. The appearance of this esterase in resistant larvae coincided with the decreased expression of a second esterase in susceptible insects. A similar pattern also was observed in larvae from field-collected strains.

Introduction

Over the past 40 years, resistance to all major classes of insecticides has been a persistent and increasing impediment to effective management of field populations of the tobacco budworm, Heliothis virescens (F.) (Sparks, 1981; Sparks et al., 1993). All three major mechanisms of insecticide resistance (i.e., reduced cuticular penetration, increased metabolic detoxication and altered sites of action) are expressed in organophosphorus (OP)-resistant H. virescens. Reduced cuticular penetration has been shown as a minor mechanism conferring low levels of resistance to malathion (Szeicz et al., 1973) and profenofos (Kanga and Plapp, 1994). In addition, decreased sensitivity of acetylcholinesterase (AChE) the target site for OPs, has also been associated with OP resistance in this pest (Brown and Bryson, 1992; Harold and Ottea, 1997).

Enhanced metabolic detoxication of insecticides by cytochrome P450-dependent monooxygenases (P450 MOs), glutathione S-transferases (GSTs) and esterases (ESTs) plays a major role in insecticide resistance (Oppenoorth, 1985; Abdel-Aal et al., 1993). Enhanced expression of P450 MO activity has been associated with metabolic resistance to OP insecticides in H. virescens (Brown, 1981; Bull, 1981) and is considered the primary cause of resistance to chlorpyrifos and chlorpyrifos methyl in some strains of this insect (Whitten and Bull, 1974). Similarly, elevated GST activities toward non-insecticide (model) substrates were measured in field-collected strains of H. virescens and were moderately correlated with frequencies of resistance to profenofos (Ibrahim and Ottea, 1995; Harold and Ottea, 1997). Finally, esterases have been found to be responsible for the hydrolysis of methyl parathion in laboratory strains of H. virescens (Konno et al., 1989; 1990). More recent studies suggest that elevated EST activities (as measured with α-naphthyl acetate [α-NA]) are associated with resistance to carbamate, OP and pyrethroid insecticides (Goh et al., 1995; Zhao et al, 1996). Activities of these enzymes are correlated with frequencies of profenofos resistance in field-collected and laboratory-selected strains of H. virescens (Harold and Ottea, 1997). The objective of this study was to explore further the utility of this EST assay as a biochemical marker to detect and monitor profenofos resistance in H. virescens.

Materials and Methods

Chemicals

Technical grade profenofos (O-(4-bromo-2-chlorophenyl)-O-ethyl-S-propylphosphorothioate; 89% purity; Novartis, Greensboro, NC) and chlorpyrifos oxon (O, O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphate; 100%; DowElanco Inc., Indianapolis, IN) were donated by respective manufacturers. Acrylamide, ethylenediaminetetraacetic acid (EDTA), and tris (hydroxymethyl)aminomethane (Tris) were purchased from Gibco BRL, (Grand Island, NY). N,N,N',N'-tetramethylethylene-diamine (TEMED), bis-acrylamide, ammonium persulfate and Coomassie Brilliant Blue R-250 were obtained from Amresco (Solon, OH). Bovine serum albumin (fraction 5), acetylthiocholine iodide (ATChI), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), Fast Blue RR salt, α-naphthyl acetate (α-NA) and β-naphthyl acetate (β-NA) were purchased from Sigma Chemical Company (St. Louis, MO). Fast Blue B salt and potassium chloride (KCl) were purchased from Aldrich Chemical Company (Milwaukee, WI).

Insects

Results of biological and biochemical assays with field-collected insects were compared with those from laboratory strains of OP-susceptible and -resistant H. virescens. For studies with field collections, eggs and larvae were collected from a domesticated stand of velvetleaf, Abutilon theophrasti Medicus, at the Louisiana State University Agricultural Center's Northeast Research Station/Macon Ridge location (MRS; Winnsboro, LA) during June (MRS Jun). Other field collections were made during August from a cotton field near Bayou Macon, LA (By Mac) that had been treated with three applications of spinosad, two applications each of cypermethrin and profenofos and one application of thiodicarb. Portions of leaves containing eggs and larvae were collected and transported to the laboratory in styrofoam coolers containing ice and placed in

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1-oz cups containing a pinto bean-based semi-synthetic diet (Leonard et al., 1988). Field-collected larvae were identified as H. virescens based on the presence of spinose cuticle and chalazas 1 and 2 on abdominal segments 1, 2, and 8, and a molar area on the oral surface of the mandible (Oliver and Chapin, 1981). Larvae were separated following head capsule slippage at the end of the fourth stadium, and fifth stadium (day 1) insects (180±20 mg) were selected for biological and biochemical assays. Adults were reared in 3.8-l cardboard cartons covered with cotton gauze as a substrate for oviposition and provided with sucrose (10% in water) as a carbohydrate source. Both larvae and adults were held at 27°C, 70% relative humidity, and a 14:10 hr (light:dark) photoperiod.

Larvae from two laboratory strains were studied. The reference susceptible strain, LSU-S, was established from field collections from cotton in 1977 (Leonard et al., 1988) and has been reared in the laboratory without intentional exposure to insecticides. A resistant laboratory strain (OPR) was originally established by repeatedly selecting larvae from a field-collection made at the Red River (RR) Research Station (Bossier City, LA) on August 23, 1995 (Harold and Ottea, 1997). Larvae from the OPR strain were reared for three generations without selection (OPR F3) and were either re-selected with profenofos (2.5 μg/larva; OPR F4S) or reared without exposure to insecticide (OPR F4). Fifth stadium larvae (day 1) from each of the strains were used for biochemical (n=30) and biological (n=30) assays.

Biological Assay

Susceptibility of H. virescens to profenofos was measured in fifth stadium (day 1) larvae following topical application of 1 μl of profenofos (in acetone) on the thoracic dorsum. The dose-mortality response of larvae was measured with at least 5 doses of profenofos (=10 larvae/dose) and replicated thrice. Treated larvae were held in 1-oz cups with diet and maintained at 27°C, 70±5% relative humidity, and a photoperiod of 14:10 (light:dark) hr. Mortality was recorded 72 hr posttreatment using absence of coordinated movement within 30 sec after being prodded with a pencil as the criterion. Data were analyzed by probit analysis (Finney, 1971) using a microcomputer-based program (SAS, 1985). Frequencies of resistance to profenofos in larvae (n=30) from field-collected strains were measured 72 hr following topical application of a single dose of profenofos (15.3 µg/larva; 10 X LD_{50} for LSU insects).

Biochemical Assays

Tissues homogenates from individual larvae were used as enzyme source for all biochemical assays. Individual larvae were weighed, decapitated, dissected and the digestive system was removed. The opened hemocoel was rinsed with ice-cold buffer (0.1 M sodium phosphate, pH 7.0) and fat bodies were obtained by gentle scraping and aspiration using a Pasteur pipette. Fat bodies were filtered using glass wool and homogenized in an all-glass homogenizer containing 200 µl of ice-cold 1.15% KCl (containing a few crystals of phenylthiourea). Individual heads were homogenized in 200 µl of 0.1 M sodium phosphate buffer (pH 8.0) containing 0.1% Triton X-100. Homogenates were centrifuged at 12,000g for 15 min and resulting supernatants were held in ice and used in enzyme assays within 30 min of preparation.

Activity of ESTs towards α-NA was measured using the assay of Gomori (1953) with modifications described previously (Harold and Ottea, 1997). Reaction mixtures were incubated at 30°C, and the rate of change in absorbance during the initial 10 min was measured at 595 nm using a Thermomax microplate reader (Molecular Devices, Palo Alto, CA). Data were corrected for non-enzymatic activity using incubations without protein as the control. Changes in OD were converted to nmol/min using an experimentally derived "extinction coefficient" (3.825 mM^{-1} 250 μl^{-1}) for the α-naphthol-Fast Blue B conjugate at 595 nm.

Native polyacrylamide gel electrophoresis (PAGE) was used to visualize ESTs from individual larvae using a vertical electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) and 5% acrylamide (Sambrook et al., 1989; Gunning et al., 1996). Protein concentration in individual fat body homogenates was adjusted to contain 30 g protein in 40 μl which was loaded onto the gel with 5 μl of 6X tracking dye (0.25% Bromophenol Blue and 40% sucrose w/v in water). Electrophoresis occurred in Tris-borate/EDTA buffer (100 mM Tris, 2.4 mM EDTA, and 100 mM boric acid, pH 8.0) at a constant voltage (150 V) until the dye marker was within 1 cm of the gel base. After electrophoresis, gels were stained in darkness at 25°C with 100 ml of 0.1M sodium phosphate buffer containing 0.5 mM α- and β -NA and 0.2% Fast Blue RR salt for 30 min. Gels were destained in distilled water and fixed in 5% acetic acid. Relative mobility (R_m) was calculated by dividing the migration distance of the specific band from the origin to the center of the band by the migration distance of the bromophenol blue tracking dye from the origin.

A "squash assay" was performed on filter paper based on earlier methods (Pasteur and Georgiou, 1989; Abdel-Aal et al., 1990) with modifications. Individual second stadium larvae (ca. 10-20 mg) were homogenized in a 1.5 ml micro-centrifuge tube (cut open at the distal end) using a pencil tip capped with a 0.5 ml micro-centrifuge tube onto a Whatman #3 filter paper moistened with phosphate buffer (0.1 mM; pH 7.0). The micro-centrifuge tubes were rinsed with distilled water between individual squashes to avoid contamination. The filter paper was incubated atop a second filter paper containing α-NA/Fast Blue substrate solution (prepared as described above) for 60 sec in darkness. The reaction was stopped by rinsing the filter paper with water followed by the addition of 5% acetic acid. Esterase activities were visualized as violet stains on the undersurface of the filter paper, which were compared with

a color scale prepared with α-naphthol standards (0-1.8 μmoles).

Protein concentrations were measured by the method of Bradford (1976) with bovine serum albumin (fraction V; concentrations corrected for impurities) as the standard. Data were subjected to analysis of variance followed by Tukey's multiple comparison test (P=0.05) using a microcomputer-based program (SAS, 1985). Linear regressions between enzyme activities and susceptibility to profenofos were estimated using the method of least squares.

Results and Discussion

Biochemical assays, including those with model substrates, can be utilized successfully for detecting and monitoring insecticide resistance in insect populations (Brown and Brogdon, 1987; Devonshire, 1987). In previous studies, resistance was shown to be associated with elevated EST activity toward naphthyl acetates in several insect pests (Abdel-Aal et al., 1993). The present study is based on a similar correlation between EST activities and frequencies of resistance to profenofos in an array of resistant field populations and laboratory strains of *H. virescens* (Harold and Ottea, 1997), and on the assumption that changes in naphthyl acetate hydrolysis reflect changes in the ability to hydrolyze insecticide esters. Such knowledge of the nature of resistance mechanisms also may serve as a foundation for the development of field kits to diagnose metabolic resistance in field populations of this pest. This would enable growers to ascertain the resistance status of H. virescens prior to insecticide application, and to choose effective insecticides for control of this pest.

Levels of profenofos resistance (based upon LD_{50} values) in laboratory-resistant strains were statistically higher than that of the susceptible LSU strain (Table 1). The highest level of resistance to profenofos (31-fold relative to LSU) was expressed in OPR larvae (LD_{50}=22.8 μg/larva). Unlike the other laboratory strains, the log-dose probit (ldp) relationship for OPR P1 plateaued at a dose of 22.3 μg/larva corresponding to 52% mortality and formed a clear deflection at higher doses of profenofos (data not shown).

In the absence of selection with profenofos, levels of resistance decreased significantly to 11.8-fold (LD_{50}=8.6 μg/larva) after 3 generations (OPR F3) and to 9.7-fold after 4 generations (OPR F4). Resistance increased significantly to 22-fold (LD_{50}=16.0 μg/larva) following selection of OPR F3 larvae with profenofos (OPR F4S). Similar to that measured for OPR P1 larvae, the ldp line for OPR F4S showed a clear plateau at a dose 40 μg/larva corresponding 85% mortality (data not shown).

Frequencies of resistance to profenofos (as determined in topical bioassays with 15.3 μg profenofos/larva) were higher in larvae from the field-collected and the laboratory-selected strains than the susceptible LSU strain (Table 1). A high frequency of resistance was determined for OPR P1 larvae (68%) but decreased to 27% in OPR F3 larvae. A further decrease in resistance frequency was measured between OPR F3 to OPR F4, but was not statistically significant. However, following selection of OPR F3 larvae with profenofos, the resistance frequency increased to 53% in OPR F4S. High frequencies of profenofos resistance also were recorded in field-collected insects (60% and 97% in MRS Jun and By Mac, respectively).

Resistance to profenofos was associated with high levels of EST activity toward α-NA. Activities of ESTs were higher in larvae of both laboratory-resistant and field-collected insects than in the susceptible LSU strain (Table 2). The highest EST activity was expressed in larvae from OPR (294 nmoles of α-naphthol formed min^{-1} mg $protein^{-1}$). Mean EST activity decreased in the absence of selection to 88.8 nmoles in OPR F3 larvae, but increased significantly (1.8-fold) in OPR F4S following selection with profenofos. In assays with field strains, larvae from By Mac expressed EST activity that was comparable with that of OPR larvae. The EST activity in MRS Jun larvae was intermediate relative to LSU and OPR P1 larvae, and did not differ significantly from that of OPR F4S.

Whereas mean levels of EST activity did not differ significantly among the OPR F3, F4 and LSU strains, differences in frequency profiles for this activity in these strains were apparent (Table 2). The frequency distribution in LSU larvae was narrow with no individuals expressing activity >90 nmoles min^{-1} mg $protein^{-1}$. In contrast, the distribution of EST activity was broad in larvae from OPR, and 97% of the individuals expressed activities greater than those of LSU larvae. In the absence of selection (OPR F3), the frequency profile narrowed, and only 20% of larvae expressed activity >90 nmoles. In profenofos-selected OPR F4S larvae, increased frequencies of individuals expressing high EST activity were detected: 83% of individuals expressed activities greater than 90 nmoles. Finally, broad frequency profiles were observed in the field strains and 62 and 87% of the individuals from MRS Jun and By Mac, respectively, expressed greater activities than those of LSU strain (Table 2).

Further evidence for an association between EST activity and profenofos resistance was measured in electrophoretic studies. Qualitative and quantitative variation was observed between OP-susceptible and resistant larvae in banding patterns of ESTs in electrophoretic gels (data not shown). Staining of ESTs was more intense with resistant larvae than those from the susceptible strain. In addition, a band (R_m= 0.65) was expressed in 100% of the individuals tested from OPR F4S but in none of the LSU individuals examined. The appearance of this band coincided with the decreased expression of a second band (R_m=0.68) in susceptible larvae. In addition, the R_m 0.65 band was expressed in 67 and 87% of the individuals from MRS Jun

and By Mac, respectively. Corresponding frequencies of resistance (60% and 97% for MRS Jun and By Mac, respectively) were measured for these strains.

Although none of the ESTs visualized in gels with OPR F4S larvae have been shown to metabolize insecticides directly, the consistent expression of these enzymes after selection suggests a potential role in profenofos resistance in H. virescens and as a biochemical marker. In a similar study, Konno et al., (1990) reported an unique EST (esterase III) that hydrolyzed methyl parathion in OP-selected H. virescens.

Differences in EST activity and electrophoretic banding patterns between susceptible and resistant H. virescens formed the basis for a "squash assay," which has potential utility in the field to assess the contribution of hydrolytic mechanisms to resistance in this pest. In preliminary assays, staining was more intense with homogenates from profenofos-resistant that -susceptible strains. The utility of this assay for detecting OP resistance in the field will be evaluated in the upcoming growing season.

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Table 1. Susceptibility of fifth stadium larvae from laboratoryand field-collected strains of H. virescens to profenofos.

Strain	n	LD50 (µg/larva)	RR	R Frequency
LSU	392	0.73[a]	-	0.00
OPR-P1	213	22.8[b]	31.2	67.7
F3	180	8.60[c]	11.7	26.8
F4	184	7.08[c]	9.70	23.3
F4S	241	16.0[b]	22.0	53.3
MRS-Jun	30	ND	ND	60.0
By Mac	30	ND	ND	96.7

Values followed by the same letter are not statistically significant. Resistance (R) frequency was defined as survival following topical application of a discriminating dose (15.3 μg/larva) of profenofos.
RR= Resistance Ratio= LD_{50} (resistant strain/LSU strain).
ND= Not Determined

Table 2. Esterase (EST) activities in fifth stadium larvae from field-collected and laboratory strains of H. virescens.

Strain	EST Activity (SD)	Freq. >LSU
LSU	71.6 (38.1)[c]	0
OPR P1	294 (127)[a]	97
OPR F3	88.8 (52.3)[c]	20
OPR F4	106 (44.5)[bc]	16
OPR F4S	163 (49.5)[b]	83
MRS Jun	167 (104)[b]	62
By Mac	237 (105)[a]	87

EST activities (-naphthol formed min^{-1}mg $protein^{-1}$) are expressed as means (±SD). Values followed by the same letter are not significantly different. Frequencies of individuals with activity greater than LSU larvae (Freq.>LSU) were calculated from frequency histograms.
LSU: laboratory-susceptible; OPR; laboratory- resistant;
MRS: Macon Ridge; By Mac: Bayou Macon