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Species Distribution and Characterization Studies of Sex-Limited Esterases Within the *virgifera* Group of *Diabrotica* (Coleoptera: Chrysomelidae)

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Ann. Entomol. Soc. Am. 81(6): 954-963 (1988)

ABSTRACT Isozyme studies revealed the existence of an autosomal, male-limited esterase locus (*Est-m*) in species of the *virgifera* group of *Diabrotica* found in the United States. The locus is characterized by the presence of active and null alleles, and particular taxa are monomorphic or polymorphic for the presumptive *Est-m* variants. Investigations of the properties of an *Est-m* form present in the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, produced data to indicate that the enzyme is a carboxylesterase and a glycoprotein with a relative molecular weight of $78,000 \pm 8,680$ SD. Additional work has shown that *Est-m* activity is localized in the accessory glands of virgin, adult males 12-14 d old. Moreover, the enzyme is passed to females during mating. The molecular properties of *Est-m*, its sex-limited expression in the accessory glands of males, and its subsequent occurrence in mated females suggest strongly that it functions in some way in reproduction.

KEY WORDS Insecta, genetics, isozyme, electrophoresis

WE HAVE BEEN USING electrophoretic and gel staining techniques to gather data on enzyme variation among members of the *virgifera* group of the genus *Diabrotica* found in the United States; i.e., *D. barberi* Smith and Lawrence, *D. cristata* (Harris), *D. lemniscata* LeConte, *D. longicornis* (Say), *D. virgifera virgifera* LeConte, and *D. v. zea* Krysan and Smith. The studies have represented part of a broadly based biosystematics research effort designed to clarify the relationships of pest species and closely related taxa in support of population management programs.

Our early investigations of adult populations of *D. longicornis* revealed a variable region of esterase banding in which electromorphs were expressed only in zymograms of adult males. Appropriate tests of inheritance were then performed; they confirmed the existence of a polymorphic and sex-limited autosome locus (designated *Est-m*) characterized by the presence of both active and null alleles. Similar electromorphs were not detected during concurrent surveys of esterases in the sibling species *D. barberi*. Nevertheless, experimental hybridization tests conducted between *D. barberi* and *D. longicornis* produced data suggesting that a null allele of *Est-m* was fixed in

native *D. barberi* populations (McDonald et al. 1982). More extensive surveys of field-collected material subsequently demonstrated the occurrence of additional allelomorphs as well as geographic clines in allelomorph frequencies in *D. longicornis*. Moreover, presumptive *Est-m* electromorphs were noted in other *virgifera* group taxa. Collectively, the data on allelic distributions (i.e., geographic and interspecific patterns) provided a basis for hypothesizing that selection was important to the evolution and maintenance of the *Est-m* variation.

Consequently, studies of the *Est-m* locus were expanded to include work on the biological role(s) of these enzymes in or among individuals or populations. We have initially examined tissue distribution patterns and various molecular properties of a putative *Est-m* found in *D. v. virgifera*. These investigations have indicated strongly that the *Est-m* enzymes function in some way in reproduction and represent the primary topic of discussion in this report. Additionally, we document data on all of the presumed *Est-m* alleles detected during our electrophoretic surveys of the *virgifera* group complex. A complete account of alleles has not appeared previously, although McDonald et al. (1982) published some information related to laboratory populations of *D. longicornis* and *D. barberi*.

Materials and Methods

Insects. Field-collected and laboratory-reared adults were surveyed for the presence or absence

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of *Est-m* electromorphs in *D. barberi*, *D. cristata*, *D. longicornis*, *D. v. virgifera*, and *D. v. zeae*, whereas only field-collected material was used for work involving *D. lemniscata*. The field samples originated from various geographic locales within the range of each taxon. All collections were made on or near known host plants during periods of adult activity in the late summer or early fall. Beetles were usually sent live to the Northern Grain Insects Research Laboratory, USDA, at Brookings, S. Dak., where they were identified and sexed. The samples were then shipped on dry ice to Fargo, where they were stored at -80°C until electrophoretic analyses were performed. All laboratory-reared specimens were obtained through J. L. Krysan at the Brookings facility.

Diabrotica v. virgifera was chosen as a model for *Est-m* characterization studies because the species was found to be monomorphic for a single *Est-m* electromorph and is recognized as an important pest on corn (i.e., the western corn rootworm) throughout much of the United States cornbelt. Additionally, *D. v. virgifera* is routinely reared in large numbers for research purposes at the Brookings laboratory. Thus, it was possible to obtain samples of live beetles for our investigations throughout the year.

Sample Preparation for Electrophoresis. Whole bodies, body parts, or tissues of adult male or female beetles were generally ground in a sample buffer (SB) containing 0.03 M Tris-glycine and 20% sucrose, pH 8.3 (4°C). The SB volumes and the insect samples varied according to the goals of the experiments performed on the *Est-m* extracts. All homogenates were centrifuged one or two times at $1,300 \times g$ in a refrigerated centrifuge (4°C). The resulting supernatants contained the *Est-m* enzymes and were stored temporarily at -80°C or were used immediately for particular procedures.

Electrophoresis and Gel Staining. Vertical polyacrylamide gel electrophoresis (PAGE) was used in conjunction with histochemical staining to assay various insect extracts or supernatant fractions for esterase activity or general protein patterns. Electrophoresis was performed in ORTEC glass analytical cells (Ortec, Inc., Oak Ridge, Tenn.) using a 0.03 M Tris-glycine buffer (pH 8.3) and a gel system described by McDonald et al. (1975). Test samples were electrophoresed at 35 mA constant current. An extract of the house fly, *Musca domestica* L., was included as a control on all gels, and a particular band was then used to measure the relative mobilities (*Rm* values) of the *Diabrotica* esterase or other protein forms. The control extracts originated from previously characterized house fly strains derived from single fertilized females (I. McDonald, unpublished data). Bromophenol blue was incorporated in the upper buffer and served as a tracking dye. Runs were normally terminated after the dye traveled 60 mm into a separating gel with exceptions as noted in the text.

Gels were stained for esterase bands by the meth-

od of Narang et al. (1976), except that the dye components were dissolved in a 0.15 M Tris buffer (pH 7.42). The gels were incubated in the dye solution at 37°C until esterase bands were detected (15–30 min). A number of protocol variations involving Coomassie Blue R-250 or G-250 (Bio-Rad Laboratories, Richmond, Cal.) were used to stain gels for general protein (Dauterman et al. 1962, Diezel et al. 1972, Reisner et al. 1975, Blakesley & Boezi 1977, Steiner & Joslyn 1979, Reisner 1984). Additionally, protein bands were resolved using variations of a Bio-Rad silver stain kit procedure on gels initially stained with Coomassie Blue G-250 by the method of Steiner & Joslyn (1979). In some cases, gels assayed for esterase activity likewise were stained for general protein using the Steiner and Joslyn technique.

Spectrophotometric Assay of Esterase Activity.

Total esterase activity in tissue extracts containing *Est-m* was measured spectrophotometrically after the enzymatic hydrolysis of the colorless substrate *p*-nitrophenyl acetate (PNPA) to yield the colored product *p*-nitrophenol (PNP; Lunday & Farmer 1983). The extracts were prepared by homogenizing 25 pairs of male accessory glands in 0.4 ml of SB. The homogenate was centrifuged (15 min at $1,300 \times g$), and 20 μl of a 1:10 dilution of the supernatant in SB was added to 960 μl of 0.15 M Tris buffer (pH 7.42). Twenty microliters of 0.1 M PNPA dissolved in absolute methanol was then mixed with the enzyme preparation, and the change in absorbance was recorded at 410 nm during a 5-min period. Cuvettes containing 1.0 ml of the 0.15 M Tris buffer were used as blanks for the assay. Also, nonenzymatic hydrolysis of the substrate was measured in cuvettes containing 20 μl of the PNPA-methanol and 980 μl of the Tris buffer. Esterase activity was calculated from the Lambert-Beer formula using a molar extinction coefficient of $1.17 \times 10^4 \text{ M}^{-1} \text{ CM}^{-1}$. One unit of esterase activity was subsequently defined as the amount of esterase solution hydrolyzing 0.1 μM of PNPA per minute under the test conditions.

In vivo Distribution Studies of *Est-m* Activity.

Initial tests to determine the location of *Est-m* activity within the insect were conducted by dissecting the heads, thoraces, abdomens, antennae, and legs from five *D. v. virgifera* males 5–10 d old. Particular anatomical structures were combined in SB, and supernatants were recovered after homogenization and centrifugation. Electrophoretic and histochemical staining procedures were then performed to assay the various supernatants for *Est-m* activity. Later, similar *Est-m* assays were conducted on supernatants derived from digestive tracts, fat bodies, hemolymph, testes, accessory glands, seminal vesicles and vasa deferentia, or ejaculatory ducts. All tests were replicated using beetles 2–3, 5–7, or 12–14 d old.

Observations of *Est-m* distribution in males prompted additional testing to determine whether or not the enzyme was passed to females during

mating. These tests consisted of interrupting copulating pairs of beetles at 15-min intervals during mating or allowing matings to proceed to completion. Whole-body or reproductive tissue extracts of the various beetles subsequently were analyzed electrophoretically for the presence or absence of *Est-m* activity.

***Est-m* Inhibition and Substrate Specificity Tests.**

Enzyme inhibition and substrate specificity tests were conducted to categorize the *Est-m* enzyme of *D. v. virgifera* according to previously employed criteria (Mounter & Whittaker 1953, Burnstone 1962, Booth et al. 1973, Shnitka 1974, Varma & Frankel 1980). The inhibition studies consisted of exposing *Est-m* containing gels to inhibitors included in the stain buffer for 15 min at 37°C before the addition of the enzyme substrate (α -naphthyl acetate) and the coupling dye (Fast Blue RR). The *Est-m* extract used for electrophoresis was prepared by pooling the supernatants from 30 pairs of male accessory glands ground separately in 200 μ l of SB; 20-microliter aliquots from the pooled sample were then electrophoresed in the wells of replicate gels.

The inhibitors employed were diisopropyl fluorophosphate (DIFP; Sigma Chemical Company, St. Louis, Mo.), eserine (Sigma), *p*-hydroxymercuribenzoate (PHMB; Sigma), Landrin (3,4,5-trimethylphenyl methyl carbamate; Shell Chemical Company, New York, N.Y.), *ethyl* parathion (Phaltz and Bauer, Inc., Stamford, Conn.), and carbaryl (Phaltz and Bauer). The inhibitors were tested at concentrations of 10^{-3} M and 10^{-4} M for eserine, Landrin, and PHMB, and 10^{-4} for the other compounds. An additional test performed with carbaryl involved a second addition of material to the dye solution midway through the gel incubation period. All stain reactions were quenched in 7% acetic acid 20 min after the addition of the substrate and coupling dye to the stain buffer. The gels were then photographed using Ektachrome Daylight 64 Film in conjunction with CC30M and CC20C gelatin filters and backlighting. Circular punches of the resulting negatives corresponding to positive and negative areas of *Est-m* banding were placed in the wells of 96-well polystyrene microtiter plates. Absorbance values were subsequently recorded for each well using a Titertek Multiskan MC microplate reader (620 nm; Flow Laboratories, McLean, Va.). Enzyme inhibition was quantified by comparing control versus inhibitor absorbance values after the subtraction of appropriate background values.

Pooled extracts like those used for the enzyme inhibition tests were likewise used for electrophoretic evaluations of *Est-m* substrate specificity. These evaluations were performed by visually comparing *Est-m* staining in zymograms developed with α -naphthyl acetate versus the following alternate substrates; β -naphthyl acetate, α - or β -naphthyl propionate, α -naphthyl myristate, α -naphthyl laurate, and indoxylacetate (all substrates from Sigma). All gels were incubated for 30 min

at 37°C in the dye solutions before the reactions were quenched. The use of visual comparisons of enzyme staining precluded problems associated with quantifying *Est-m* bands that differed in color according to which substrate was employed in the dye mixture.

Tests for Lipoprotein or Glycoprotein in *Est-m* Preparations. Extracts of *Est-m* were prepared from the abdomens of individual *D. v. virgifera* males, and 20 μ l of each were electrophoresed in separate gels. Gel samples were stained for esterase to locate the *Est-m* electromorph, and corresponding unstained gel sections were then analyzed for lipoprotein or glycoprotein by the methods of Brown et al. (1977). The lipoprotein tests were conducted by staining the gels for 24 h in 100 ml of an aqueous solution containing 0.55 g Sudan black, 7.5 ml acetic acid, and 70 ml ethanol. Destaining was then accomplished at room temperature using two to three changes of 7.5% acetic acid. Glycoproteins were revealed by first fixing the unstained gel portions in 7.5% acetic acid at room temperature, then incubating in 0.2% aqueous periodic acid for 45 min at 4°C. The gels were stained for 45 min in Schiff reagent prepared according to Humason (1972), except that 10 g of pararosaniline was substituted for basic fuchsin in the stain solution. Destaining was conducted at room temperature using two or three changes of 10% acetic acid.

A modified lectin overlay assay (Glass et al. 1981, Clegg 1982) directed against *Est-m* immobilized on nitrocellulose (NC) was used as a further test for glycoprotein. Male *D. v. virgifera* accessory gland extracts containing 4.7 units of total esterase activity were initially subjected to electrophoresis for the assay. *Est-m* was then electroeluted from gel slices onto nitrocellulose (NC) using an electroblot apparatus available from Bio-Rad Laboratories, Richmond, Calif. The transblots were conducted for 24 h at 30 V (500 volt-h) using a 0.05 M Tris-glycine buffer system (4°C). Circular punches of NC (6 mm) containing *Est-m* were next placed in the wells of 96-well flat-bottom microtiter plates. Nonspecific protein binding sites in the wells were blocked (1 h) with 0.2 ml of 10% bovine serum albumin in phosphate-buffered saline (Garvey et al. 1977), which was further diluted 1:10 with distilled water for use (all phosphate-buffered saline employed for the assays additionally contained 1 mM CaCl_2 and 1 mM MgCl_2). The blocking solution was removed and the saturated NC samples were incubated for 1 h at room temperature with 0.2 ml of a concanavalin A-horseradish peroxidase conjugate (Sigma).

The plates were subsequently subjected to five 5-min washes with phosphate-buffered saline to remove any unbound material. Finally, 0.2 ml of peroxidase substrate containing a 1:1 mix of ABTS solution [2,2'-azino-di-(3-ethyl-benzothiazoline sulfonate); Kirkegaard and Perry Laboratories, Gaithersburg, Md.] and hydrogen peroxide was added to each well. The development of green color in

the wells was diagnostic for the presence of glycoprotein. Control wells containing NC without *Est-m* were used to check for background staining caused by glycoprotein impurities often present in commercially available bovine serum albumin. Another control involved the addition of 50 μ l of 0.1 M PNPA to a blocked well; the development of a yellow color in the well then indicated the presence of esterase capable of hydrolyzing the acetate to form PNP.

Relative Molecular Weight Determination. The molecular weight (mol wt) of *Est-m* from *D. v. virgifera* was estimated by gel filtration on a Sephacryl S-400 column (2.6 by 105 cm; Pharmacia, Inc., Piscataway, N.J.). The column was calibrated with the protein standards ribonuclease A, mol wt 3,700; chymotrypsinogen A, mol wt 25,000; ovalbumin, mol wt 43,000; bovine serum albumin, mol wt 68,000; catalase, mol wt 232,000; and Ferritin, mol wt 440,000 (Pharmacia). All calibration proteins were equilibrated in elution buffer (0.15 M Tris, pH 7.42) and chromatographed alone or in pairs. Blue Dextran 2,000 was used to determine the void volume of the column. Elution profiles were monitored with a UV photometer (280 nm) and given elution volumes were estimated from the peak fractions. The elution volume parameter, K_{av} , was then calculated for each standard and plotted against the corresponding log mol wt to generate a standard curve. The K_{av} value of *Est-m* was subsequently derived by the same procedure, and the mol wt was determined from the standard curve.

Partial Purification and Concentration of *Est-m*. Results from the gel staining and lectin binding assays for glycoprotein directed against *Est-m* suggested that lectin affinity columns might be used for enzyme purification. Thus, a column procedure was devised to recover glycoproteins from *D. v. virgifera* male accessory glands using the affinity support Affi-Gel Con A (Bio-Rad). Crude tissue extracts were prepared for the separations by first homogenizing 25 pairs of accessory glands in 1 ml of a 0.15 M Tris application buffer (pH 7.42) containing 0.1 M CaCl_2 and 0.1 M MnCl_2 . The homogenates were next centrifuged twice for 15 min at $15,000 \times g$ (4°C); the resulting pellets were discarded each time. The final supernatant contained *Est-m* and was diluted to 5 ml with the Tris buffer for use. The Affi-Gel Con A column (1 by 16 cm) was prewashed with about 100 ml of application buffer. One 5-ml sample of the *Est-m* extract was subsequently applied to the support material, and washing was continued until the OD of eluted fractions (255 nm) was the same as that of the application buffer. A second *Est-m* extract was loaded on the column and the washing and monitoring process was repeated. Specifically bound materials were then eluted (3-ml fractions) with application buffer containing 0.2 M α -D-methylmannopyranoside. PAGE was performed on 20- μ l portions of each final wash fraction to assay for the presence of *Est-m* and other proteins. Selected fractions con-

taining *Est-m* were then combined and simultaneously dialyzed (against application buffer) and concentrated to ≈ 1 ml using a collodion dialysis membrane (avg retention 25,000 mol wt) in conjunction with a vacuum apparatus (Schleicher and Schuell, Inc., Keene, N.H.). PAGE was again performed to test 20- μ l samples of each concentrate for the presence or absence of *Est-m* and other proteins.

An enzyme immunosorbent assay (EIA) was used to estimate the recovery of *Est-m* activity in samples concentrated from selected column fractions. The assay was initiated by incubating separate 25- μ l portions of concentrated and crude accessory gland extracts (24 h at 4°C) in the wells of 96-well round bottom polystyrene plates to adsorb *Est-m* to the well surfaces. The crude extract served as a control and contained approximately 1.5 units of total esterase activity. The plates were emptied, and nonspecific protein binding sites in each well were blocked for 40 min at 4°C with 0.3 ml of 10% bovine serum albumin in phosphate-buffered saline diluted 1:15 with distilled water. The blocking solution was removed and 50- μ l samples of mouse ascites fluid containing an anti-*Est-m* monoclonal antibody (IgM; I. McDonald et al., unpublished data) were added to the wells. The plates were then incubated with shaking for 10 min at room temperature followed by an additional 50-min incubation at 4°C. The supernatants were decanted, and each well was washed twice with 0.3 ml of a wash solution (0.02 M imidazole-buffered saline with 0.2% Tween 20; Kirkegaard and Perry) diluted 1:20 with distilled water to remove unbound primary antibody.

Lyophilized goat-antimouse antibody (0.5 mg, IgG and IgM; Kirkegaard and Perry) conjugated with horseradish peroxidase was rehydrated in 2.0 ml of distilled water. The reconstituted material was further diluted 1:100 with 10% bovine serum albumin in phosphate-buffered saline (diluted 1:10 with distilled water), and 0.2-ml portions of the latter were incubated in each test well for 1 h at room temperature. The wells were again emptied and rinsed five times by filling each well with wash solution and quickly inverting the plates. Finally, 0.1 ml of peroxidase substrate solution containing ABTS and hydrogen peroxide (1:1) was added to each well. The development of green color in the wells was diagnostic for the presence of *Est-m*. Absorbance values in all wells were recorded with a Titertek Multiskan MC microplate spectrophotometer (414 nm). The values obtained for wells containing the crude accessory gland extracts were then compared with those from wells containing concentrated fractions to derive estimates of *Est-m* recovery. Negative (10% bovine serum albumin was incubated in the wells instead of *Est-m*) and positive controls were included on all plates to delineate background staining and to test the performance of the substrate and peroxidase labeled antibody, respectively.

Table 1. Data on *Est-m* heterogeneity recorded among members of the *virgifera* group of *Diabrotica*

Taxon	No. presumed alleles (null) ^a	Electromorph relative mobilities $\pm$ SD ^b	No. populations surveyed (lab)
<i>D. barberi</i>	0 (1)		27 (1)
<i>D. cristata</i>	0 (—)		4 (1)
<i>D. lemniscata</i>	3 (1)	1.03 $\pm$ 0.062	3
		0.90	
		0.70 $\pm$ 0.026	
<i>D. longicornis</i>	5 (1)	1.02 $\pm$ 0.055	24 (1)
		0.91 $\pm$ 0.023	
		0.80 $\pm$ 0.018	
		0.72 $\pm$ 0.022	
		0.59 $\pm$ 0.031	
<i>D. v. virgifera</i>	1 (1)	0.34 $\pm$ 0.014	12 (2)
<i>D. v. zaeae</i>	1 (1)	0.35 $\pm$ 0.029	2 (1)

^a Presence of a null allele was assumed when zymograms lacking *Est-m* activity were observed in those taxa where *Est-m* electromorphs existed; see text for information on *D. barberi*.

^b Migration distance of house fly standard protein = 10.2 $\pm$ 0.62 mm.

Results

Est-m Variation Within the *virgifera* Group.

Electrophoretic surveys of *virgifera* group populations revealed putative null alleles in conjunction with three versus five active alleles of *Est-m* in *D. lemniscata* and *D. longicornis*, respectively (Table 1). The locus was likewise polymorphic in *D. v. virgifera* and *D. v. zaeae*, but the variation was characterized by the presence of an apparent null allele and a single active electromorph in both taxa. The common electromorphs of *D. lemniscata* (the 0.90 *Rm* variant was observed only once in the populations examined) and seemingly comparable electromorphs of *D. longicornis* exhibited slightly different mobilities when mixed or when separate extracts of the two species were electrophoresed on the same gel. In contrast, the enzymatic forms of *D. v. virgifera* and *D. v. zaeae* were indistinguishable by similar mixed-extract or side-by-side gel comparisons. *D. barberi* and *D. cristata* were classed as lacking *Est-m* enzymes, although zymograms of *D. barberi* males sometimes exhibited slight esterase activity in areas of gels where the faster migrating enzymes of *D. longicornis* or *D. lemniscata* occurred. McDonald et al. (1982) discussed evidence from interspecific hybridization tests between *D. barberi* and *D. longicornis* which suggested that a null allele of *Est-m* is fixed in *D. barberi* populations. However, there are presently no data to indicate that the *Est-m* locus occurs in *D. cristata*.

***Est-m* in vivo Distribution Studies.** Initial PAGE analyses of selected anatomical structures showed that *Est-m* activity was localized in the abdomens of virgin male *D. v. virgifera* (Table 2). Later investigations of abdominal tissues further demonstrated that the enzyme activity was restricted almost exclusively to the accessory glands, although zymograms of seminal vesicle and vasa deferentia extracts exhibited faint *Est-m* banding activity

Table 2. *Est-m* activity in tissues of virgin *D. v. virgifera* males

Fraction ^a	<i>Est-m</i> activity ^b
Antennae	--
Heads	--
Thoraces	--
Legs	--
Abdomens	+++
Digestive tracts	--
Fat bodies	--
Hemolymph	--
Accessory glands	+++
Seminal vesicles plus vasa deferentia	+
Testes	--
Ejaculatory ducts	--

^a Body parts or tissues from five males were combined for electrophoresis.

^b -- to +++ equals no activity to strong activity, respectively.

(Table 2). There were no differences in the distribution of *Est-m* in beetles 2–3, 5–7, or 12–14 d old.

Electrophoretic analyses of whole-body extracts clearly demonstrated the presence of *Est-m* in females that had been in copulation for $\approx$ 1.5 h or longer (Fig. 1). Enzyme activity was likewise apparent in females assayed immediately after completing the mating process. In contrast, *Est-m* band intensities appeared to be reduced in females electrophoresed 24 to 36 h after mating.

***Est-m* Characterization Tests.** The organophosphates DIFP and ethyl parathion essentially suppressed *Est-m* gel-staining activity as evidenced by absorbance readings which were reduced more than 98% compared with a control when test gels were exposed to 10^{-4} M concentrations of either inhibitor (Table 3). Conversely, PHMB appeared to have a "synergistic" effect on *Est-m* activity because absorbances recorded for treated gels were 10.7 and 24.1% greater than those of the controls when this material was present at concentrations of 10^{-4} M or 10^{-3} M, respectively. The carbamates, eser-

Fig. 1. *Est-m* activity in whole-body extracts of *D. v. virgifera* adults. (A) Virgin beetles. (B–D) Beetle pairs interrupted during mating at 15-, 60-, and 105-min intervals, respectively. (E and F) Beetle pairs processed immediately or 24 h after mating, respectively. Arrows indicate *Est-m* activity.

Table 3. Comparison of *Est-m* staining intensities in polyacrylamide gels exposed to selected enzyme inhibitors

Material and concentration	Absorbance (620 nm) minus background ^a	% Change in absorbance
Carbaryl		
Control	0.704	
10 ⁻⁴ M	0.520	-26.1
10 ⁻⁴ M + 10 ⁻⁴ M ^b	0.357	-49.3
DIFP		
Control	0.605	
10 ⁻⁴ M	0.011	-98.2
Eserine		
Control	0.776	
10 ⁻⁴ M	0.588	-24.2
10 ⁻³ M	0.259	-66.6
Landrin		
Control	0.741	
10 ⁻⁴ M	0.493	-33.5
10 ⁻³ M	0.422	-43.0
Ethyl parathion		
Control	0.515	
10 ⁻⁴ M	0.002	-99.6
PHMB		
Control	0.516	
10 ⁻⁴ M	0.578	+10.7
10 ⁻³ M	0.680	+24.1

^a Gels were photographed and absorbencies were recorded from the resulting negatives (620 nm). Values shown were derived by averaging absorbencies from two to three gel runs and subtracting a corresponding gel background value.

^b A second addition of material was made to the dye solution midway through the gel incubation period.

ine, and Landrin caused partial, although progressively greater, reductions in *Est-m* stain activity depending on whether gels were incubated with 10⁻⁴ M or 10⁻³ M concentrations of these materials; carbaryl exhibited a similar pattern of inhibition when one versus two additions of this carbamate were made to the dye solutions.

Est-m staining reactions (Fig. 2) obtained with the substrate α -naphthyl propionate (brown bands) were comparable with or slightly more intense than those obtained with α -naphthyl acetate (brown

Fig. 2. *Est-m* staining in acrylamide gels incubated with varying substrates. (A) α -naphthyl acetate control. (B) α -naphthyl propionate. (C) α -naphthyl butyrate. (D) Indoxylacetate. (E) β -naphthyl propionate. (F) β -naphthyl acetate. Arrows indicate *Est-m* activity in individual electrophoretic lanes; only positive staining reactions are shown.

Fig. 3. Lectin-binding assay for the presence of carbohydrate in *Est-m* immobilized on nitrocellulose. Rows A and C show color development in replicate test wells 1-3 or 4-6 incubated with 0.1 or 0.01% solutions of a concanavalin A, horseradish peroxidase conjugate, respectively. Rows B and D show corresponding control wells lacking *Est-m*.

bands). *Est-m* bands were further resolved in tests using α -naphthyl butyrate (brown bands), β -naphthyl acetate (pink bands), β -naphthyl propionate (pink bands), or indoxylacetate (blue bands), but band intensities were reduced to varying degrees relative to the α -naphthyl acetate control. No bands were observed in tests with α -naphthyl laurate or α -naphthyl myristate.

Sudan black did not stain *Est-m* in acrylamide gels, thereby indicating that the enzyme was not a lipoprotein. However, a light pink band was resolved in the *Est-m* area of gels subjected to the Schiff's staining procedure for glycoprotein. The lectin overlay assay directed against *Est-m* immobilized on NC provided further evidence to indicate that the enzyme is a glycoprotein because test wells developed a more intense green color in comparison with control wells following the addition of the concanavalin-A horseradish peroxidase conjugate and appropriate substrate (Fig. 3).

The standard curve constructed for molecular weight determinations by the sephacryl gel filtration technique is shown in Fig. 4. The relative molecular weight of *Est-m* from *D. v. virgifera* was estimated to be $78,680 \pm 8,680$ SD. This estimate is a mean derived from six replicate experiments.

Partial Purification and Concentration of the Enzyme. Crude accessory gland extracts of *D. v. virgifera* males contained *Est-m* along with a second esterase and twelve other proteins as assayed by PAGE and gel-staining techniques (Fig. 5). Electrophoretic analyses further showed that *Est-m* was consistently eluted from the Affi-Gel Con A affinity column only when α -D-methylmannopyranoside was included in the column wash buffer. Subsequently, *Est-m* and two other protein bands (neither stained for esterase) were detected via electrophoretic assays of the dialyzed and concen-

Fig. 4. Gel filtration standard curve used to estimate the relative molecular weight of *Est-m* from *D. v. virgifera*. K_{av} = protein elution volume - column void volume (Blue Dextran 2000 elution volume)/total bed volume - column void volume. Text lists molecular weights of standards.

trated *Est-m* samples (Fig. 5). Twenty-five-microliter portions of crude accessory gland extracts (about 1.5 units of esterase activity) produced absorbance values approximating 0.400 in the EIA directed against *Est-m*. Absorbance values similarly obtained with the semipurified and concentrated samples approximated 0.575, thereby indicating at least a 30% difference between the enzyme concentrations of the test versus control wells. By extrapolation the test wells contained the equivalent of about 2.2 units of esterase activity. This extrapolation is based on the assumption that *Est-m* was primarily responsible for the hydrolysis of PNPA in the enzyme activity assays conducted against the crude accessory gland extracts.

Discussion

The tissue distribution studies showed that *Est-m* enzyme activity was localized in the accessory glands of virgin *D. v. virgifera* males 12–14 d old. Thus, it seems likely that the enzyme is synthesized in the glands, although we cannot exclude the possibility that it is manufactured elsewhere and is transported to the glands before or just after adult eclosion. The enzymatic function of *Est-m* in the reproductive process is suggested by the sex-limited occurrence of the enzyme in the accessory glands and by its passage from males to females during mating. Unfortunately, we have no knowledge regarding the potential significance of null alleles at the *Est-m* locus. However, the involvement of accessory gland secretions in sperm transfer, sperm activation, inhibition of female remating, or other aspects of insect reproduction is well documented (Leopold 1976). Importantly, proteins and carbohydrates represent ubiquitous components of accessory gland secretions in Coleoptera (Anderson 1950, Gerber et al. 1971, deLoof & Lagasse 1972,

Fig. 5. Gel staining of affinity-purified concentrates or crude accessory gland extracts containing *Est-m* activity. (A) Affinity-purified concentrates stained for esterase. (B) Affinity-purified concentrates stained for esterase and general protein using Coomassie Blue G-250. (C) Crude accessory gland extracts stained for esterase and general protein using Coomassie Blue G-250. Arrows indicate areas of *Est-m* activity, dashes indicate protein bands, and asterisk indicates area exhibiting three protein bands in the absence of esterase stain; the three bands are also present in *Est-m* null phenotypes.

Huignard & Lamy 1972) or other insects (Davey 1958, Leopold 1970, Cantacuzene 1971), and most workers agree that these components are probably complexed as muco- or glycoproteins (Leopold 1976).

We are categorizing the *Est-m* enzyme of *D. v. virgifera* as a carboxylesterase based on the varying effects of inhibitors on its activity. Thus, the *Est-m* response pattern was similar to those observed previously with carboxylesterases (Mounter & Whittaker 1953, Burnstone 1962, Booth et al. 1973, Shnitka 1974, Varma & Frankel 1980) in that enzyme activity was completely inhibited by DIFP and ethyl parathion (both organophosphates), but not by carbaryl, Landrin, eserine (all carbamates), or PHMB at those concentrations tested. The substrate specificity tests provided evidence to further characterize *Est-m* as acting primarily on esters having side chains two to three carbons long. Also, some steric effects on enzyme activity were suggested because the gel-staining reactions of the α -esters were more intense than their β -ester counterparts. The classification of *Est-m* is artificial and the *in vivo* substrates are unknown. However, enzymes similarly classed as carboxylesterases are widespread in insects and may be highly poly-

morphic in natural populations. Moreover, these enzymes have been implicated as functioning in diverse processes such as digestion (Hippis & Nelson 1974), chemical detoxification (Dauterman & Hodgson 1978), reproduction (Richmond et al. 1980), or the regulation of juvenile hormone titers (Whitmore et al. 1975).

The combined results of the Schiff's staining and concanavalin-A overlay assays indicated that *Est-m* of *D. v. virgifera* is most likely a glycoprotein. Both assays are diagnostic for the presence of glucose or mannose in carbohydrates, although positive Schiff's tests also are obtained against galactose and the methyl pentose, fucose (Sharon & Lis 1972, Culling 1976). The specificity of concanavalin-A for *Est-m* was further demonstrated by the retention of the enzyme on the Affi-Gel Con A affinity column and its subsequent elution via column washings with buffer containing α -D-methylmannopyranoside. Soluble glycoproteins are commonly present in cells or in the extracellular fluids of organisms. Evidence suggests that the carbohydrate substituents may function to stabilize protein configurations or they may serve as recognition markers in physiological processes such as cellular uptake (Sharon 1981). A protective role for the carbohydrate also has been suggested based on the observation that glycoproteins are more resistant to proteolysis in vitro than are nonglycosylated proteins (Sharon & Lis 1981). Carbohydrate moieties appear capable of modifying various physical properties of proteins (e.g., electrical charge) without affecting their biological activities (e.g., see Cochrane & Richmond 1979). Thus, it is possible that the intra- or interspecific mobility differences of *Est-m* electromorphs may reflect variations in the carbohydrate portions of the enzymes.

Our investigations have demonstrated that affinity chromatography using concanavalin-A as a ligand represents a readily useable technique for recovering semipurified extracts of *Est-m*. Moreover, the methodology, as described herein, has revealed two other presumptive glycoproteins of *D. v. virgifera* accessory gland extracts that may be worthy of further study. An obvious goal of future work will be to combine the column procedure with other techniques to obtain pure *Est-m* preparations for more detailed analyses of the enzyme's biochemical and molecular properties. In this regard, we note here that the Bio-Rad Coomassie Blue G-250 assay was used to obtain early estimates of total protein in various *Est-m* extracts. However, we have been unable to resolve *Est-m* in acrylamide gels with either Coomassie Blue or silver stain, even though a number of staining protocols have been used and tests have been conducted against a variety of semipurified or crude *Est-m* preparations (e.g., containing material from up to 180 insects). We cannot completely exclude the possibility that the observed lack of staining is the result of low concentrations of *Est-m* in the gels. Nevertheless, we currently favor the hypothesis that the carbo-

hydrate portion of the enzyme, or possibly other structural peculiarities, somehow prevent Coomassie Blue or silver stain from reacting with enzyme moieties essential to stain uptake. The inability to stain *Est-m* in gels has suggested that estimates of total protein recorded for given extracts by the Bio-Rad assay fail to reflect the *Est-m* content. Thus, we are currently investigating alternate approaches for estimating protein present in samples at successive stages of enzyme purification.

Est-m represents the first documented instance of sex-limited esterase inheritance in Coleoptera. Furthermore, reports of sexual dimorphism in esterase expression are rare among insects in general, even though esterase studies have encompassed a wide variety of insect species. Steiner et al. (1983) genetically characterized a sex-linked esterase of the caddisfly, *Trienodes tardus* Milne, in which enzyme activity was restricted to electrophoretic zymograms of females. In contrast, male-only esterase expression was reported in *Anopheles stephensi* Liston (Freyvogel et al. 1968) and *A. funestus* Giles (Green 1977). The last-named enzymes were described as sex-limited, although it was not determined whether they were sex-linked. Importantly, the enzyme activity was shown to be restricted to the accessory glands in *A. funestus*.

Male-limited esterases have further been reported to occur in the ejaculatory ducts of various *Drosophila* species (Johnson & Bealle 1968, Sheehan et al. 1979). In this regard, a polymorphic *EST-6* system of *D. melanogaster* Meigen has been studied extensively and shares characteristics analogous with those of *Est-m*. Thus, the *EST-6* locus has been mapped to an autosome (chromosome 3; Wright 1963), and the variant enzymes have been classed as carboxylesterases as well as glycoproteins having molecular weights in the range of 60–65,000 (Sheehan et al. 1979, Mane et al. 1983a). The expression of *EST-6* appears to be mediated by regulatory loci (Cochrane & Richmond 1979, Tepfer et al. 1982) and may be further modulated by juvenile hormone titers (Stein et al. 1984). The presence of the enzyme(s) in the male ejaculate has been associated with effects on sperm utilization, female fecundity, and the timing of female remating (Richmond et al. 1980; Gilbert 1981; Gilbert et al. 1981a,b; Gilbert & Richmond 1982). It was suggested earlier that *EST-6* hydrolyzed a male-produced acetate ester to its alcohol in the female and that the alcohol then acted to reduce the attractiveness of mated females to courting males (Mane et al. 1983b). However, Vander Meer et al. (1986) have questioned whether such a mechanism of pheromone production operates in vivo based on direct chemical analyses of the postulated system components in the reproductive tracts or cuticle of mated insects. Oakeshott et al. (1987) recently cloned the *EST-6* gene and characterized the enzyme as a serine hydrolase. Enzymes identified as serine hydrolases possess a serine residue

at their active site and appear to be functionally related. Various carboxyl-, choline-, and aliesterases fall within the group along with the homologous family of serine proteases (Davis et al. 1985, Rogers 1985, Schumacher et al. 1986).

Finally, an *S-esterase* found among various members of the *virilis* group of *Drosophila* is coded for by a structural gene on autosome 2 and is likewise a carboxylesterase (Korochkin et al. 1976a,b, 1978a). The activity levels of *S-esterase* vary among strains and species within the group, and Korochkin et al. (1978a,b, 1981) reported evidence to indicate that enzyme expression may be modified by genes acting at both the levels of transcription and post-transcription. Also, Korochkin et al. (1976a,b) noted that *S-esterase* exhibits lipase activity and theorized that it somehow promotes fertilization via the breakdown of lipid materials in the female reproductive system.

Acknowledgment

The research reported was performed in partial fulfillment of the requirements for an M.S. degree in microbiology by Susan K. Ruud. This paper is published with the approval of the Director of the North Dakota Agricultural Experiment Station as journal article no. 1674.

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Received for publication 30 October 1987; accepted 25 July 1988.