
John A. Harold and James A. Ottea*

Department of Entomology, Louisiana State University Agricultural Center, Baton Rouge

The utility of microplate and electrophoretic assays for detection of biochemical and physiological mechanisms underlying resistance to profenofos in the tobacco budworm, *Heliothis virescens* (F.), was assessed. Esterase (EST) activities were significantly higher in profenofos-resistant than -susceptible larvae, and activities were highly correlated ($r^2 = 0.87$) with resistance to profenofos. Both qualitative and quantitative variation was observed in electrophoretic gels stained with $\alpha$- and $\beta$-naphthyl acetates. Staining of ESTs was more intense with resistant larvae than those from a susceptible strain. In addition, a band (designated $A'$) was expressed in larvae from profenofos-resistant strains, but not in larvae from an insecticide-susceptible strain. The frequency of expression of $A'$ increased following selection with profenofos and was detected in 100% of the individuals from a profenofos-selected strain. The appearance of this band coincided with the decreased expression of a second band (designated $A$). A similar pattern (overexpression of $A'$ and underexpression of $A$) also was observed in larvae from field-collected strains. Finally, reduction in the activity or the sensitivity of acetylcholinesterase to inhibition by chlorpyrifos oxon was observed in laboratory-selected and field-collected larvae that expressed resistance to profenofos. These results suggest that microplate and electrophoretic assays can be utilized as complementary tools for detecting and monitoring profenofos resistance in *H. virescens*. Arch. Insect Biochem. Physiol. 45:47–59, 2000. © 2000 Wiley-Liss, Inc.

Key words: insecticide; resistance; organophosphate; detection; *Heliothis virescens*

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 detoxification and altered sites

*Correspondence to: Dr. James A. Ottea, Department of Entomology, 402 Life Sciences Building, Louisiana State University, Baton Rouge, LA 70803. E-mail: jottea@unix1.sncc.lsu.edu

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of action) are expressed in organophosphorus (OP)-resistant *H. virescens*. Reduced cuticular penetration has been shown as a minor mechanisms 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 and carbamates, has also been a cause of 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 is 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 (Whitte 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 are 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), and are correlated with frequencies of profenofos resistance in field-collected and laboratory-selected strains of *H. virescens* (Harold and Ottea, 1997).

Knowledge and biochemical and physiological mechanisms of resistance is essential for the rational development and effective implementation of insecticide resistance management (IRM) strategies (Brown and Brogdon, 1987). Since the success of IRM relies on the ability to diagnose the nature, frequency, and evolution of insecticide resistance in the target population, rapid and sensitive techniques to detect resistance mechanisms are needed (ffrench-Constant and Roush, 1990). Although results of biological assays provide verification that resistance is present in field populations, they fail to provide information about the underlying resistance mechanism(s). Rapid, preliminary diagnosis of metabolic mechanisms of resistance may be obtained using bioassays with insecticide synergists. Similarly, quantitative biochemical assays based on spectrophotometric determination of enzyme activities toward model substrates have been used as indicators of metabolic resistance (Brown and Brogdon, 1987; Rose et al., 1995). However, since detoxifying enzymes exist in multiple forms (Oppenoorth, 1985; Schoknecht and Otto, 1989) with differing sensitivities to synergists, a single synergist may not inhibit the enzymes responsible for resistance (Brown et al., 1996a). Therefore, absence of synergism cannot be considered as proof that a particular metabolic mechanism is absent (ffrench-Constant and Roush, 1990). Similarly, results from studies using model substrates are inconclusive unless enzyme activities measured are consistently correlated with the expression of resistance (Brown and Brogdon, 1987; Ibrahim and Ottea, 1995). Since EST activities toward α-NA were found previously to be correlated with frequencies of profenofos resistance (Harold and Ottea, 1997), the objective of this study was to explore further the utility of this EST assay as a biochemical market to detect and monitor profenofos resistance in *H. virescens*.

**MATERIALS AND METHODS**

**Chemicals**

Technical grade profenofos (O-(4-bromo-2-chlorophenyl)-O-ethyl-S-propyl phosphorothioate: 89%; 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′-tetramethylethylenediamine (TEMED), bis acrylamide, ammonium persulfate and Coomasie 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, β-naphthyl acetate (β-NA), and α-NA were purchased from Sigma Chemical Company (St. Louis, MO). Fast Blue B salt and potassium chloride (KCl) were pur-
chased 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 (150–250) 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) on June 12, 1997 (MRS Jun). Other field collections were made on August 22 and 25, 1997, 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 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, 70–5% relative humidity, and a photoperiod of 14:10 (light:dark) hr.

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 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 selected with profenofos (15.3 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 Assays**

Susceptibility of *H. virescens* to profenofos was measured in fifth stadium (day 1) larvae following application of 1 µl of acetone containing varying doses of profenofos onto 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 following topical application 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 laboratory and field-collected strains were measured 72 hr following topical application of a single dose of profenofos (15.3 µg/larva; approximately 20× the LD₅₀ for LSU insects). This dose was high enough to kill all susceptible larvae but low enough to allow differences in frequencies among the resistant strains to be resolved.

**Tissue Preparation**

Tissue homogenates from individual larvae were used as enzyme sources 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 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. The resulting supernatants were filtered through glass wool then held in ice.
and used in enzyme assays within 30 min of preparation.

Biochemical Assays

Activity of esterases towards α-NA was measured using the assay of Gomori (1953) with modifications (van Asperen, 1961; Grant et al., 1989; Ibrahim and Ottea, 1995). Reaction mixtures in individual wells of a microtiter plate contained 240 µl of substrate solution (2.13 mM and 0.56%, final concentrations of α-NA and Fast Blue B, respectively), and 10 µl of fat body homogenate (containing 0.05 tissue equivalent; 0.016 ± 0.007 mg protein). The substrate solution was prepared by adding 600 µl of α-NA (0.113 M dissolved in 50% acetone in water) to a solution of Fast Blue B salt (18 mg in 30 ml of 0.1 M phosphate buffer, pH 7.0), and then filtered using Whatman no. 3 filter paper. 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 PAGE was used to visualize EST 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). The protein concentrations of homogenates from individual fat bodies were adjusted to contain 30 µg protein in 40 µl, which was loaded onto a gel with 5 µl of 6× 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 0.1 M sodium phosphate buffer (pH 8.0) 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 (Rm) 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 Georghiou, 1989; Abdel-Aal et al., 1990) with modifications. Individual second stadium larvae (approximately 10–20 mg) were homogenized in a 1.5 ml microcentrifuge tube (cut open at the distal end) using a pencil tip capped with a 0.5 ml microcentrifuge tube onto a Whatman no. 3 filter paper moistened with phosphate buffer (0.1 mM; pH 7.0). 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 strains on the undersurface of the filter paper, which were compared with a color scale prepared with α-naphthol standards (0–1.8 µmol).

Sensitivity of AChE to chlorpyrifos oxon was measured using head homogenates from individual larvae following the method of Ellman et al. (1961) with modifications (Moores et al., 1988; Byrne and Devonshire, 1991, 1993; Ibrahim and Ottea, 1995). As profenofos in an indirect inhibitor of AChE (Wing et al., 1998; Byrne and Devonshire, 1993), it could not be used for these studies. Homogenates of individual heads (30 µl containing 0.15 tissue equivalent; 0.070–0.083 mg protein) were incubated for 10 min at 30°C in wells of a microplate containing 69 µl of sodium phosphate buffer (0.1 M, pH 8.0) and 1 µl of chlorpyrifos oxon (148.8 nM final concentration). The concentration of chlorpyrifos oxon used in these studies was the I₉₀ for the LSU strain as determined in preliminary experiments (data not shown). Following preincubation, reactions were initiated by adding 200 µl of ATChI/DTNB (0.5 mM/0.05 mM, final concentration), and the rate of change in OD at 405 nm at 30°C was recorded. Data were corrected for nonenzymatic activity using incubations without protein as the control and expressed as miliOD min⁻¹ mg protein⁻¹. Activities of AChE measured in the absence of chlorpyrifos oxon served as the control. Percentage inhibition was calculated as 1-(activity with inhibitor/activity without inhibitor) × 100.
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

Profenofos Resistance

Profenofos resistance in laboratory-resistant strains was statistically higher than in the susceptible LSU strain (Table 1). The highest level of resistance to profenofos (31-fold relative to LSU) was expressed in OPR larvae (LD50 = 22.8 µg/larva). In the absence of selection with profenofos, levels of resistance decreased significantly to 11.8-fold (LD50 = 8.6 µg/larva) after 3 generations (OPR F3) and to 9.7-fold (LD50 = 7.1 µg/larva) after 4 generations (OPR F4). Resistance increased significantly to 22-fold (LD50 / 16.0 µg/larva) following selection of OPR F3 larvae with profenofos (OPR F4S).

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 laboratory-selected strains than the susceptible LSU strain (Table 1). A high frequency of resistance was measured for OPR larvae (68%) but decreased to 27% in OPR F3 larvae. Resistance frequency decreased further between OPR F3 to OPR F4, but this difference was not statistically significant. However, following selection of OPR F3 larvae with profenofos, 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).

Esterase Activities

Activities of ESTs were higher in larvae from both laboratory-selected and field-collected insects than in the susceptible LSU strain (Table 2). The highest mean EST activity (294 nmol α-naphthol formed min⁻¹ mg protein⁻¹) was expressed in larvae from the OPR strain. Mean EST activity de-

### TABLE 1. Profenofos Susceptibility in Larvae From Laboratory and Field-Collected Strains of *H. virescens*

<table>
<thead>
<tr>
<th>Strain</th>
<th>N</th>
<th>LD50 (95% CL)</th>
<th>Slope ± SE</th>
<th>Resistance ratio</th>
<th>χ²</th>
<th>Percentage survival @ diagnostic dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSU</td>
<td>392</td>
<td>0.73 (0.65–0.81)</td>
<td>3.72 ± 0.32</td>
<td>—</td>
<td>3.34</td>
<td>0.00</td>
</tr>
<tr>
<td>OPR</td>
<td>213</td>
<td>22.8 (18.8–27.5)</td>
<td>2.46 ± 0.30</td>
<td>31.2</td>
<td>3.42</td>
<td>67.7</td>
</tr>
<tr>
<td>OPR F3</td>
<td>180</td>
<td>8.60 (6.78–10.6)</td>
<td>2.44 ± 0.30</td>
<td>11.7</td>
<td>1.57</td>
<td>26.8</td>
</tr>
<tr>
<td>OPR F4</td>
<td>184</td>
<td>7.08 (5.50–8.86)</td>
<td>2.23 ± 0.28</td>
<td>9.70</td>
<td>1.97</td>
<td>23.3</td>
</tr>
<tr>
<td>OPR F4S</td>
<td>241</td>
<td>16.0 (13.1–19.1)</td>
<td>2.44 ± 0.28</td>
<td>22.0</td>
<td>0.85</td>
<td>53.3</td>
</tr>
<tr>
<td>MRS Jun</td>
<td>30</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>60.0</td>
</tr>
<tr>
<td>By Mac</td>
<td>30</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>96.7</td>
</tr>
</tbody>
</table>

†Susceptibility was measured 72 hr following topical application of 1 µl of acetone containing profenofos onto fifth stadium (day 1) larvae. Resistance ratio = LD50 of resistant strain/LD50 of LSU strain. Percentage survival @ diagnostic dose = survival following topical application of 15.3 µg of profenofos/larva (n = 30) in fifth stadium (day 1) larvae. ND, not determined.

*Expressed as µg/larva; values followed by the same letter are not statistically different (Tukey’s; P = 0.05).

### TABLE 2. Enzyme Activities and Sensitivity of AChE to Inhibition by Chlorpyrifos Oxon in Field-Collected and Laboratory Strains of *H. virescens*

<table>
<thead>
<tr>
<th>Strain</th>
<th>EST (SD)</th>
<th>AChE activity (SD)</th>
<th>AChE inhibition (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSU</td>
<td>71.6 (38.1)</td>
<td>0.25 (0.05)</td>
<td>89.8 (2.72)</td>
</tr>
<tr>
<td>OPR</td>
<td>294 (127)</td>
<td>0.33 (0.16)</td>
<td>69.6 (14.8)</td>
</tr>
<tr>
<td>OPR F3</td>
<td>88.8 (52.3)</td>
<td>0.20 (0.18)</td>
<td>78.0 (12.0)</td>
</tr>
<tr>
<td>OPR F4</td>
<td>106 (44.5)</td>
<td>0.16 (0.06)</td>
<td>88.3 (4.79)</td>
</tr>
<tr>
<td>OPR F4S</td>
<td>163 (49.5)</td>
<td>0.12 (0.05)</td>
<td>87.4 (5.83)</td>
</tr>
<tr>
<td>MRS Jun</td>
<td>167 (104)</td>
<td>0.20 (0.09)</td>
<td>74.9 (11.4)</td>
</tr>
<tr>
<td>By Mac</td>
<td>237 (105)</td>
<td>0.10 (0.04)</td>
<td>83.2 (10.7)</td>
</tr>
</tbody>
</table>

*Activities were measured in assays with individual larvae (n ≥ 30) from laboratory-susceptible (LSU), -resistant (OPR), and field-collected (MRS: Macon Ridge, By Mac: Bayou Macon) strains of *H. virescens*. Values followed by the same letter are not significantly different (Tukey’s; P = 0.05). Mean EST activities (±SD) expressed as nmols of α-naphthol formed min⁻¹ mg protein⁻¹. Mean AChE activities (±SD) toward ATPChI expressed as miliOD min⁻¹ mg protein⁻¹. Mean percent inhibition of AChE (±SD) measured following preincubation of head homogenates with chlorpyrifos oxon for 10 min.
creased in the absence of selection to 88.8 nmol in OPR F3 larvae, but increased significantly (1.8-fold) in OPR F4S larvae 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 larvae, and did not differ significantly from that of OPR F4S.

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

**Electrophoretic and Filter Paper Analysis**

Qualitative and quantitative variation in banding patterns of ESTs in electrophoretic gels was observed between OP-susceptible and -resistant larvae. Staining of ESTs appeared more intense with resistant larvae than those from the susceptible strain (Figs. 3–5). In addition, a band (designated A\(^{\prime}\); \(R_m = 0.65\)) was expressed in some OPR larvae, but not in larvae from the LSU strain (Fig. 3). In most cases, the appearance of this band coincided with the decreased expression of a second band (designated A) with \(R_m = 0.68\). In addition, after selection of OPR larvae with a high dose of profenofos (OPR F4S), expression of band A\(^{\prime}\) (and underexpression of band A) was measured in 100% of the individuals tested (Fig. 4). Finally, this pattern (i.e., overexpression of A\(^{\prime}\) and underexpression of A) was observed in gels with the majority of field-collected larvae from MRS Jun (67%) and By Mac (87%) (Fig. 5).

![Graph](image-url)  
**Fig. 1.** Frequency distribution of EST activity (expressed as \(\alpha\)-naphthol formed min\(^{-1}\) mg protein\(^{-1}\)) from fat body homogenates of individual, fifth stadium larvae (day 1; \(n = 30\)) in laboratory-susceptible and -resistant strains of *H. virescens*. 


Quantitative differences in EST activity between LSU and OPR F4S larvae were also apparent in a filter paper assay using individual, second stadium larvae (Fig. 6). The color developed using susceptible larvae ranged from no color to moderate violet color, and staining was more intense with homogenates from resistant than susceptible larvae. In individual homogenates from 15 LSU larvae, intensity of staining corresponded to < 0.8 μmol of α-naphthol. In contrast, intense staining was observed with homogenates from OPR F4S larvae and amounts of α-naphthol produced were > 0.8 μmol for 14 of the 15 homogenates.

**Activity of AChE and Sensitivity to Inhibition by Chlorpyrifos Oxon**

Activities of AChE from individual larvae were variable in both laboratory and field-col-
The highest activity (0.33 miliOD min$^{-1}$ mg protein$^{-1}$) were measured in OPR larvae. Activities decreased significantly in the absence of selection to 0.20 and 0.16 miliOD min$^{-1}$ mg protein$^{-1}$ in the OPR F3 and F4 strains, respectively. Although activity was significantly lower in OPR F4S than LSU larvae, activities did not differ between OPR F4 (unselected) and OPR F4S (selected). The lowest activity was measured in the field-collected By Mac strain.

Sensitivity of AChE (expressed as percent inhibition following incubation with chlorpyrifos oxon) was significantly lower in larvae from the OPR strain than from the LSU strain (Table 2). Sensitivity increased significantly in the absence of selection for both the OPR F3 (78%) and OPR F4 (88%) strains. However, as with AChE activity, there were no differences in AChE sensitivity between OPR F4 and OPR F4S larvae. Reduced sensitivity of AChE also was measured in field-collected MRS Jun insects (75%), but there was no significant difference in AChE sensitivity between By Mac (83%) and LSU (90%) larvae.

Frequency profiles for AChE inhibition also reflected differences among strains (Fig. 7). The sensitivity of AChE in LSU larvae was normally distributed with more than 88% inhibition measured in 97% of individuals. For OPR and OPR F3 strains, 80 and 30% of individuals, respectively, expressed AChE with decreased sensitivity (i.e., <88% inhibition). Broad frequency profiles of AChE sensitivity were measured with larvae from the field-collected strains, and decreased sensitivity of AChE was expressed in 67 and 27% of individuals from MRS Jun and By Mac, respectively.
Fig. 6. Filter paper assay measuring the intensity of color developed using crude homogenates of second stadium larvae from susceptible (LSU) and resistant (OPR F4S) strains of *H. virescens*. **Bottom:** Color scale developed with known concentration of α-naphthol.

Fig. 7. Frequency distribution of AChE sensitivity in profenofos-susceptible (LSU) and -resistant (OPR, OPR F3, F4, and F4S) strains of *H. virescens*. Sensitivity is expressed as percentage inhibition of AChE activity measured in head homogenates of individual, fifth stadium larvae (day 1; n = 30) following 10 min preincubation with 149 nM chlorpyrifos oxon.
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). Such knowledge of the nature of resistance mechanisms 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. The basis for the present study is a correlation reported between EST activities and frequencies of profenofos resistance in an array of resistant field populations and laboratory strains of *H. virescens* (Harold and Ottea, 1997).

Elevated EST activities toward model substrates have been associated with OP resistance in a number of insect pests (Abdel-Aal et al., 1993). In previous studies, elevated activities of ESTs toward permethrin, α-NA (Dowd et al., 1987) and methyl parathion (Konno et al., 1989, 1990) have been documented in larvae from laboratory strains of OP-resistant *H. virescens*. More recently, EST activities toward α-NA from mass homogenates of thiodicarb-selected *H. virescens* were suggested to be responsible for cross-resistance among carbamate, OP, and pyrethroid insecticides (Goh et al., 1995; Zhao et al., 1996). In the present study, a high level of resistance to cypermethrin (17-fold at the LD₅₀) was measured in OPR larvae (data not shown), suggesting that metabolic mechanisms expressed in this strain may confer cross-resistance between cypermethrin and profenofos.

Results presented in this study provide indirect evidence for the role of ESTs in OP resistance in *H. virescens* and illustrate the utility of EST activity toward α-NA as a biochemical marker for profenofos resistance in this insect. Frequencies of profenofos resistance in 21 field-collected and laboratory-selected strains of *H. virescens* were highly correlated (r² = 0.90) with mean levels of EST activities toward α-NA (this study and Harold and Ottea, 1997). In addition, EST activity toward α-NA decreased in the absence of selection (i.e., from OPR to OPR F4) but increased following one generation of selection (i.e., from OPR F3 to OPR F4S). Finally, frequencies of profenofos resistance and EST activities were significantly higher in two field populations of *H. virescens* than in the LSU strain.

In addition to elevated EST activities, a unique EST was expressed in larvae from profenofos-resistant laboratory colonies, and the frequency of expression increased following selection with profenofos. Further, this EST was detected in field-collected strains and was expressed in 67 and 87% of the individuals from MRS Jun and By Mac, respectively. Corresponding frequencies of resistance measured in these strains were 60 and 97% for MRS Jun and By Mac, respectively. Since detoxifying enzymes exist in multiple forms (Schoknecht and Otto, 1989), the mean interstrain difference in total activity with α-NA might overlap between susceptible and resistant strains (Devonshire, 1989), masking the expression of a resistance-associated esterase. However, results from the present study suggest that such esterases can be accurately and rapidly detected in individual larvae using electrophoretic separation. In a similar study, discrimination between susceptible and moderately resistant aphids (whose total activity overlapped with that of susceptible aphids) was made possible by electrophoretic separation of esterase-4 (Sawicki et al., 1978). Since estimations based on intensity of electrophoretic bands are subjective, electrophoresis may best be viewed as a complementary tool with spectrophotometric determinations of total EST activities (Devonshire and Field, 1991).

Although no ESTs from the profenofos-resistant larvae used for this study have been shown to metabolize insecticides directly, the consistent expression of a unique enzyme in resistant insects suggests a potential role in profenofos resistance in *H. virescens* and as a biochemical marker. In a similar study, Konno et al. (1990) reported a unique EST (esterase III) that hydrolyzed methyl parathion in OP-selected *H. virescens*. In the current study, elevated EST activity and electrophoretic banding patterns in resistant *H. virescens* formed the basis for a preliminary version of a “squash assay,” which has potential utility in the field to assess the contribution of ESTs to resistance in this pest. In the prelimi-
nary assays reported here, staining was more in-
tense with homogenates from profenofos-resistant 
than -susceptible strains.

Altered expression and sensitivity of AChE 
were measured in the OP-resistant strains. High 
frequencies (>50%) of the individuals tested from 
field-collected and resistant laboratory strains 
expressed both increased EST activity and altered 
AChE (data not shown). Similar enhancement of 
esterase-based insecticide resistance by insensi-
tive AChE has been observed in the aphids, 
Myzus persicae and M. nicotianae (Moores et al., 
1994). In the present study, both decreased 
anal activity of AChE and reduced sensitivity to inhibition 
by chlorpyrifos oxon were measured at high 
frequencies in larvae from field-collected strains: 
38 and 87% of individuals from MRS Jun and By 
Mac, respectively, expressed lower AChE activ-
ity than LSU larvae. In addition, as in the OPR 
strain, AChE with decreased sensitivity to inhibi-
tion by chlorpyrifos oxon was expressed in 67 
and 27% of MRS Jun and By Mac larvae, respec-
tively. Whereas reduced sensitivity of AChE to OP 
inhibitors in H. virescens has been documented 
(Brown, 1991; Brown and Bryson, 1992; Kanga 
and Plapp, 1994; Wolfenbarger, 1996; Brown et 
.al., 1996b), decreased activity of AChE was not. 
Lower hydrolyzing efficiency, in conjunction with reduced sensitivity of AChE, has also been ob-
served in azinphosmethyl-resistant Colorado po-
tato beetles, Leptinotarsa decemlineata (Zhu and 
Clark, 1995), and several other insect species (re-
viewed in Oppenoorth, 1985).

In conclusion, the results of this study 
showed that both enhanced esterase activity and altered 
expression of AChE are associated with 
profenofos resistance in H. virescens. Elevated 
EST activities toward α-NA in OP-resistant lar-
vae were measured using a microplate assay and 
were associated with the appearance of a unique 
electrophoretic band. These data suggest that 
microplate and electrophoretic assays can be uti-
lized as complementary tools for detecting and 
monitoring mechanisms of profenofos resistance 
in H. virescens.

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REFERENCES

esterases: biochemical aspects and importance in 
the diagnosis of insecticide resistance. Pestic 
Biochem Physiol 38:255–266.

Detection methodology of esterase-mediated insecticide 
resistance from bioassay to biotechnology. Rev Pestic 
Toxicol 2:13–33.

quantitation of microgram quantities of protein util-
izing the principle of protein-dye binding. Anal Chem 


Brown TM. 1991. Resistant acetylcholinesterase of the to-
bacco budworm, Heliothis virescens. Rev Pestic Toxicol 
1:185–195.

Brown TM, Brogdon WG. 1987. Improved detection of in-
secticide resistance through conventional and molecular 

parathion-resistant acetylcholinesterase from Heliothis 

propynyl aryl ethers in permethrin-resistant tobacco bud-

Brown TM, Bryson PK, Arnette F, Roof M, Mallette JLB, 
Graves JB, Nemec SJ. 1996b. Surveillance of resis-
tant acetylcholinesterase in Heliothis virescens. In: 
Brown TM, editor. Molecular genetics and evolution 
of pesticide resistance, Vol 645. Washington, DC: 
American Chemical Society. p149–159.

Bull, DL. 1981. Factors that influence tobacco budworm re-
stance to organophosphorus insecticides. Bull Entomol 

and acetylcholinesterase activities by profenofos treat-
ments in the tobacco whitefly, Bemisia tabaci (Genn.): 
implications for routine biochemical monitoring of these 

Byrne FJ, Devonshire AL. 1993. Insensitive acetylcholin-
esterase and esterase polymorphism in susceptible and 
resistant populations of the tobacco whitefly, Bemisia 
tabaci (Genn.). Pestic Biochem Physiol 45:34–42.

Devonshire AL. 1987. Biochemical studies of organophospho-
rus and carbamate resistance in house flies and


Sparks TC. 1981. Development of insecticide resistance in...


