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Partial purification and characterization of a methyl-parathion resistance-associated general esterase in *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)

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Abstract

Increased hydrolytic metabolism of organophosphate insecticides has been associated with resistance among Nebraska western corn rootworm populations. In this study, resistance-associated esterases were partially purified by differential centrifugation, ion exchange, and hydroxyapatite column chromatography, with a final purification factor of 100-fold and recovery of approximately 10%. Kinetic analysis of the partially purified enzyme indicated that the K_m of the group II esterases was identical for the two populations, although V_{max} was consistently threefold higher in the resistant population. A putative esterase, DvvII, was further purified to homogeneity by preparative polyacrylamide gel electrophoresis. DvvII is a monomer with a molecular weight of approximately 66 kDa, although three distinct isoforms with similar p*I*s were evident based on isoelectric focusing gel electrophoresis. Immunoassays with the *Myzus persicae* E4 antiserum indicated that group II esterases from *D. v. virgifera* were cross-reactive and expressed at much higher titers in the resistant population relative to the susceptible counterpart. These results suggest that the resistance is likely associated with overproduction of an esterase isozyme in resistant *D. v. virgifera* populations.
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Keywords: Esterase; Insecticide resistance; Purification; Characterization; *Diabrotica virgifera virgifera*

1. Introduction

The western corn rootworm, *Diabrotica virgifera vigifera* LeConte, is a serious pest of field corn, *Zea mays* (L.) in the United States [1,2]. Crop rotation and chemical control have been the primary management strategies [2], although *D. v. virgifera* has become increasingly difficult to control because of its remarkable ability to adapt to these management practices. Chlorinated hydrocarbons were the first synthetic

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insecticide class to be introduced in Nebraska for larval control in the late 1940s [3]. However, as a consequence of their broadcast application and extreme persistence, resistance to cyclodienes was detected in less than a decade [3,4].

In certain areas of the Platte Valley of south central Nebraska, adult rootworm control was adopted after the development of cyclodiene resistance [5]. Microencapsulated methyl-parathion (Penncap-M, Elf Atochem North America, Philadelphia, PA) has been the most commonly used organophosphate insecticide to control adult *D. v. virgifera* in Nebraska because of its low cost and relatively long persistence. Penncap failures were first reported in the 1990s [5], and preliminary studies implied the involvement of hydrolytic metabolism in resistant rootworm populations [6]. In vivo metabolism experiments with ^{14}C-labeled ethyl-parathion, synergism bioassays with the esterase inhibitor DEF[1] (*S*,*S*,*S*-tributyl phosphorotrithioate), and esterase activity assays with model substrates suggested a common esterase-mediated resistance in both larvae and adult *D. v. virgifera* [6,8–11].

Recent investigations have identified three distinct esterase isozyme groups (I, II, and III) from *D. v. virgifera* based on their mobility in native polyacrylamide gel electrophoresis (PAGE) [11]. Elevated activity of group II esterase isozymes is highly correlated with resistance and the activity of these enzymes is strongly inhibited by a variety of organophosphate and carbamate insecticides [7, unpublished]. These enzymes have provided a reliable biochemical marker for detection of methyl-parathion resistance in individual *D. v. virgifera* adults [10]. In this investigation, we report the purification and characterization of the methyl-parathion resistance-associated group II esterases and provide a basis for defining a molecular mechanism of resistance.

[1] *Abbreviations used:* DEF, *S*,*S*,*S*-tributyl phosphorotrithioate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; IEF, isoelectric focusing; PNPA, *p*-nitrophenyl acetate; Mops, 3-(*N*-morpholino) propanesulfonic acid; PVDF, polyvinyldiene difluoride.

2. Materials and methods

2.1. D. v. virgifera populations

Two populations were collected in Nebraska during the summer of 1998 and 1999. The Saunders County population was collected from areas where adult management is not practiced and has previously been shown to be highly susceptible to methyl-parathion. The resistant Phelps County population originated from an area where continuous corn production and adult control practice have been used for more than two decades and where field control failures have been reported. Topical bioassays with methyl-parathion indicated a 16.4-fold level of resistance in the Phelps County population relative to the Saunders County population [5]. In addition, the Saunders and Phelps County populations exhibited 97.0 and 7.0% mortality, respectively, after exposure to a diagnostic concentration of methyl-parathion [10]. Field populations were collected before seasonal application of adulticides had been initiated. The field populations were maintained in the laboratory at ambient temperature on a 14:10 h (L:D) photoperiod and were fed on a standard diet of head lettuce and sweet corn before being harvested and stored at −80 °C.

2.2. Chemicals

DEAE-Sepharose, hydroxyapatite, and all other reagents and solvents were purchased from Sigma Chemical (St. Louis, MO) unless stated otherwise. Electrophoresis reagents and ampholytes were purchased from Bio-Rad Laboratories (Hercules, CA). Protein assay reagents and bovine serum albumin were purchased from Pierce (Rockford, IL).

2.3. Protein and esterase assays

Protein concentration was determined with a commercially available bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as the standard. During purification, esterase activity was estimated by colorimetric measurement of α-naphthol formation from

α-naphthyl acetate. The approach of Van Asperen [12] was adapted to a 96-well microplate format. The assay mixture contained 188 μl of 0.02 M sodium phosphate buffer (pH 7.0), 2 μl 25 mM α-naphthyl acetate (in acetone), and 10 μl enzyme preparation. The reaction was stopped by the addition of 33.2 μl of fast blue BN (tetrazotized *O*-dianisidine) (0.3% in 3.5% sodium lauryl sulfate). The plate was incubated at 30 °C for 15 min, the absorbance was recorded at 600 nm using a Bio Kinetics Reader (BIO-TEK, Winooski, VT). Formation of product was determined from a standard curve of α-naphthol.

2.4. Electrophoresis

Continuous non-denaturing PAGE was performed in a vertical Mini Protean II electrophoresis apparatus (Bio-Rad, Richmond, CA) using a 7.5% separating gel and 4% stacking gel with a continuous Tris/glycine running buffer system (50 mM, pH 8.3). Samples were diluted 1:1 with 2× sample buffer [20% sucrose (w/v), 0.1% bromophenol blue in 50 mM Tris/glycine running buffer (pH 8.3)]. Gels were run at 120 V constant voltage for 75 min at 4 °C. Esterase bands were then visualized by incubating the gels in 100 ml of 0.02 M sodium phosphate buffer (pH 7.0), with 2% (v/v) 30 mM α-naphthyl acetate dissolved in acetone at 30 °C for 15 min before adding 0.04% (w/v) fast blue BN salt (tetrazotized *O*-dianisidine).

Denaturing SDS (sodium dodecyl sulfate)–PAGE was used to examine protein composition of crude homogenates and column fractions during the purification process. SDS–PAGE was performed along with molecular size standard proteins (Kaleidoscope Prestained Standards; M_r, 6700–205,000; Bio-Rad, Hercules, CA) using a Bio-Rad Mini-Protean II electrophoresis unit and a 10% acrylamide concentration as described by Laemmli [13]. Coomassie brilliant blue G was used for staining protein on SDS–PAGE gels. The molecular weight of purified esterase was determined using a calibration curve of the protein standards.

Isoelectric focusing electrophoresis (IEF) was performed on a vertical unit (Model MGV-100, Scientific, Del Mar, CA) using a 7.5% polyacrylamide gel with narrow pH range ampholytes (5–7). Gels were run at ambient temperature and at 200 V for the first 2 h followed by 400 V for an additional 2 h. Esterase bands were visualized by the method previously described for non-denaturing PAGE. In order to determine the pH values at various points across the gel, an empty lane (50 mm in width) adjacent to the sample was excised and used as a pH ladder. Ten evenly spaced segments (2 mm × 50 mm) from the pH ladder were excised and placed in 0.5 ml double distilled water for at least 30 min. The pH of the ampholytes eluted from each segment was measured by an AR15 pH meter (Fisher Scientific, Bohemia, NY), which was standardized with a buffer of known pH (Fisher Certified Buffer). Sample lanes were stained with esterase activity as described previously, and the p*I* of each isozyme band was determined based on a calibration curve of pH versus distance from the cathode [14].

2.5. Enzyme purification

Unless stated otherwise, all purification procedures were performed at 4 °C. An initial purification involving differential centrifugation was employed since the group II esterase isozymes are primarily cytosolic in nature [11]. Abdomens of 20 rootworm beetles (stored at −80 °C) were homogenized in 1.5 ml ice-cold 0.1 M sodium phosphate homogenization buffer (pH 7.8) with a Potter–Elvehjem homogenizer. The homogenate was centrifuged in an Eppendorf microcentrifuge (Brinkman Instruments, Westbury, NY) at 10,000*g* for 20 min. The 10,000*g* supernatant was filtered through glass wool to remove lipids and centrifuged in a Beckman Optima TLX-1 M-1 ultracentrifuge (Allendale, NJ) at 100,000*g* for 1 h. The resulting supernatant was applied to a DEAE-Sepharose ion exchange column (Pharmacia 1.6 cm ID × 40 cm length) equilibrated with 30 mM MOPS [3-(*N*-morpholino) propanesulfonic acid] buffer (pH 8.0). Esterase activity was eluted with a salt gradient (0–0.5 M NaCl in MOPS buffer) at 1 ml/min for 100 min. Fractions of 2 ml were collected and elution profiles determined for esterase activity and protein. Peak fractions were assayed by non-denaturing PAGE, and the

fractions containing group II esterase isozymes were combined and concentrated in an Amicon 8050 stirred cell (Beverly, MA) with a 10,000 kDa ultrafiltration membrane. The concentrated fraction was then desalted with 30 mM Mops buffer (pH 8.0) in the same stirred cell.

The desalted and concentrated ion exchange fraction was applied to a hydroxyapatite column (Pharmacia 1 cm ID × 12 cm length) equilibrated with 10 mM sodium phosphate buffer (pH 6.8). Group II esterase isozymes were eluted with a phosphate gradient (10–500 mM, pH 6.8) at 1 ml/min for 70 min and fractions of 1 ml were collected. Elution profiles were determined for esterase activity and protein. Hydroxyapatite chromatography was performed at ambient temperature. Esterase activity was eluted in one major peak, and peak fractions were examined by non-denaturing PAGE. The fractions containing group II esterase activity were combined, concentrated, and exchanged with 30 mM MOPS buffer (pH 8.0) as previously described.

The concentrated hydroxyapatite fraction was further concentrated to less than 0.1 ml with NANOSEP microconcentrators (10,000 kDa cut-off, Pall, Ann Arbor, MI), and subjected to preparative electrophoresis. The concentrated sample was examined by SDS–PAGE and protein bands were visualized by zinc staining (Bio-Rad, Hercules, CA). The bands of interests were excised, destained, and then eluted overnight with SDS running buffer in a Bio-Rad Model 422 Electro-Eluter (Bio-Rad, Hercules, CA). Eluted samples were dialyzed for 24 h against three changes of 5 mM Tris buffer (2 L) in individual Slide-A-Lyzer Dialysis Cassettes (10,000 kDa cutoff, Pierce, Rockford, IL). The dialyzed samples were concentrated in a Centrivap Concentrator (Labconco, Kansas City, MO) and the purity examined by SDS–PAGE.

2.6. Kinetic analysis

Michaelis–Menten constants (K_m) and maximal velocities (V_{max}) for the partially purified group II esterase (post-ion exchange fraction) from resistant and susceptible populations were determined by measuring activities toward a series of *p*-nitrophenyl acetate (PNPA) concentrations (2.6–168.8 mM). The 210 μl assay mixture consisted of 5 μl PNPA in acetonitrile, 195 μl of 0.02 M sodium phosphate buffer (pH 7.0), and 10 μl enzyme preparation. The assay was initiated by the addition of PNPA, and the change in absorbance was recorded at 405 nm. An extinction coefficient of 6.53 $mM^{-1} cm^{-1}$ was used to convert the absorbance in mOD/min to nanomoles of *p*-nitrophenol produced per minute.

2.7. Gel filtration

After ion exchange chromatography, the native molecular weight of the group II esterase was determined using a Superose 12 HR 10/30 column (LKB Pharmacia, Bromma, Sweden) equilibrated with 20 mM Mops buffer (pH 8.0). The column was calibrated at 4 °C with standard proteins (M_r, 12,400–160,000) (LKB Pharmacia, Bromma, Sweden). A 0.2 ml sample volume was applied with a flow rate of 0.4 ml/min and fractions of 0.4 ml were collected and elution profiles were determined for esterase activity and protein. The native molecular weight corresponding to the group II esterases was determined from a standard curve of the elution volume of standard proteins plotted against the logarithmic value of their molecular weight.

2.8. Western blotting

The polyclonal antiserum prepared from the E4 esterase of *Myzus persicae* (provided by A. Devonshire, IRAC Rothamsted, UK) was tested for cross-reactivity with the *D. v. virgifera* group II esterase by Western blotting. SDS–PAGE was performed in a vertical Mini Protean II electrophoresis apparatus using a 10% separating gel and 4% stacking gel. Individual wells were loaded with equal amounts of proteins and gels were run at constant 100 V for 90 min at ambient temperature. Each gel was then transferred to a polyvinyldiene difluoride (PVDF) membrane (Millipore, Boston, MA) and probed with a polyclonal antiserum to *M. persicae* E4. Transfer was made using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad, Hercules, CA) with 120 mM Tris, 40 mM glycine, and 20% methanol (v/v) as transfer buffer. Procedures for probing the blot with primary and

secondary antibody were according to instructions for the Western-Light and Western-*Star* System, a chemiluminescent immunoblot detection system with CDP-*Star* substrate (Applied Biosystems, Bedford, MA). The chemiluminescence was visualized with a Fluor-S Multiimager (Bio-Rad, Hercules, CA).

2.9. Amino acid sequence analysis

Approximately 100 pmol of the post-preparative electrophoresis fraction, partially purified group II esterases, were subjected to 10% SDS–PAGE and electroblotted onto PVDF membrane (Millipore, Bedford, MA). The blotted proteins were submitted to the W.M. Keck Foundation, Biotechnology Resource Laboratory, Yale University and analyzed by automated Edman degradation methods to determine NH_2-terminal amino acid sequences of the putative group II esterase.

2.10. Statistical analyses

Standard curves for protein, α-naphthol, molecular weight, and isoelectric focusing point were determined by least squares linear regression analysis.

3. Results

3.1. Purification of a group II esterase

Data from a representative purification of resistance-associated esterases are provided in Table 1. Ion exchange chromatography resulted in approximately 10-fold purification with 56.6% recovery. The ion exchange elution profiles obtained from the resistant and susceptible populations (Fig. 1) indicated similar chromatographic behavior for protein and esterase activity in both strains although the activity peak corresponding to group II esterases was significantly larger from the resistant population. These results are consistent with denaturing PAGE results, in which peak fractions containing group II esterase activity from the resistant population were elevated relative to the susceptible population (Figs. 2A and B). Analysis of esterase isozyme composition by non-denaturing PAGE revealed that the three peak fractions corresponded to the three esterase groups previously identified. The group I esterases did not appear on the separating gel (Fig. 2A) because the majority of their activity remained in the stacking gel [10,11]. Hydroxyapatite column chromatography gave one major activity peak, which corresponded to the group II esterases based on non-denaturing PAGE (data not shown) and resulted in an additional 11-fold purification with 9.89% recovery.

Post-hydroxyapatite fractions containing group II esterases were subjected to preparative SDS–PAGE to isolate individual proteins. Examination of peak fractions from both ion-exchange and hydroxyapatite chromatography by SDS–PAGE revealed three protein bands of approximately 60, 25, and 20 kDa that exhibited increased staining intensity in the resistant strain (Fig. 3), in which a single protein band of expected size (ca. 60 kDa) was identified as the putative group II esterase based on molecular weight of other resistance associated esterases (Table 2) and cross-reactivity with the E4 esterase antiserum from *M. persicae* (see below). The 60 kDa band was purified to homogeneity following preparative electrophoresis, zinc staining, excision, destaining, and electroelution. The pure protein, referred to as DvvII,

Table 1
Purification of group II esterase from a resistant *D. v. virgifera* population

Purification step	Protein (mg)	Specific activity[a]	Units[b]	Recovery (%)	Purification factor
350,000*g* supernatant	28.29	397.82	11254.33	100.00	1.00
DEAE-Sepharose column	1.62	3946.13	6373.00	56.63	9.92
Hydroxyapatite column	0.08	42393.65	3338.50	9.89	106.56

[a] Specific activity is expressed as nmol/min/mg protein.
[b] Units are expressed as nmol/min.

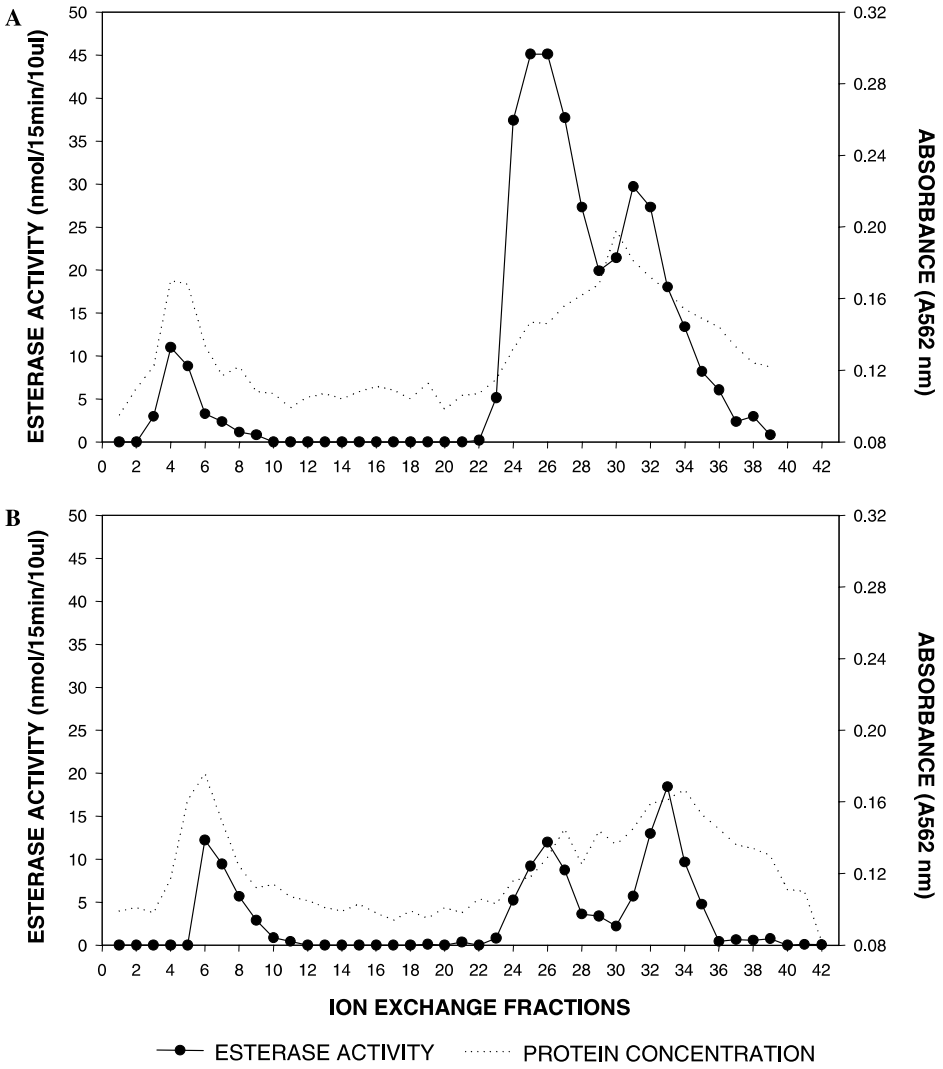

Fig. 1. Elution profiles for protein and esterase activity using α-naphthyl acetate as substrate and a DEAE-Sepharose ion exchange column. (A) Resistant population. (B) Susceptible population. See text for details of chromatography.

was concentrated for subsequent amino terminal sequencing. The purity and molecular weight of DvvII as determined by SDS–PAGE after the final purification procedure is presented in Fig. 3.

3.2. Physical properties of group II esterase

Isoelectric focusing of the partially purified group II esterases revealed three different esterase isozymes in the resistant population with isoelectric points (p*I*) of 5.32 ± 0.04, 5.21 ± 0.01, and 5.07 ± 0.02, respectively (Fig. 4). The monomeric molecular weight of the putative group II esterase, DvvII, estimated by SDS–PAGE was 65.6 ± 3.3 kDa (mean ± SE, $n = 3$). The mass of the native form of this group II esterase estimated by gel filtration chromatography was 65.6 kDa ($R^2 = 0.99$, data not shown). These data in combination suggest that the native form of this esterase is monomeric.

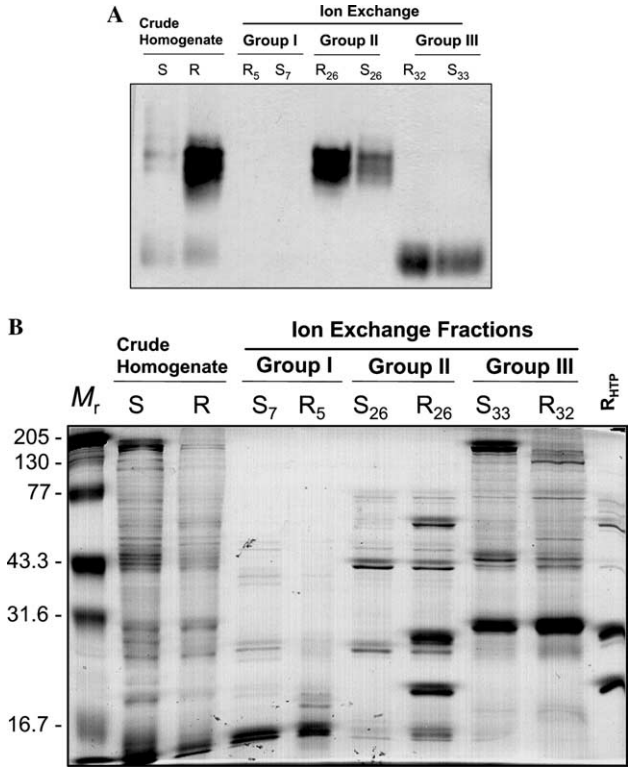

Fig. 2. (A) Non-denaturing PAGE stained for esterase activity toward α-naphthyl acetate of crude homogenates and three ion exchange peak fractions (Group I, II, and III) from both susceptible (S) and resistant (R) populations and (B) SDS–PAGE stained with Coomassie blue of peak ion exchange fractions (Group I, II, and III) from both susceptible (S) and resistant (R) populations and the resistant hydroxyapatite fraction (R_{HTP}).

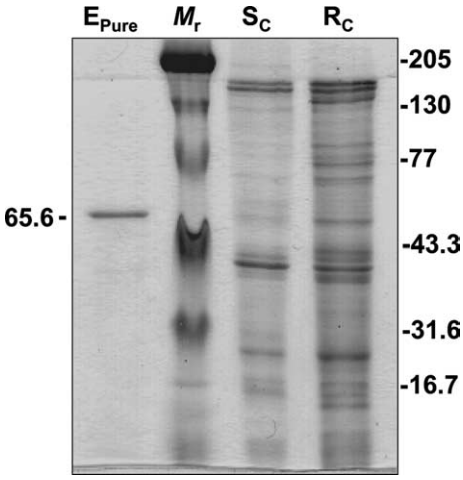

Fig. 3. Molecular weight of the partially purified group II esterase on SDS–PAGE. M_r, molecular mass marker; E_{PURE}, purified group II esterase; Sc and Rc, crude homogenates from the susceptible and resistant populations, respectively.

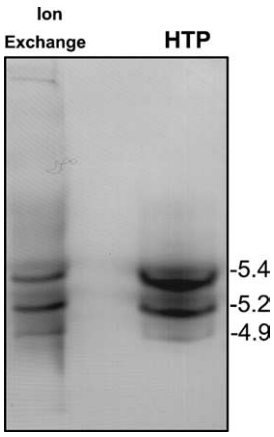

Fig. 4. Isoelectric point (p*I*) of the native group II esterase from a resistant *D. v. virgifera* population. Peak fractions from ion exchange chromatography and hydroxyapatite chromatography (HTP) used as enzyme source.

Table 2
Physical properties of insect esterases from selected resistant species

Insect species/esterases	Order	p*I*	M_r (kDa), native	M_r (kDa), denatured	Molecular form	References
Culex quinquefasciatus A2	Diptera	5.2	67.0	67.0	Monomer	[31]
Culex quinquefasciatus A2	Diptera	NA*	58.0–67.0	58.0–67.0	Monomer	[32]
Culex quinquefasciatus B2	Diptera	5.0	60.0	60.0	Monomer	[33]
Culex quinquefasciatus B2	Diptera	5.0	NA	63.0	Monomer	[34]
Culex quinquefasciatus Est β1	Diptera	4.8	66.0	66.0	Monomer	[35]
Culex tarsalis						
Susceptible	Diptera	6.8	59.0	NA	NA	[36,37]
Resistance		6.8/6.2	59.0	NA	NA	
Culex pipiens pallens	Diptera	4.6	NA	66.0	NA	[38]
Anopheles albimanus	Diptera	4.6	60.0	NA	NA	[39]
Lucilia cuprina	Diptera	5.5	60.5	60.5	Monomer	[40,41]
Myzus persicae						
E4	Homoptera	NA	65.0	57.0/65.0	Monomer	[42,43]
FE4		NA	66.0	58.0/66.0	Monomer	
Myzus persicae						
E4	Homoptera	NA	65.0	58.8	Monomer	[44]
FE4		NA	66.0	60.2	Monomer	
Myzus nicotianae	Homoptera	4.6	67.3	66.0	Monomer	[45]
Nilaparvata lugens E_1–E_3	Homoptera	4.7–4.9	NA	62.0–64.0	Monomer	[46]
Nilaparvata lugens	Homoptera	4.7–5.0	NA	66.0–68.0	NA	[30]
Laodelphax striatellus	Homoptera	4.7–5.3	NA	66.0–70.0	NA	[47]
Schizaphis graminum Type II	Homoptera	4.8	102.0	52.0/56.0	Dimer	[26]
Pseudoplusia includens	Lepidoptera	4.6–4.8	NA	80.0	NA	[48]
Leptinotarsa decemlineata	Coleoptera	4.8	46.0–48.0	46.0–48.0	Monomer	[49,50]
		4.5	57.0–59.0	30.0–33.0	Dimer	
Leptinotarsa decemlineata	Coleoptera	6.2	NA	NA	NA	[51]
Tribolium castaneum						
Resistant	Coleoptera	7.3	62.0	NA	Monomer	[52]
Susceptible		6.6	61.7	NA	Monomer	
Oryzaephilus surinaemensis	Coleoptera	NA	130.0	65.0	Dimer	[53]
Oryzaephilus surinaemensis	Coleoptera	NA	NA	71.0	Monomer	[54]
Diabrotica virgifera virgifera	Coleoptera	5.1–5.3	65.6	65.6	Monomer	This study
Nephotettix cincticeps E1–E4	Hemiptera	4.8–5.1	NA	E3: 58.6	NA	[55]
Anisopteromalus calandrae	Hymenoptera	5.2	NA	NA	NA	[56]
Blatella germanica E6	Dictyoptera	4.8	55.0	55.0	Monomer	[57]

*NA, not available.

3.3. Kinetic analysis

Results of kinetic analysis of partially purified group II esterases from the resistant and susceptible population are presented in Fig. 5. Similar K_m values were obtained for the two populations although the V_{max} was consistently two- to threefold higher in the resistant population. K_m and V_{max} values for the resistant and susceptible population were 8.40×10^{-4} M and 1.09 μmol/min/ml, and 7.39×10^{-4} M and 0.304 μmol/min/ml, respectively. Similar K_m values indicate that enzymes from susceptible and resistant populations interact similarly with the model substrate, *p*-nitrophenyl acetate. The higher V_{max} of the resistant strain suggests that there are greater amounts of the group II esterase in the resistant strain.

3.4. Cross-reactivity of *M. persicae* E4 antiserum with *D. v. virgifera* group II esterases

Fig. 6 shows that the *M. persicae* E4 antiserum was cross-reactive with a group II esterase isozyme weighing approximately 66 kDa in size from both

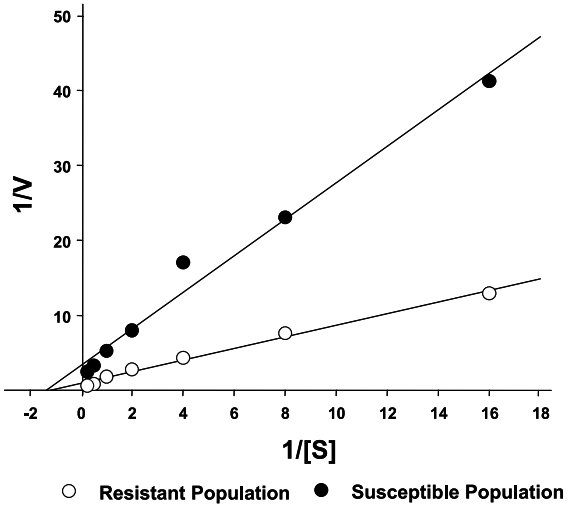

Fig. 5. Linerweaver–Burk plot of partially purified group II esterases from the resistant and susceptible populations.

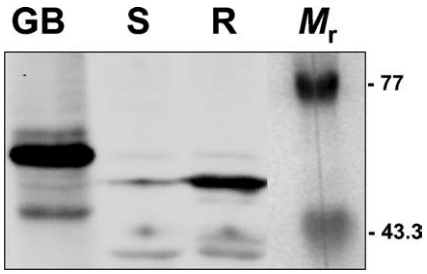

Fig. 6. Cross-reaction of *D. v. virgifera* group II esterases with *M. persicae* E4 polyclonal antiserum. GB, type I Greenbug, *S. graminum* (a positive control for *M. persicae* E4 antiserum); R, resistant Phelps County population, NE; S, susceptible Saunders County population, NE;. M, Kaleidoscope Prestained Standards (Bio-Rad, Hercules, CA).

resistant and susceptible *D. v. virgifera*. However, the enzyme preparation from the resistant population exhibited much stronger signal intensity than preparations from the susceptible counterpart. A crude homogenate prepared from the greenbug, *Schizaphis graminum*, was used as a positive control as it was previously shown that greenbug esterase isozymes exhibit strong cross-reactivity with *M. persicae* E4 antiserum [15].

3.5. N-terminal amino acid analysis

The first 16 N-terminal amino acid residues of the putative group II esterase DvvII were identified as: Asp, Ile, Val, Leu, Asn, Lys, Lys, Arg, Phe, Pro, Lys, Asp, Phe, Met/Gly, Phe, and Gly. Comparisons of these 16 residues with N-terminal regions from other resistance-associated insect esterase revealed little or no homology with other known esterase sequences (Fig. 7).

Species	**1**				**5**					**10**					**15**	
Hva1	D	D	E	-	R	E	**V**	R	T	A	Q	**G**	P	L	R	**G**
CuB1	S	L	E	S	L	T	**V**	Q	T	K	Y	**G**	P	V	R	**G**
DmE6	T	D	D	P	L	L	**V**	Q	L	P	Q	**G**	K	L	R	**G**
MpE4/FE4	-	S	N	T	P	K	**V**	Q	V	H	S	**G**	E	I	A	**G**
BesB	-	-	E	S	P	R	**V**	T	V	K	H	**G**	T	L	-	**G**
N1-Est1	N	S	V	P	V	V	H	D	T	A	S	**G**	D	L	S	**G**
GbT2Est (56)	-	-	N	P	-	V	**V**	R	I	T	N	**G**	A	I	R	**G**
HH012f/MH130f	E	N	D	F	P	F	**V**	S	T	K	L	**G**	D	I	K	-
7012/malRR	-	N	D	F	P	F	**V**	S	T	K	L	-	G	I	GA	-
DvvII	D	I	V	L	N	K	K	R	F	P	K	D	F	M/G	F	**G**

Fig. 7. Comparison of the first 16 N-terminal sequences of the *D. v. virgifera* group II esterase DvvII with other amino terminal ends from: *Heliothis virescens* (Hva1), *Culex* spp. esterase B1 (CuB1), *Drosophila melanogaster* esterase-6 (DmE6), *Myzus persicae* E4 and FE4 (MpE4/FE4), *Bombyx mori* blood esterase B (BesB), *Nilaparvata lugens* esterase (N1-Est1), 56 kDa subunit of Type II *Schizaphis graminum* esterase [GbT2Est (56)], and esterases from two different organophosphate-resistant *Oryzaephilus surinamensis* populations (HH012f/MH130f, and 7012/malRR). The sequences were aligned by hand using results of PILEUP analysis (Wisconsin Package Version 10.2, Genetics Computer Group, Madison, WI). Letters in bold denote amino acid residues conserved among the 10 sequences.

4. Discussion

A putative *D. v. virgifera* group II esterase, DvvII, was partially purified by the combined techniques of differential centrifugation, ion exchange chromatography, hydroxyapatite chromatography, and preparative SDS–PAGE. The molecular form of DvvII is consistent with most resistance-associated esterases from other insect species (Table 2). The isoelectric point as well as the molecular weight estimated by both SDS–PAGE and gel filtration lie within the range of pH 4–7 and 55–70 kDa in size, respectively, which are typical of other insect esterases (Table 2) [16–18]. Although DvvII has similar physical properties with many other resistance-associated esterases (Table 2), it exhibits little homology at the N-terminal sequence. However, it should be noted that an esterase isolated from another organophosphate-resistant Coleopteran, *Oryzaephilus surinamensis*, also exhibits little homology with N-terminal sequences from other insect esterases [19].

The combined results of this investigation indicate that the differences in group II esterase activity between resistant and susceptible *D. v. virgifera* populations are the result of quantitative differences in the amount of enzymes [20–25]. Following purification, a 66 kDa band was clearly visible after Coomassie staining in the resistant population, but barely visible in the susceptible population. The difference in intensity between populations indicates a quantitative mechanism of resistance involving over-production of the same enzyme that is present in susceptible insects. This conclusion is supported by results of western blotting using the E4 antiserum of *M. persicae* where increased signal intensity of the 66 kDa band was observed in the resistant strain. The cross-reactivity exhibited between the 66 kDa band from both resistant and susceptible *D. v. virgifera* with *M. persicae* E4 antiserum suggests that the resistance associated esterase is not immunologically distinct from the susceptible esterase and the intensity difference is likely the result of the quantitative rather than qualitative modification of group II esterases [26–29].

If resistance were the result of a mutant ali-esterase with increased catalytic efficiency, qualitative differences in physical and chemical properties of the enzyme between the resistant and susceptible population should have been evident. Similarities between the resistant and susceptible population in chromatographic behavior, physical and immunological properties of group II esterases suggest the existence of identical esterases in both populations. However, it is still not clear whether the genetic basis of this esterase-mediated resistance is the result of multiple gene copies (i.e., gene amplification), or if the overproduction is caused by a change in the sequences of regulatory DNA outside the protein coding region that leads to increased protein synthesis.

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