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Elevated Esterases Exhibiting Arylesterase-like Characteristics in an Organophosphate-Resistant Clone of the Greenbug, *Schizaphis graminum* (Homoptera: Aphididae)

Kun Yan Zhu*,[1] and Fengqin He†

**Department of Entomology, Kansas State University, Manhattan, Kansas 66506; and*
†State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, CAS, Beijing 100080, China

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The profiles of esterase activity in an organophosphate (OP)-susceptible (OSS) clone and an OP-resistant (OR-2) clone of the greenbug (*Schizaphis graminum*) were compared using nondenaturing polyacrylamide gel electrophoresis (PAGE) coupled with esterase assays after gel fractionations. A distinct peak of esterase activity was found in the esterase profiles of the OR-2 clone but not in the OSS clone. The peak represents the elevated esterases identified previously and constitutes a major biochemical difference between the OSS and the OR-2 clones. The esterases within that peak hydrolyzed five substrates, including phenyl acetate (PA), β-naphthyl acetate (β-NA), α-naphthyl butyrate (α-NB), *p*-nitrophenyl acetate (*p*-NA), and α-naphthyl acetate (α-NA). The most preferred substrate was PA followed by β-NA, α-NB, *p*-NA, and α-NA. Nondenaturing PAGE revealed that the major esterase peak was contributed by three different esterase bands. These esterases showed a similar affinity to β-NA and were highly sensitive to inhibition by both paraoxon and *p*-hydroxymecuribenzoic acid. In addition, the enzyme activity was slightly to moderately activated by Ca^{2+}, but significantly inhibited by Triton X-100. These characteristics suggested that the elevated esterases in the OR-2 clone were arylesterases or arylesterase-like esterases with certain biochemical properties resembling phosphoric triester hydrolase. However, these arylesterase-like esterases were not able to efficiently utilize paraoxon and chlorpyrifos-oxon as their substrates. Thus, the elevated esterases identified in the OR-2 clone appeared to contribute to OP resistance by sequestrating OP molecules.

Key Words: insecticide resistance; esterase; arylesterase; enzyme kinetics; greenbug; *Schizaphis graminum*.

INTRODUCTION

The greenbug, *Schizaphis graminum* (Rondani), is a major insect pest of sorghum, wheat, and other small-grain crops worldwide (1). In the midwestern United States, infestations of the greenbug are common and sometimes become extremely destructive. Organophosphate insecticides (OP)[2] have been used for many years as a major control measure for the greenbug, which, consequently, has developed high resistance to many of them (2–5). For example, laboratory bioassays have revealed that three field-derived resistant clones of the greenbug are resistant to all seven OP insecticides examined, ranging from 3- to 327-fold, compared with the susceptible greenbugs (J.-R. Gao and K.Y. Zhu, unpublished). Numerous studies on OP resistance in the greenbug have suggested the involvement of multiple resistance mechanisms, including

[1] To whom correspondence and reprint requests should be addressed. Fax: 785-532-6232. E-mail: kzhu@oz.oznet.ksu.edu.

[2] Abbreviations used: OP, organophosphate insecticide; AChE, acetylcholinesterase; ATC, acetylthiocholine iodide; BCA, bicinchoninic acid solution; BSA, bovine serum albumin; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); EST, esterase; α-NA, α-naphthyl acetate; β-NA, β-naphthyl acetate; *p*-NA, *p*-nitrophenyl acetate; α-NB, α-naphthyl butyrate; OSS, organophosphate-susceptible greenbug clone; OR-1 and OR-2, organophosphate-resistant greenbug clones with different patterns of increased esterase levels; PA, phenyl acetate; PAGE, polyacrylamide gel electrophoresis; PHMB, *p*-hydroxymercuribenzoic acid; SDS, sodium dodecyl sulfate.

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enhanced esterase levels (6–12) and alterations of acetylcholinesterase (AChE) (6, 7, 13).

Detailed studies on general esterases have indicated several striking differences in enzyme kinetics (6, 7, 9, 10, 12) and electrophoretic mobility (11, 14) in the two OP-resistant strains OR-1 and OR-2 (formerly referred to as Type I or Pattern I and Type II or Pattern II, respectively). Nondenaturing polyacrylamide gel electrophoresis (PAGE) using α-naphthyl acetate as a substrate showed several minor esterase bands and a major band with slower electrophoretic mobility from OR-1 greenbugs. This major esterase apparently was present but probably expressed at low level in OP-susceptible (OSS) greenbugs (12). In contrast, nondenaturing PAGE showed several strongly stained esterase bands with faster electrophoretic mobility from the OR-2 greenbugs and these esterases were absent or expressed at very low levels in the OSS greenbugs (12). Antiserum raised against a purified Type II esterase was specific for that enzyme but did not cross-react with the Type I esterase, indicating that these two esterases found in the greenbug were immunologically distinct (15).

When phenyl acetate (PA) was used as a substrate, the maximal velocity of the PA-hydrolyzing esterases was approximately 12-fold higher in the OR-2 strain than in the OSS strain (12). These esterases appeared to have some characteristics of A-esterases with respect to their substrate specificity. Although the major Type II esterases have been purified to homogeneity and their biochemical properties have been characterized partially (16), the nature of these elevated esterases in the OR-2 greenbugs and their roles in conferring OP resistance are still unclear. The objectives of this study were to (1) compare esterase profiles in OSS and OR-2 clones of the greenbug, (2) determine substrate specificity of major esterases separated by nondenaturing PAGE, and (3) classify these esterases based on their substrate and inhibitor specificities.

MATERIALS AND METHODS

Insects

The OSS and OR-2 clones of the greenbug were isolated from the OSS and OR-2 strains, respectively. They were maintained on the stems of 1- to 2-month-old sorghum plants supplied with water in 2-liter flasks covered with a nylon screen (13). The OR-2 greenbugs possessed esterases with the Type II banding pattern as previously described (11, 14). An OP-resistant colony of the lesser grain borer (*Rhyzopertha dominica*) collected from Brazil in 1993 was used as a positive control for determinations of phosphoric triester hydrolase activity (17).

Chemicals

Acetylthiocholine iodide (ATC), bicinchoninic acid solution (BCA), 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), fast blue B salt (*O*-dianisidine, tetrazotized), fast garnet GBC salt (2-methyl-4-[(2-methylphenyl)-azo]benzenediazonium salt), *p*-hydroxymercuribenzoic acid (PHMB), α-naphthol, β-naphthol, α-naphthyl acetate (α-NA), β-naphthyl acetate (β-NA), α-naphthyl butyrate (α-NB), *p*-nitrophenyl acetate (*p*-NA), paraoxon (diethyl *p*-nitrophenyl phosphate, 90% pure), rabbit serum, sodium dodecyl sulfate (SDS), and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide, bovine serum albumin (BSA), *N*,*N*′-methylene-bis-acrylamide, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, and Tris(hydroxymethyl)aminomethane were purchased from Bio-Rad Laboratories (Hercules, CA). Chlorpyrifos-oxon [*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphate, 97% pure] and phenyl acetate (PA) were purchased from Chem Service (West Chester, PA) and Aldrich Chemical Co. (Milwaukee, WI), respectively.

Effects of Triton X-100, $CaCl_2$, and pH on PA-Hydrolyzing Esterases

To study the effects of Triton X-100 on PA-hydrolyzing esterase activity, batches of 100 apterous greenbug adults were homogenized in 2 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.0) containing 1 mM $CaCl_2$. After the homogenates were centrifuged at 15,000*g* for 15 min at 4°C, the supernatants were transferred to fresh tubes and used as enzyme sources. The PA-hydrolyzing esterase activity was determined

based on the method of Kitchen *et al.* (18) with some modifications (12). Briefly, the reaction mixture (750 μl) contained 1 mM $CaCl_2$, 4 mM PA, different concentrations (0, 0.0002, 0.001, 0.005, or 0.025%) (vol/vol) of Triton X-100, and 40 μl of enzyme preparation in 0.1 M phosphate buffer (pH 7.0). After the reaction was initiated by adding the enzyme preparation, the absorbance was recorded continuously at 270 nm for 1 min at approx 24°C using a UV/visible spectrophotometer (Ultrospec 3000; Pharmacia Biotech Ltd., Cambridge, UK). The molar extinction coefficient of 1310 M^{-1} cm^{-1} was used for calculation of the PA-hydrolyzing esterase activity (19). To study the effects of $CaCl_2$ and pH on the PA-hydrolyzing esterases, the enzyme activity was measured in the presence or absence of 1 mM $CaCl_2$ under six different pH conditions (6.5, 7.0, 7.5, 8.0, 8.5, and 9.0).

Electrophoretic Analysis of Esterases

Nondenaturing PAGE of the esterases from the OSS and OR-2 greenbug clones was carried out with a Penguin P8DS dual-gel electrophoresis system (Owl Scientific, Woburn, MA) coupled with a cold-water circulating system as previously described (12). Batches of the greenbug were homogenized in 0.1 M phosphate buffer (pH 7.5) at a ratio of 1:10 (weight:volume). After the homogenates were centrifuged at 15,000*g* for 15 min at 4°C, the supernatants were used as enzyme sources for PAGE analysis. Each lane was loaded with a normalized amount of total protein (35 ng) from each greenbug clone. The gel (4 and 7.5% acrylamide in stacking and separating gels, respectively) was run at a constant voltage of 200 V for 75 min and stained for esterase activity using β-NA as a substrate and fast garnet GBC as a chromogenic agent (20). Briefly, after the gel was soaked in 0.25 M boric acid for 15 min, the gel was incubated in a reaction mixture consisting of 99 ml 0.1 M phosphate buffer (pH 7.2) and 1 ml β-NA stock (60 mg in 4 ml acetone) for 2 min at room temperature. To visualize the esterase bands, 60 mg fast garnet GBC salt was added to the mixture, and the gel was incubated again for 4 min at room temperature. After the gel was rinsed with tap water, it was soaked in the tap water for 30 min and dried with a gel dryer coupled to a vacuum pump.

Fractionation of Esterases by Nondenaturing PAGE

Following nondenaturing PAGE, the gel was soaked in 0.1 M phosphate buffer (pH 7.5) for 5 min. It then was sliced into 33 blocks including 5 from the stacking gel. To extract esterases, each gel block was homogenized in 400 μl of ice-cold 0.1 M phosphate buffer (pH 7.5). After the homogenates were centrifuged at 15,000*g* for 15 min at 4°C, 200 μl of supernatant was recovered from each gel block. The gel pellets then were recentrifuged under the same conditions, and another 50 μl of the supernatant was recovered from each one. The supernatants recovered from two rounds of centrifugation were combined (total 250 μl) and used as enzyme sources for studies of esterase profiles and substrate specificity of the esterases.

Examinations of Esterase Profiles Using Selected Substrates

Profiles of the fractionated esterases were determined with six selected substrates, including α-NA, α-NB, β-NA, *p*-NA, PA, and ATC (Fig. 1). For the first three substrates, 15 μl of appropriately diluted enzyme preparation from each gel block was incubated in a final reaction volume of 150 μl in 0.1 M phosphate buffer (pH 7.5) containing 0.54 mM α-NA, 0.27 mM α-NB, or 0.27 mM β-NA at 37°C for 30 min. Reactions were stopped by adding 50 μl of fast blue B-SDS solution (21). After 15 min, absorbance was determined at 600 nm for α-NA and α-NB and at 560 nm for β-NA using a microplate reader (Molecular Devices, Menlo Park, CA) (22).

For the substrate *p*-NA, esterase activity in each fraction was determined based on the method of Frohlich *et al.* (23) with some modifications. Briefly, 15 μl of enzyme preparation was mixed with 185 μl of 0.27 mM *p*-NA solution in 0.1 M phosphate buffer (pH 7.0). The

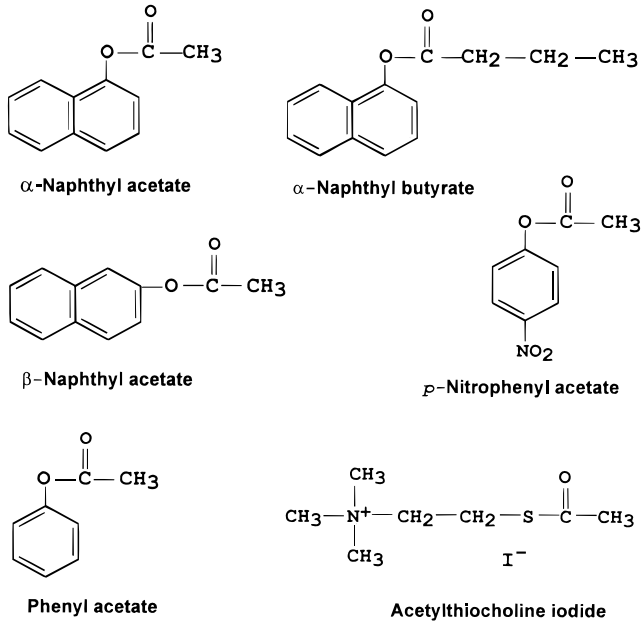

FIG. 1. Chemical structures of selected esterase substrates used in this study: α-naphthyl acetate, α-naphthyl butyrate, β-naphthyl acetate, *p*-nitrophenyl acetate, phenyl acetate, and acetylthiocholine iodide.

final volume and substrate concentration in the reaction were 200 μl and 0.25 mM, respectively. The change of absorbance was recorded immediately at 405 nm for 2 min with the microplate reader at approximately 24°C. Enzyme activity was calculated based on the molar extinction coefficient of 16,240 M^{-1} cm^{-1} (24).

For the substrate ATC, the enzyme activity was determined in 0.1 M phosphate buffer (pH 7.5) containing 0.5 mM ATC and 0.04 mM DTNB in a final volume of 150 μl according to Zhu and Gao (13). Absorbance was recorded at 405 nm for 2 min at approx 24°C with the microplate reader.

For the substrate PA, enzyme activity was assayed in a way similar to that described above. The reaction mixture consisted of 4 mM PA and 40 μl enzyme preparation in a final volume of 750 μl in 0.1 M phosphate buffer (pH 7.0) containing 1 mM $CaCl_2$. The change of absorbance was recorded at 270 nm for 1 min at approx 24°C using the UV/visible spectrophotometer (12).

Kinetic Analysis of Three Major Esterase Isozymes

After esterases were separated by 7.5% nondenaturing PAGE, three major esterase isozymes in the gel were located by staining the gel for 1 to 2 min or until they were just visible using β-NA as a substrate and fast garnet GBC as a chromogenic agent. Slices containing the esterase isozymes were excised individually from the gel, and each isozyme was extracted from the corresponding gel slice as described above. Esterase activities were determined by the method of van Asperen (21) using β-NA as the substrate with the microplate assay format as previously described (12). Briefly, aliquots of 15 μl of enzyme preparations were incubated with β-NA at six different concentrations in a final volume of 150 μl in 0.1 M phosphate buffer (pH 7.5) for 20 min at 37°C. The enzyme reactions were stopped by the addition of 50 μl fast blue B-SDS solution, and absorbance was determined 15 min later at 560 nm using the microplate reader. The initial velocity of the

enzyme reaction was obtained and the Michaelis constant (K_m) and maximal velocity (V_{max}) were estimated by Hanes transformations (25).

Inhibition of Three Major Esterase Isozymes

The three major esterase isozymes were isolated from a nondenaturing PAGE gel by the method described above. Two different inhibitors (i.e., paraoxon and PHMB) were used to classify these PA-hydrolyzing esterase isozymes from the OR-2 greenbug clone. The inhibition reaction was started by incubating 20 μl of enzyme with 20 μl of each inhibitor at approx 24°C for 5 min. The remaining PA-hydrolyzing esterase activity then was determined immediately. The IC_{50} (the inhibitor concentration required to inhibit 50% of the enzyme activity) value for each inhibitor was determined based on log (inhibitor concentration) vs probit (percentage inhibition) linear regression.

Assay of OP-Hydrolyzing Activity in Five Esterase Isozymes

OP-hydrolyzing activities in each of five esterase isozymes (i.e., the three major isozymes described above plus two others) were measured using paraoxon and chlorpyrifos-oxon as substrates according to the methods of Furlong *et al.* (26, 27) with some modifications. Individual esterase isozymes were extracted from the nondenaturing PAGE. Twenty microliters of 60 mM paraoxon or 30 mM chlorpyrifos-oxon in acetone was added to 0.96 ml of 0.1 M Tris–HCl buffer (pH 8.5) containing 2 mM $CaCl_2$ and 1.5 M NaCl in a 1.5-ml cuvette. The final concentration in the reaction mixture was 1.2 mM for paraoxon or 0.6 mM for chlorpyrifos-oxon. The reaction was initiated by adding 20 μl of each extracted esterase isozyme to the mixture. The absorbance was recorded in the UV/visible spectrophotometer at 405 nm for paraoxon or 310 nm for chlorpyrifos-oxon for 2 min at approx 24°C. Molar extinction coefficients of 18,050 and 5562 M^{-1} cm^{-1} were used for calculations of the paraoxon- and chlorpyrifos-oxon-hydrolyzing activities, respectively.

Determination of Protein Contents

Protein contents of the enzyme preparations were determined according to Smith *et al.* (28) using BSA as a standard. The measurement was performed with the microplate reader at 560 nm (29).

RESULTS

Effects of Triton X-100, $CaCl_2$, and pH on Esterase Activity

Figure 2 shows the profile of residual PA-hydrolyzing activity of esterases from OR-2 greenbugs in the presence of Triton X-100. Low concentrations (i.e., 0.0002 and 0.001%, vol/vol) of Triton X-100 did not have any significant effect on the PA-hydrolyzing esterase activity ($P > 0.05$). However, Triton X-100 at concentrations of 0.005 and 0.025% in the reaction mixtures had significant effects on enzyme activity ($P < 0.05$), reducing it by approximately 35 and 70%, respectively.

The increase of pH from 6.5 to 9.0 in the reaction mixtures had very little effect on the enzyme activity (Fig. 3), indicating that the PA-hydrolyzing esterases can tolerate a broad range of pH. However, 1 mM $CaCl_2$ in the reaction mixtures slightly or moderately enhanced the enzyme activity at all pH conditions examined (Fig. 3). The average increase in activity was 17.5%, and the highest value was 35% at pH 6.5 ($P < 0.05$).

Electrophoretic Analysis of Esterases

The nondenaturing PAGE analysis of esterases from both the OSS and the OR-2 clones of the greenbug displayed multiple bands of esterase activity when β-NA was used as substrate (Fig. 4). At least eight different esterase bands were detected from the OR-2 clone using this method. The bands indicating esterase activity for the OR-2 clone were much more intense than those for the OSS clone. This difference was particularly obvious for those identified as ESTs 4, 5, 6, 7, and 8.

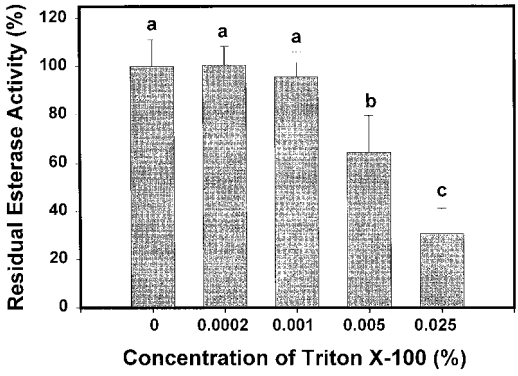

FIG. 2. Effects of Triton X-100 on PA-hydrolyzing activity of esterase prepared from the OR-2 clone of the greenbug. Data for the percentage of remaining esterase activity represent the means of five determinations ($n = 5$) for each concentration of Triton X-100. Vertical bars indicate standard errors of the mean. Different letters on the bars indicate that the means are significantly different from that of the control (i.e., Triton X-100 = 0%) ($P < 0.05$) in Duncan's multiple range test (48).

Esterase Profiles and Substrate Specificity

Esterase profiles for the OSS clone did not exhibit any distinct peak of activity for any substrate used (Fig. 5), which agreed well with results of the nondenaturing PAGE analysis (Fig. 4). Absence of such a peak of activity in the OSS clone suggested that the esterases that constituted the peak activity in the OR-2 clone were responsible for the increased esterase activity observed in those greenbugs. In both OSS and OR-2 clones, enzyme activity was hardly detectable in any fraction when ATC was used as a substrate (data not shown). Because ATC is a rather specific substrate for AChE, an undetectable or extremely low level of the activity indicated that the esterases identified by nondenaturing PAGE (Fig. 4) were not able to hydrolyze ATC.

Generally, all remaining substrates generated similar activity profiles and showed a distinct peak of esterase activity in the same position for the OR-2 clone (Fig. 5). However, PA was the preferred substrate followed by β-NA, α-NB, *p*-NA, and α-NA. The peak activity (i.e., gel slice

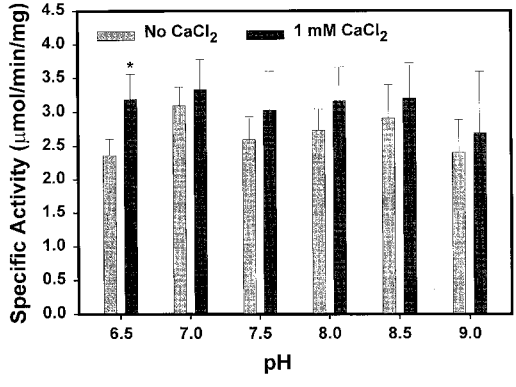

FIG. 3. Effects of pH and $CaCl_2$ on PA-hydrolyzing activity of esterases prepared from the OR-2 clone of the greenbug. Data for the enzyme specific activity represent the means of five determinations ($n = 5$) under each condition. Vertical bars indicate standard errors of the mean. The asterisk on the bar indicates that the mean is significantly different from the control with no $CaCl_2$ under the same pH condition.

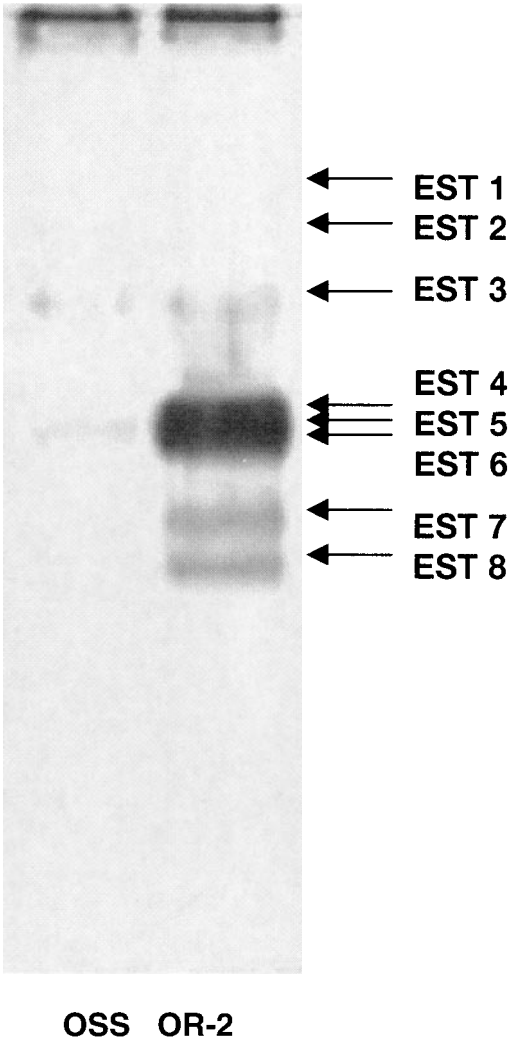

FIG. 4. Comparison of the esterase profiles of the OSS and OR-2 clones of the greenbug from nondenaturing PAGE. Each lane was loaded with 35 ng of total protein from each greenbug clone. The gels (7.5% separating gel and 4% stacking gel) were run at the constant voltage of 200 V for 75 min, and the esterases were stained for their activity using β-NA as substrate in fast garnet GBC solution. (The esterases EST 4, EST 5, and EST 6 could be seen easily if the gel was stained briefly).

No. 19) of the esterases in hydrolyzing PA was approximately 94-fold higher than that in hydrolyzing α-NA. Results indicated that the increased esterase activity in the OR-2 greenbug clone was due mainly to a few esterases with similar electrophoretic properties.

Kinetics and Inhibition of Three Major Esterase Isozymes

Nondenaturing PAGE analysis using a brief staining procedure revealed that the major peak (gel slices Nos. 18–20) observed in the esterase profiles (Fig. 5) actually contained three distinct bands suggesting three isozymes (i.e., EST 4, 5, and 6). Kinetic analysis did not indicate statistically significant differences in the K_m values among the esterase isozymes ($P > 0.05$) but showed a significant difference in the V_{max} value between ESTs 4 and 5 ($P < 0.05$; Table 1). These results suggested no significant difference in the affinity between enzymes and substrate β-NA but a significant difference in catalytic efficiency between EST 4 and EST 5 (Fig. 6).

The esterase isozymes appeared to be highly sensitive to inhibition by both paraoxon and PHMB but did not differ significantly among the isozymes (Fig. 7). There was no statistically significant difference in sensitivity to inhibition by either paraoxon or PHMB among the esterase isozymes. Paraoxon was a relatively more potent inhibitor than PHMB for these isozymes. The IC_{50} values for paraoxon were estimated to be 0.20, 0.40, and 0.37 μM for EST 4, 5, and 6, respectively, whereas the IC_{50} values for PHMB were 0.90, 2.6, and 4.3 μM for EST 4, 5, and 6, respectively.

OP-Hydrolyzing Activity in Five Esterase Isozymes

No hydrolytic activity toward either paraoxon or chlorpyrifos-oxon was detected in any of the five esterase isozymes (i.e., ESTs 4–8 in Fig. 4). In contrast, when rabbit serum and enzyme preparation from an OP-resistant strain of the lesser grain borer were used as enzyme sources, respectively, very high to moderate levels of chlorpyrifos-oxon-hydrolyzing activity were observed (Table 2). These results suggested that the assay procedures worked and the lack of the enzyme activity in the OR-2 greenbugs was due

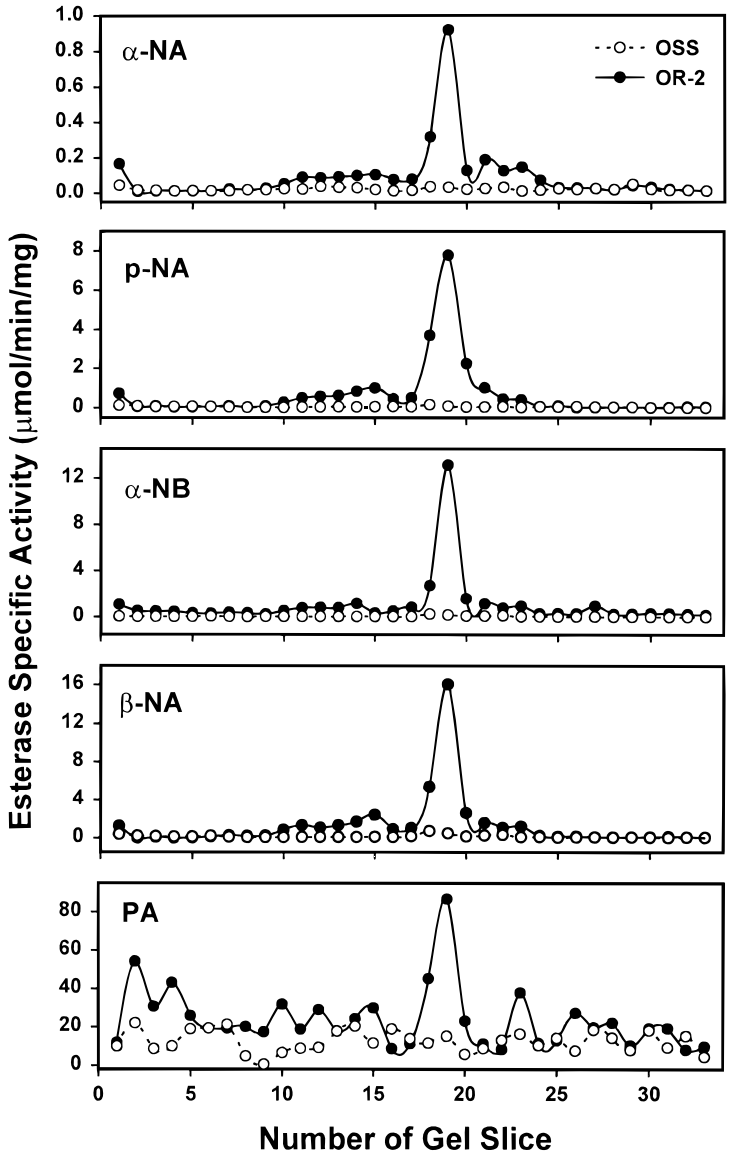

FIG. 5. Comparison of esterase profiles of the OSS and OR-2 greenbug clones. Gel slices Nos. 1–5 were from the stacking gel and the slices Nos. 6–33 were from the separating gel. The esterase activity was determined in the enzyme extraction from each gel slice using six selected esterase substrates (Fig. 1). Because the use of the substrate acetylthiocholine did not result in much enzyme activity in both the OSS and the OR-2 greenbug clones, those profiles are not presented.

to absence or extremely low levels of enzyme activity toward these OP compounds.

DISCUSSION

The interaction of esterases with OPs has long been accepted as a useful tool for the separation of esterases into different types (30). The A-esterases are not inhibited by OPs but degrade these insecticides as their substrates, whereas the B-esterases are inhibited readily by OPs (31). The C-esterases, the third type that was later added to the classification, do not interact with

TABLE 1
Comparison of Kinetic Parameters of Three Esterases Isolated from the OR-2 Greenbug Clone Using β-NA as a Substrate[a]

Esterase	K_m (μM)	V_{max} (μmol/min/mg)	$r(P)^b$
EST 4	40.9 ± 20.0 a	0.69 ± 0.18 a	>0.97 (<0.01)
EST 5	43.4 ± 8.8a	1.56 ± 0.40 b	>0.98 (<0.01)
EST 6	50.5 ± 21.7 a	0.96 ± 0.36 ab	>0.99 (<0.01)

[a] Results are the means ± SE of three determinations. Means within columns followed by the same letter are not significantly different ($P > 0.05$) using Duncan's multiple range test (48).
[b] Regression coefficient (r) and significance of the r value (P).

OPs (32). More recently, however, the name arylesterase (EC 3.1.1.2) was recommended for the esterases hydrolyzing the substrate PA to phenol and acetate (33, 34). The arylesterase can be either unaffected or inhibited by OP compounds in a time-independent manner (33). In contrast, the name phosphoric triester hydrolase was introduced for all esterases capable of hydrolyzing OP compounds. These esterases include paraoxonase (EC 3.1.8.1) and DFPase (EC 3.1.8.2) (35).

Based on the new criteria as outlined above, the PA-hydrolyzing esterases found in the OR-2 greenbugs should be classified as arylesterases or arylesterase-like esterases because they utilized PA as their preferred substrate. For the OR-2 greenbug clone, the peak PA-hydrolyzing activity of the esterases recovered from gel slice No. 19 was 5.4-, 6.6-, 11-, and 94-fold higher than the peak activities in hydrolyzing β-NA, α-NB, p-NA, and α-NA, respectively (Fig. 5). These results explain our earlier observation as to why the maximal velocity of the esterases from the OR-2 greenbugs was only 2.4-fold higher for α-NA but was 12-fold higher for PA compared to that of esterases from the OSS greenbugs (12).

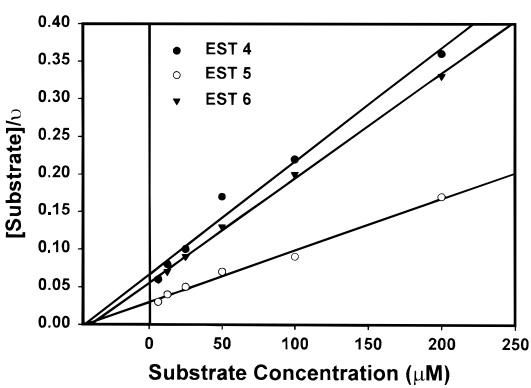

FIG. 6. Hanes plots of [s] vs [s]/v for three esterases isolated from nondenaturing PAGE for the OR-2 clone using β-NA as a substrate. Each point represents the mean of three replicate reactions ($n = 3$). All regression coefficients (r) > 0.97 ($P < 0.01$).

When paraoxon and chlorpyrifos-oxon were used as substrates, the arylesterase-like esterases from the OR-2 greenbugs did not show any detectable activity, probably because of extremely low or no OP-hydrolyzing activity. In fact, these esterases were highly sensitive to inhibition by paraoxon in crude enzyme preparations (12), PAGE-fractionated esterases (Fig. 7), and purified esterases (16). All these findings clearly indicate that the elevated arylesterase-like esterases in the OR-2 greenbugs are different from those phosphotriesterases found in other insecticide-resistant insects (17, 36). For example, a methyl paraoxon hydrolase purified from the tobacco budworm (*Heliothis virescens*) exhibited very low sensitivity to inhibition by methyl paraoxon and relatively high efficiency in hydrolyzing it as a substrate (36). Thus, the elevated arylesterase-like esterases in the OR-2 greenbugs appeared to be arylesterases that were sensitive to inhibition by OP compounds (33).

Although the elevated esterases in the OR-2 greenbugs had some of the important characteristics of arylesterases described by Aldridge *et al.* (33), several questions remain as to the properties of these esterases. First, these arylesterase-like esterases could be slightly or moderately activated by the metal ion Ca^{2+} (Fig. 3). Such a phenomenon has been found commonly in

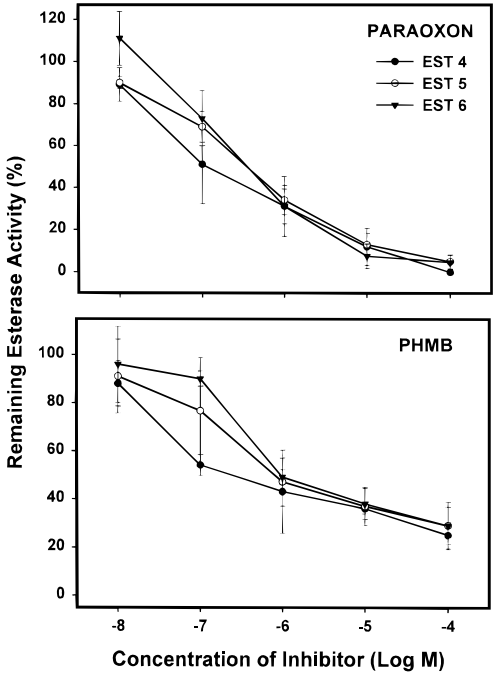

FIG. 7. Inhibition of three esterases by selected esterase inhibitors (paraoxon and PHMB). Each point represents the mean of three replicate inhibition reactions ($n = 3$). The IC_{50} values were estimated to be 0.2, 0.4, and 0.4 μm for paraoxon and 0.9, 2.6, and 4.3 μM for PHMB, for EST 4, EST 5, and EST 6, respectively.

mammalian paraoxonase (33, 37). Human and rabbit paraoxonases appear to have two distinct Ca^{2+}-binding sites, one required for stability and one required for catalytic activity, with different affinities (37). However, we do not know the mechanism by which Ca^{2+} can enhance the arylesterase-like esterase activity in the OR-2 greenbugs.

Second, the arylesterase-like esterases from the OR-2 greenbugs were highly sensitive to inhibition by the nonionic detergent Triton X-100 at 0.005–0.025% (v/v) (Fig. 2). This property also has been documented in phosphotriesterases of the bacterium *Flavobacterium* sp. However, the bacterial phosphotriesterases were able to efficiently hydrolyze paraoxon as a substrate (38), whereas the greenbug enzymes were not. We do not know whether having one response in common implies any structural similarity between the two groups of esterases.

Finally, the arylesterase-like esterases were highly sensitive to inhibition by both paraoxon and PHMB. High sensitivity of these esterases to paraoxon might explain why they did not show any detectable hydrolytic activities toward paraoxon and chlorpyrifos-oxon. On the other hand, high sensitivity of these esterases to inhibition by PHMB suggested the presence of sulfhydryl group(s). Such groups have been found commonly in phosphoric triester hydrolases, including the *Flavobacterium* phosphotriesterases (38) and mammalian paraoxonase (37). Nevertheless, a free sulfhydryl group is not required for either paraoxonase or arylesterase activity (37), and the presence of sulfhydryl groups does not seem to guarantee hydrolytic activity toward paraoxon as suggested in this study.

All these characteristics of the elevated esterases in the OR-2 greenbug clone indicated that they were not carboxylesterases, although they were highly sensitive to OP compounds such as paraoxon. However, these esterases possessed some properties of phosphoric triester hydrolases such as paraoxonase. Similar results have been found in birds (39). Among 14 species of birds, all showed considerable arylesterase

TABLE 2
Chlorpyrifos-Oxon Hydrolyzing Activity in Rabbit Serum and an OP-Resistant Colony of the Lesser Grain Borer in Contrast to Five Elevated Esterase Isozymes from the OR-2 Clone of the Greenbug[a]

Enzyme source	Specific activity (nmol/min/mg)
Rabbit serum	244.3 ± 45.5
Lesser grain borer	28.5 ± 2.3
EST 4	ND[b]
EST 5	ND
EST 6	ND
EST 7	ND
EST 8	ND

[a] Results are the means ± SE of three determinations, each with three replicates.

[b] Not detectable.

activity toward the substrate PA, but only 4 species displayed low activity toward the substrates paraoxon and pirimiphos-methyloxon, and the remaining 10 species did not show any detectable phosphoric triester hydrolase activity. Apparently, the elevated esterases in the OR-2 clone of the greenbug are arylesterases or arylesterase-like esterases with no detectable hydrolytic activity toward paraoxon and chlorpyrifos-oxon, although the OR-2 clone was highly resistant to their parent compounds, parathion and chlorpyrifos (J.-R. Gao and K. Y. Zhu, unpublished).

Esterases-mediated insecticide resistance has been documented in many insect species. In peach–potato aphid (*Myzus persicae*) and southern house mosquito (*Culex quinquefasciatus*), increased carboxylesterase activities are due to the overproduction of unaltered enzymes as a result of gene amplification (40–44). These enzymes are B-esterases with a very limited ability to hydrolyze the insecticide esters. They cause insecticide resistance primarily via sequestration of insecticides by large amounts of esterases present in resistant insects (44, 45). In contrast, OP resistance in the sheep blowfly (*Lucilia cuprina*) is associated with the loss of carboxylesterase (E3) activity determined using standard esterase substrates as a result of a single amino acid substitution ($Gly^{137} \rightarrow Asp$) in the enzyme (46). Such a substitution allows the E3 to acquire OP-hydrolyzing activity, leading to insecticide resistance in the sheep blowflies.

In the greenbug, however, the role of the arylesterase-like esterases in conferring OP resistance in the OR-2 clone is still unclear. Although biochemical studies have suggested an overproduction of an esterase that might be involved in insecticide sequestration (8), immunological assays indicate that the esterase is produced in equal quantities in the resistant and susceptible greenbugs (47). Our study clearly shows that these esterases possess certain characteristics resembling phosphoric triester hydrolase. However, they are not able to hydrolyze paraoxon and chlorpyrifos-oxon as substrates despite the fact that these insects showed 54- and 327-fold resistance to chlorpyrifos and parathion, respectively (J.-R. Gao and K. Y. Zhu, unpublished). Thus, the elevated arylesterase-like esterases identified in the OR-2 clone appeared to contribute to OP resistance by sequestrating OP insecticides. Molecular analysis of these esterases will be necessary to better clarify their nature and roles in conferring OP resistance in OR-2 greenbugs.

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