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Characterization of the Esterase Isozymes of *Ips typographus* (Coleoptera, Scolytidae)

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Four esterase isozymes hydrolyzing α-naphthyl acetate (α-NA) were detected screening whole body homogenates of larvae and adults of *Ips typographus* by electrophoresis. Two of the four isozymes (isozymes 3 and 4) were not detected by α-NA staining in the pupal stage, but topical application of juvenile hormone III (JH III) on the pupa induced these isozymes. The JH esterase (JHE) activity on the gel was associated with the proteins of isozyme 2. The compounds OTFP, PTFP, and DFP inhibited this catalytic activity of isozyme 2 on the gel at low concentrations, whereas the proteins of isozyme 3 and 4 were affected only at higher concentrations. A quantitative developmental study was performed to characterize which of the esterases hydrolyzed JH III, using a putative surrogate substrate for JH (HEXTAT) and α-NA. The I_{50} of several esterase inhibitors and the JH metabolites were also defined. All findings supported the results that a protein associated with isozyme 2 is catabolizing JH and that isozymes 3 and 4 are the main contributors to the general esterase

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activity on α-NA. The JHE from *Tenebrio molitor* was purified by affinity chromatography. Although the recovery was low, an analytical isoelectric focusing gel showed that the JHE activity of the purified enzyme *T. molitor* cochromatographed at the same pI as the JHE activity of *I. typographus.* Arch. Insect Biochem. Physiol. 34:203–221, 1997.

Key words: ***Ips typographus; Tenebrio molitor;*** **developmental profile; juvenile hormone esterase**

INTRODUCTION

The eight-spined spruce bark beetle, *Ips typographus*, is a major pest in forests of *Picea abies* (Postner, 1974). Enzyme electrophoresis was used in this analysis of *I. typographus* esterases (EC.3.1.1.1). Four isozymes were found when gels of *I. typographus* homogenates were stained with α-naphthyl acetate (α-NA*) (Stauffer et al., 1992). Populations showed high polymorphism in these loci, and 18 alleles were genetically associated with isozyme *Est-2* (Stauffer, 1994). The polymorphism may reflect the physiological range of the esterases which are supposedly involved in detoxification processes and / or in the hydrolysis of juvenile hormone (JH) (Terriere, 1984).

As we show in this study, homogenates of *I. typographus* pupae do not stain for esterase isozymes 3 and 4 on native polyacrylamide gel with α-NA as substrate. However, the two proteins and their activities are induced by topical application of JH III, leading to the initial hypothesis that the two loci could be coding for juvenile hormone esterase (JHE). JHE is involved in the hydrolysis of JH in critical stages of the metamorphic development in most insects (Slade and Zibitt, 1972; Hammock, 1985). This enzyme has been studied intensively at the molecular level in recent years (Hanzlik and Hammock, 1987; Venkatesh et al., 1988; Hanzlik et al., 1989; Wroblewski et al., 1990; Valaitis, 1991; Schelling and Jones, 1995). Most of the studies on JHE have been performed in species of Lepidoptera. Besides studies on *Leptinotarsa decemlineata* (Kramer and de Kort, 1976; de Kort et al., 1983; Lefevre, 1989) and *Tenebrio molitor* (Sparks and Hammock, 1980; Connat, 1983), little information about JHE is available in Coleoptera.

The aims of this study were to determine the biochemical and physiological characteristics of the esterase isozymes in *I. typographus* and then to ask which if any of these isozymes hydrolyze JH III and which isozymes are involved in other detoxification processes. In order to assess the generality of our results, we

*Abbreviations used: α-NA = α-naphthyl acetate; BBA = n-butyl boronic acid; BSA = bovine serum albumin; *Est-1–Est-4 = esterase isozyme 1–isozyme 4;* DFP = diisopropyl fluorophosphate; IEF = isoelectric focusing; JH = juvenile hormone; JHE = juvenile hormone esterase; JHEH = juvenile hormone epoxide hydrolase; HEXTAT = methyl hexylthio acetothioate; HPLC = high performance liquid chromatography; MTFO = 8-mercapto-1,1,1-trifluoro-2-octanone; OG = n-octyl-β-D-glucopyranoside; OTFP = 3-Octylthio-1,1,1-trifluoro-2-propanone; PAGE = polyacrylamide gel electrophoresis; paraoxon = O,O-diethyl-*p*-nitrophenyl phosphate; PMSF = phenylmethylsulfonylfluoride; PTFP = 3-pentylthio-1,1,1-trifluoro-2-propanone; TLC = thin layer chromatography; TX100 = Triton X-100.

also examined *Tenebrio molitor*. This species feeds on stored products such as flour. The ontogenetic development of this family has been investigated endocrinologically (Edwards and Rowlands, 1977; Connat, 1983) and genetically (Beeman et al., 1990; Brown et al., 1990). In contrast to *T. molitor*, endocrinological studies with scolytids are problematic because of problems of rearing.

MATERIALS AND METHODS

Chemicals

Tritium-labeled (10-^{3}H(N))-JH III was purchased from New England Nuclear Research Products (Boston, MA) and diluted with unlabeled JH III (Sigma, St. Louis, MO) to yield solutions in absolute ethanol of 5×10^{-3} M (3.6 MBq/μl) for thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) and of 5×10^{-4} M (1.2 MBq/μl) for partition assays. Experimental sample was added to give a final concentration of 5×10^{-6} M JH III in each case. 3-Octylthio-1,1,1-trifluoro-2-propanone (OTFP) (Abdel-Aal and Hammock, 1985a), 8-mercapto-1,1,1-trifluoro-2-octanone (MTFO) (Shiotsuki et al., 1994), 3-pentylthio-1,1,1-trifluoro-2-propanone (PTFP) (Shiotsuki et al., 1994), and methyl hexylthio acetothioate (HEXTAT) (McCutchen et al., 1993) were available from previous synthesis. Precoated EM Silica Gel 60 TLC plates (250 micron; F254; VWR Scientific, San Francisco, CA) were used for TLC, and ampholine PAG plates (4.0–6.5 and 4.0–9.0; LKB-Pharmacia, Piscataway, NJ) were used for isoelectric focusing (IEF). All other chemicals were of high purity grade.

Insects

I. typographus were reared in spruce logs (25 ± 2°C, 16:8 h light:dark; humidity 70%); individuals were randomly collected by cutting them out of the bark for staging. The larvae die shortly after removing them from the logs. No artificial diet is known. The larvae were staged by measuring the head capsule under a microscope (Wiener, 1988), and late pupae were distinguished from other pupae by their brown head capsule. They were frozen at –70°C. For comparative purposes, larvae of *T. molitor* were reared in the laboratory in Davis on oatmeal (30 ± 2°C; 18:6 h light:dark; humidity 60%). To avoid juvenilizing effects, frass was periodically removed by sieving. One-day-old pupae (0–24 h old) were collected for analysis. The prepupae were determined by weight, lack of movement, and eye stage (Stellwaag-Kittler, 1954) and then held separately in trays with a thin layer of bran and a banana peel to monitor for pupation. For the experiments involving topical application of JH III, the hormone was dissolved in acetone or in olive oil and applied topically on pupae in 2 μl drops (10 ng JH III/mg body weight of *I. typographus*). Control pupae were treated with acetone or olive oil. Treated and control pupae were homogenized 2–18 h after application and analyzed for esterase activity by native polyacrylamide gel electrophoresis (PAGE).

Homogenate Preparation

Twenty beetles of *I. typographus* were pooled, the wet weight was taken, and the whole bodies were homogenized in ice-cold 50 mM Tris buffer (pH 8.0) containing 0.5% 1-phenyl-2-thiourea as a tyrosinase inhibitor. The homo-

genate was centrifuged (2,500*g*, 5 min), and, after the supernatant was removed to another tube, the pellet was extracted a second time. The supernatants were combined, and the final volume of buffer was adjusted to 12 times the weight of the insects homogenized (v / w). The supernatants were filtered through a Sephadex G-25 (20–50 mm) column. The same protocol was performed with five pupae of *T. molitor*. For native PAGE and IEF runs, individual *I. typographus* were homogenized in 200 µl buffer containing 38 mM glycine, 5 mM Tris, 10% sucrose, and 0.5% bromphenol blue at pH 7.4. Unless otherwise noted, similar amounts of protein (25–35 mg) were loaded onto each slot (PAGE) or application wick (IEF). The protein concentration for all studies was determined by the dye-binding method of Bradford (1976) as modified by BioRad® using a microtiter plate reader. Bovine serum albumin (BSA) Fraction V was used as a protein standard.

Hydrolysis of JH III—JH Esterase Assay

For routine monitoring, JHE activity was determined by a previously described partition method (Hammock and Sparks, 1977). Dilutions with Tris buffer (50 mM; pH 8.0) were performed until the linear range of hydrolysis (20–40% of the maximal counts) was achieved. For the data presented here, aliquots (100 µl) of the diluted homogenates were incubated with tritium-labeled JH III (5×10^{-6} M final concentration, added in 1 µl ethanol) for 30 min at 30°C. Under these conditions the hydrolysis data were linear with both time and protein concentration. To halt the reaction, 50 µl of basic methanol (methanol:distilled water:ammonium hydroxide, 10:9:1) and 300 µl of isooctane were added to the 10 × 75 mm borosilicate tubes, and the contents of the tubes were mixed vigorously (30 s) and centrifuged (2,500*g* 5 min). An aliquot (75 µl) was removed from the aqueous phase and mixed with 2.5 ml of scintillation fluid (CytoScint; ICN Biomedicals Inc., Irvine, CA) in scintillation vials before counting for 2 min.

Esterase Hydrolysis of α-NA—General Esterase Assay

The hydrolysis of α-NA was measured by mixing 278 µl of sodium phosphate buffer (0.05 M, pH 7.4) containing Fast Blue RR (2.5 mg / ml) with 20 µl of enzyme solution and 2 µl of α-NA solution (75 mM stock solution diluted to 0.5 mM final concentration). The change in absorbance was monitored for 4 min at 450 nm (Grant et al., 1989).

Esterase Hydrolysis of HEXTAT

The hydrolytic activity for HEXTAT, a putative surrogate substrate for JH, was measured according to the method of McCutchen et al. (1993).

Effect of Inhibitors on Esterase Activity

A solution of the respective inhibitor (1 µl) in ethanol was added to the diluted enzyme preparation (100 µl) and incubated for 10 min at 30°C prior to the addition of the substrate. Semilog plots of percentage inhibition against inhibitor concentration were used to estimate the I_{50} (inhibitor concentration that results in a 50% reduction of the enzyme activity). Controls were incubated with ethanol instead of the inhibitor solution (Sparks and Hammock, 1980).

Native Polyacrylamide Gel Electrophoresis

Native PAGE was performed with 0.75 mm polyacrylamide gels (10%; pH 8.9) using a vertical slab instrument (Mighty Small®; Hoefer, San Francisco, CA). Homogenate (15 µl) was loaded and the gels were run at 30 mA constant current and with continuous cooling at 0–3°C. Staining of esterases hydrolyzing α-NA was carried out according to Stauffer et al. (1992). The sensitivity of these esterases to five inhibitors was assessed by incubating the gel, after the run, with a washing solution containing inhibitor (10 min at 30°C) prior to staining. JHE activity in the gel was evaluated by assaying Tris buffer (300 µl) which had been incubated with 2.5 mm homogenized gel slices for 30 min. High recovery of esterase activity was obtained. Determination of the esterase activity on JH III followed the same procedure as described above.

Isoelectric Focusing

IEF was conducted for 2 h on precast 1 mm horizontal slab gels (LKB, Los Angeles, CA) first with wide (4.0–9.0) and then with narrow (4.0–6.5) pH gradients after a prefocusing period of 45 min. JHE activity and pH profiles of these gels were generated by assaying Tris buffer (300 µl) and 20 mM KCl solution (300 µl), respectively, which had been incubated with 2.5 mm gel slices for 30 min (Hanzlik and Hammock, 1987). The IEF gel was also stained for protein with Coomassie Brilliant Blue R-250 and for esterase activity with a solution of Fast Blue RR containing α-NA as substrate.

Thin Layer Chromatography

TLC was performed according to the method of Casas et al. (1991). Whole body homogenates (100 µl) were incubated with tritium-labeled JH III for 30 min at 30°C in 10 × 75 mm borosilicate tubes. Reactions were terminated by the addition of saturated brine (100 µl) and ethyl acetate (200 µl). Following vigorous vortexing and centrifugation at 2,500*g* to break the emulsion, the organic layer was removed with a 100 µl syringe, and the extraction was repeated. The combined ethyl acetate fractions were concentrated to 5–10 µl under a blanket of nitrogen, and 30 µl of chloroform containing either authentic metabolite standards or methoprene and methoprene acid as mobility markers was added to each tube prior to spotting on TLC plates. TLC was developed in equilibrated glass tanks containing hexane:ethyl acetate (7:4). After chromatography the positions of the authentic standards or the methoprene and methoprene acid were marked under UV light to determine the relative positions of the metabolites. Positions of radioactive metabolites were determined with a Positron TLC scanner (System 200 Imaging Scanner; Bioscan, Washington, DC). Each lane was counted for 15 min. Following subtraction of background, the ratios of the metabolites were calculated. Similar results were obtained when the lanes were subsequently scraped and the samples were analyzed using a liquid scintillation counter. Changes in relative mobility of the metabolites were monitored by TLC by microchemical modification of the combined radioactive and unlabeled material using n-butyl boronic acid (BBA), diazomethane, and dilute acid (Hammock et al., 1994).

High Performance Liquid Chromatography

HPLC was used to support the TLC-based tentative identification of JH metabolites (Morello and Agosin, 1979; Halarnkar and Schooley, 1990). A Vydac (Hesperia, CA) reverse phase C_{18} column (250 × 4.6 mm) and a Perkin Elmer (Norwalk, CT) series 410 B 10 LC pump were used with 60% methanol in H_2O at a flow rate of 1 ml/min with a run time of 30 min. Radioactivity was detected by a β-Ram 1B radioactive flow detector (IN/US Systems, Inc., Tampa, FL) with IN-FLOW BD scintillation fluid running at 3 ml/min.

Chemical Derivatization

JH III acid and JH III diol standards were generated by incubating JH III with affinity-purified JHE from *Manduca sexta* (Abdel-Aal and Hammock, 1985b) and affinity-purified cytosolic epoxide hydrolase from mouse liver (Prestwich and Hammock, 1985), respectively. The JH III acid diol standard was generated by incubating JH III with both enzymes. Identification of enzymatically derived metabolites was carried out by previously published methods using comparison with synthetic standards (Hanzlik and Hammock, 1988). These mixtures (100 µl) were extracted three times with 150 µl of hexane to remove the unmetabolized JH III. Following initial extraction, 75 µl of the aqueous phase was removed and extracted twice with 200 µl ethyl acetate. The ethyl acetate extract solution was divided into three parts. One part was analyzed by TLC and HPLC without derivatization, and the second part was analyzed after derivatization of JH III acid with 500 µl of diazomethane-ether solution to regenerate the methyl ester. The final part was analyzed for derivatization of JH III diol with 50 µl of 1% 1-butaneboronic acid (w/v) in ethyl acetate to form the boronic diester of the diol. In each case the synthetically and enzymatically generated standards cochromatographed before and after chemical modification. For subsequent routine chromatography with the metabolites of *I. typographus* and *T. molitor*, the enzymatically produced standards were used.

Affinity Chromatography

JHE was purified from a whole body homogenate of *T. molitor* pupae. All procedures were carried out at 4°C, and a sodium phosphate buffer (0.1 M, pH 7.4, containing 5% sucrose and 0.02% sodium azide) was used as purification buffer. The MTFO-affinity gel (5 ml bed volume) was packed into a column with a fluoropolymer frit at the bottom (Econo-column; BioRad®, Hercules, CA). The column was washed thoroughly with 50% ethanol, distilled water, and finally purification buffer. The enzyme solution (20.5 ml) was added and shaken gently for several hours; then the solution was removed and enzymatic activity determined. After loading, the column was washed with purification buffer and then with purification buffer containing 0.5% n-octyl-β-D-glucopyranoside (OG). The enzyme was eluted from the affinity gel by gentle shaking for several hours with purification buffer containing 0.5% OG and 1 mM PTFP. This step was repeated three times with fresh eluting solution. The eluates were combined and concentrated in a SPECTRA/POR molecular membrane tubing dialysis bag (m.w. cutoff = 12,000–14,000; Spectral Medical Industries, Inc., Los Angeles, CA) coated with polyethylene glycol (m.w. = approximately 10,000). Further concentration was

performed using Centricon-30 micro concentrators (Amicon, Beverly, MA) (4,000*g*, 30 min). Concentrated eluate was dialyzed against the purification buffer to remove PTFP.

RESULTS

Separation of Esterase Isozymes

The proteins of the different ontogenetic stages of *I. typographus* were separated by 10% native PAGE and stained with α-NA (Fig. 1). Four esterase isozymes could be stained in adult and in second and third instar homogenates. The two faster running isozymes were not detected in pupae (Fig. 1, lanes 3, 4). Topical application of JH III onto the pupa resulted in a zymogram with four isozymes after 2 h, with expression lasting up to 15 h (Fig. 1, lanes 6, 7). Apparent degree of induction was the same whether JH III was applied in acetone or olive oil. Acetone or olive oil alone did not induce these proteins.

One lane of the native gel was cut into 2.5 mm pieces, and after extraction the JHE activity from each gel slice was measured with JH III as substrate. No JHE activity was detected in isozymes 3 and 4. The activity observed with JH III was associated solely with the proteins of isozyme 2 on the native gel (Fig. 1b).

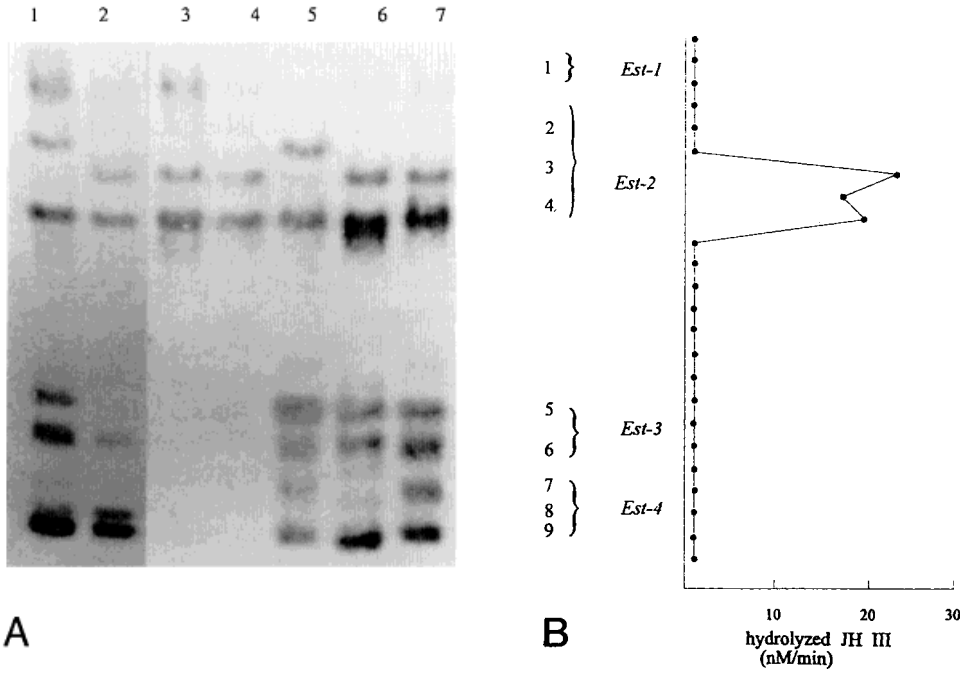

Fig. 1. **A:** Developmental zymogram profile of esterase isozymes from *I. typographus,* separated by native PAGE and hydrolyzing α-NA. Lane 1: Second larval instar. Lane 2: Third larval instar. Lanes 3, 4: Pupa. Lane 5: Adult; Lane 6: JH III diluted in olive oil applied onto pupa. Lane 7: JH III diluted in acetone applied onto pupa. Est-1–Est–4 = esterase isozyme 1–isozyme 4. **B:** The diagram represents the JHE activity isolated from polyacrylamide gel slices of an adult with the same pattern as shown in lane 7. All JHE activity is associated with the *Est-2* isozyme.

TABLE 1. Inhibitory Effect of Five Chemicals on the Esterase Isozymes Hydrolyzing α-NA, From the Pupa of *I. typographus*, Separated by Native PAGE. Inhibitory Potencies Are Shown as Maximal Concentrations (M) for no Inhibition Measured in the Gel

	OTFP	PTFP	DFP	TX100	Paraoxon
Isozyme 1	10^{-11}	10^{-8}	10^{-11}	No inhibition	10^{-11}
Isozyme 2	10^{-10}	10^{-10}	10^{-11}	No inhibition	10^{-10}
Isozyme 3	10^{-3}	10^{-6}	10^{-5}	No inhibition	10^{-8}
Isozyme 4	10^{-3}	10^{-5}	10^{-4}	No inhibition	10^{-8}

[a]No inhibition detected up to concentrations of 1.0% v / v.

Triton X-100 (TX100) at a concentration of 1.0% showed no inhibition of the activity of any of the four isozymes (Table 1). Isozymes 1 and 2 were inhibited by OTFP, PTFP, and diisopropyl fluorophosphate (DFP) at concentrations between 10^{-8} and 10^{-11} M. However, isozymes 3 and 4 were much less sensitive towards these inhibitors. All α-NA–staining esterases were inhibited by O,O-diethyl-*p*-nitrophenyl phosphate (paraoxon) at concentrations between 10^{-8} and 10^{-11} M.

Characterization of JH Metabolites

JH III was metabolized to more polar products when incubated with diluted whole body homogenates of *I. typographus* or *T. molitor.* Metabolites of JH III were tentatively identified by HPLC and TLC using a combination of inhibitors, standards, and chemical derivatization (see Materials and Methods). Following incubation of JH III with whole body homogenates of the two species, only two radioactive peaks of R_f 0.60 and 0.92 were observed on TLC. The TLC R_f values of the standards were 0.92, 0.60, 0.39, and 0.16 for JH III, JH III acid, JH III diol, and JH III diol acid, respectively. The position of the radioactive materials and the quantitation of the radioactive materials were done with a Positron TLC scanner. These scanners are highly sensitive and reproducible with ^{14}C, and with care can be quantitative with ^{3}H as well. In this case the data generated by scanning the plate matched the data generated by subsequent scraping and counting the plate, which in turn matched the partition data.

On HPLC, the retention time of JH III was 20 min and that of the JH III acid was 5 min under the conditions described. When the putative JH III acid was incubated with diazomethane, a reagent able to reesterify the acid, both the polar UV standard and the radioactive peaks at 5 min were eliminated. Instead, the radioactivity as well as the unlabeled JH III acid standard eluted as a single peak at 20 min, as expected for the methyl ester. The standard JH III diol eluted at 9 min under these conditions, and its retention time was not influenced by diazomethane. The BBA-derivitized diol cochromatographed with JH III, but the mobility of the radioactive metabolite was not changed on either TLC or HPLC by the addition of BBA. OTFP and PTFP at concentrations between 10^{-3} and 10^{-5} M inhibited the esterases hydrolyzing JH III, and no JH III acid peak was detected either by TLC or HPLC following incubation with these inhibitors.

Esterase Activities and Inhibitor I_{50} Values From Ontogenetic Stages of the Two Species

Three different substrates were used to detect esterase activity in homogenates from developmental stages of *I. typographus* and *T. molitor:* JH

III, HEXTAT, and α-NA (Table 2). JH III activity in *I. typographus* increased from the second to the third larval instar. Activity fluctuated little (75–95 pmol/min/mg) in subsequent stages except for the young brown adult, whose activity decreased more than sixfold to 12.1 pmol/min/mg. In contrast, the pupa of *T. molitor* had almost four times the JHE activity of the prepupa.

HEXTAT has been optimized as a colorimetric surrogate substrate for JHE in larvae of the lepidopterous insect, *Heliothis virescens* (McCutchen et al., 1993). The pupal and the adult stages of *I. typographus* had less enzyme activity on HEXTAT than the third larval stage, the prepupa, and the late pupa. The *T. molitor* prepupae had about fourfold the activity of the pupae.

The level of α-NA activity during the development of *I. typographus* was on average 10^4 higher than the activity for JH III. Activity on α-NA increased during the larval phases and peaked in the third instar (520 nmol/min/mg). The activity decreased to 60 nmol/min/mg in the pupa and increased again in the late pupa to 417 nmol/min/mg. In brown and black adults, activity was below 200 nmol/min/mg. A similar trend was observed in *T. molitor:* the pupa had less than 50% of the activity of the prepupa. The I_{50} values for six esterase inhibitors measured in solution are listed in Table 3. Paraoxon was a surprisingly poor inhibitor of JH III hydrolysis in *T. molitor,* and in both species it was a more potent inhibitor of α-NA hydrolysis than on JH III hydrolysis. This suggests that at least some of the enzymes comigrating as esterase-2 on native PAGE and hydrolyzing α-NA can be inhibited without reducing JHE activity.

TABLE 2. Specific Esterase Activity of the Developmental Stages of *I. typographus* and *T. molitor* With JH III, HEXTAT, and α-NA[a]

Stage	Number of samples tested	JH III (pmol/min/mg)	HEXTAT (nmol/min/mg)	α=NA (nmol/min/mg)
Ips typographus[a]				
Larva 2	4	25.5 ± 5.1[b]	6.06 ± 0.67	257 ± 45
Larva 3	5	76.2 ± 9.8[c,d]	9.11 ± 1.78 n.s.	520 ± 78[d]
Prepupa	7	95.8 ± 3.8[d]	11.82 ± 2.34 n.s.	276 ± 27[d]
Pupa	6	80.5 ± 2.4[d]	1.42 ± 0.96[d]	60 ± 8[d]
Late pupa	9	82.6 ± 8.9 n.s.	7.11 ± 2.23[d]	417 ± 23[d]
Brown adult	11	12.1 ± 1.3[d]	1.92 ± 0.97[d]	197 ± 52[d]
Black adult	12	75.2 ± 8.6[d]	1.64 ± 0.22 n.s.	150 ± 32[e]
T. molitor				
Prepupa	8	1,700 ± 200	89.2 ± 7.7	134 ± 23
Pupa	7	6,700 ± 1,300[d]	24.3 ± 4.2[d]	61 ± 8[d]

*Data expressed in means ± S.D.

[a]Data are presented from homogenates of pooled organisms. The esterase activities of ontogenetic stages were also monitored on homogenates of single individuals with similar results.

[b]Results are from partition assay supported by TLC analysis from same samples.

[c]Mean values of the ontogenetic stages were compared by the Student-Newman-Keuls procedure. Each mean value was compared with the one of the previous stage.

[d]Significant at the 0.001 level.

[e]Significant at the 0.005 level.

TABLE 3. I_{50} of Six Inhibitors Acting on Enzymes Hydrolyzing JH III, HEXTAT, and α-NA From *I. typographus (I.t.)* and *T. molitor (T.m.)*[a]

	I_{50}[a] in M					
	JH III		HEXTAT		α-NA	
	T.m.	*I.t.*	*T.m.*	*I.t.*	*T.m.*	*I.t.*
OTFP	9×10^{-10}	6×10^{-7}	6×10^{-8}	4×10^{-7}	2×10^{-2}	8×10^{-2}
PTFP	2×10^{-8}	8×10^{-6}	9×10^{-8}	2×10^{-7}	4×10^{-3}	8×10^{-2}
DFP	4×10^{-3}	5×10^{-3}	3×10^{-4}	8×10^{-8}	4×10^{-4}	2×10^{-6}
PMSF	3×10^{-8}	8×10^{-5}	n.d.[b]	n.d.[b]	8×10^{-3}	5×10^{-3}
Paraoxon	9×10^{-2}	8×10^{-5}	n.d.[b]	n.d.[b]	2×10^{-5}	7×10^{-8}
TX100[c]	0.2%	0.6%	0.5%	0.3%	1.2%	2.4%

*Homogenate of whole pupae was used.
[a]Standard deviation did not exceed 8% of the reported values.
[b]Not determined.
[c]Percent (v/v) needed for 50% inhibition. Semilog plots of percentage inhibition against inhibitor concentration were used to estimate the I_{50}.

IEF of Crude Homogenates and Affinity Purified JHE

JHE was purified by affinity chromatography using the MTFO gel (Shiotsuki et al., 1994) resulting in a 49-fold purification; however, only 1.56% of the total JHE activity was recovered (Table 4). The purified proteins focused on IEF plates in two bands stained with Coomassie Blue (not shown) at pI 4.21 and 4.34, and α-NA staining for esterases catabolizing JH III revealed two bands between pIs 4.21 and 4.34 (Fig. 2). Due to the difficulty of measuring the pI of bands from analytical IEF precisely, these pIs should be taken as only relative indicators of mobility. When the gel was sliced into 2.5 mm pieces and JH III activity was measured, JHE activity was observed only at pI 4.34 for *I. typographus* and *T. molitor* homogenates and for the purified JHE of *T. molitor* (Fig. 2a). The same results were obtained on wide-range IEF (pI 4–9 gel) following analysis with α-NA and JH III and with protein staining by Coomassie Blue (not shown).

DISCUSSION

Induction of Esterases by JH

Of the four esterase isozymes active on α-NA that were found in the larval and adult homogenates of *I. typographus*, the proteins of isozymes 3 and 4 were absent in pupae but could be induced by topical application of JH III. JH induction of esterases is not without precedent. Whitmore et al. (1972)

TABLE 4. Affinity Purification of JHE From Whole Body Homogenate of *T. molitor*

	Total protein (mg)	Specific activity (μmol/min/mg)	Total activity (μmol/min)
Homogenate	855	7.1	6,070
After purification	0.27	350[a]	94.5

[a]Enzyme is likely partially inhibited by the trifluoroketone used to remove it from the affinity column.

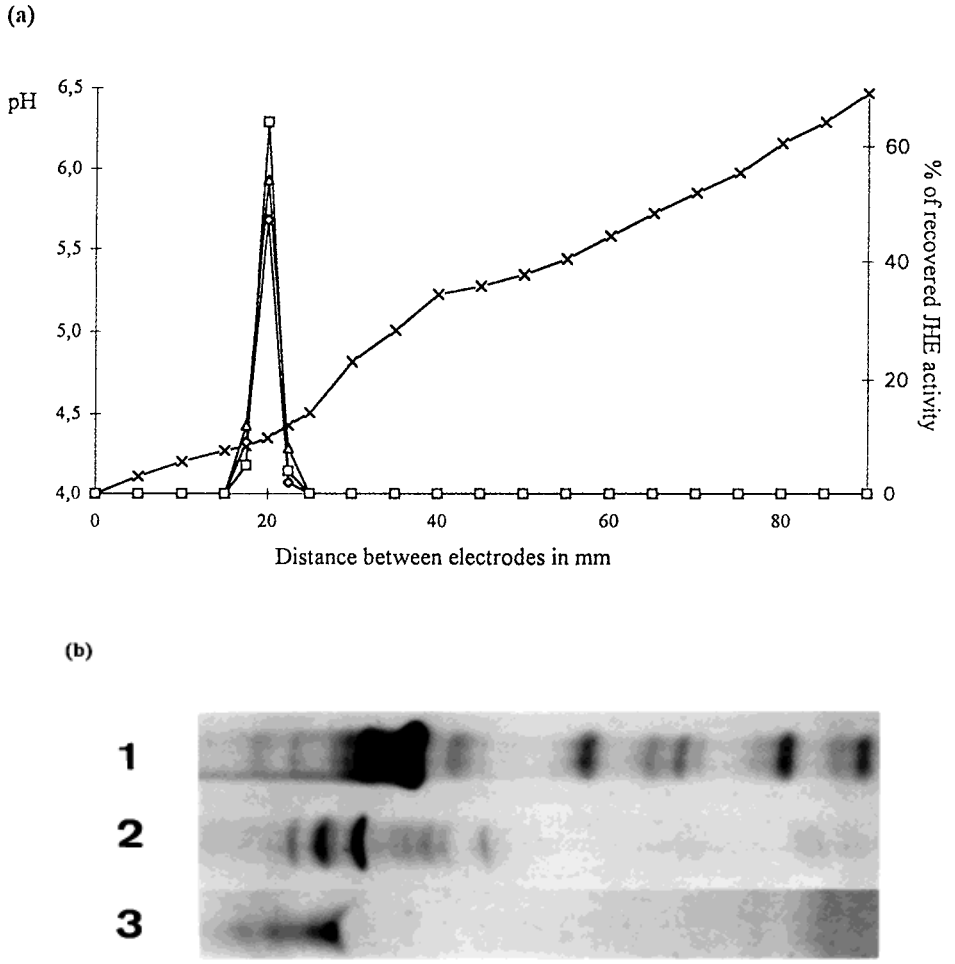

Fig. 2. Narrow range IEF stain and JHE activity. **a:** IEF analysis of whole body homogenates of pupae of *I. typographus* (◇) and of *T. molitor* (△) and affinity-purified JHE of *T. molitor* (□). Samples were focused on a narrow range (pH 4.0–6.5) gel, and gel slices were incubated for 2 h either with assay buffer containing 0.1 mg/ml BSA for JHE activity determination or with 20 mM KCl solution for pH (x) determination. Over 80% of the catalytic activity applied was recovered. **b:** Zymogram of whole body homogenates of pupae of *I. typographus* (lane 1) and of *T. molitor* (lane 2) and affinity-purified JHE of *T. molitor* (lane 3). Samples were focused on a narrow range (pH 4.0–6.5) gel. Approximately 40 μg of protein was loaded on lanes 1, 2. Gels were stained with Fast Blue RR using α-NA as substrate.

observed that in *Hyalophora gloveri* JH III induced the appearance of specific multiple molecular forms of carboxyl esterase that degraded α-NA. Sparks and Hammock (1979) investigated an artificially induced peak as well as a naturally occurring peak of JHE in last instar larvae of *Trichoplusia ni.* The two peaks appeared to be largely due to one esterase distinct from the majority of the esterases acting on α-NA. Campbell et al. (1992) investigated JHE and esterases hydrolyzing α- and β-NA from *Drosophila melanogaster* us-

ing native PAGE and inhibitor studies. They found no similarities between JHE and the esterase isozymes hydrolyzing α- and β-NA. Parenthetically, those authors also noted that the activity of carboxyl esterases in *D. melanogaster* can be increased by application of olive oil, whereas no quantitative or qualitative effects of the solvents olive oil or acetone were detected on the esterases of *I. typographus*.

In our study, the JH-inducible isozymes 3 and 4 were more heavily stained with α-NA than isozymes 1 and 2; isozymes 3 and 4 were visible after only 3 min, while isozymes 1 and 2 became evident after 15 min of exposure to α-NA. In *L. decemlineata*, a group of JH esterases was clearly separated from the other carboxylesterases by native PAGE, and these JHE proteins stained weakly with α-NA (Kramer and de Kort, 1976). In fact, in most investigations with lepidopteran insects, α-NA had a poor K_m with JHE but was slowly turned over (Hanzlik and Hammock, 1987). Thus, staining for α-NA activity does not mean that an enzyme is incapable of metabolizing JH, but the correlation is not positive. On the other hand, the pupae of most insects are thought to have no JH during normal metamorphosis to the adult stage (Nijhout and Williams, 1974), and this induction of isozymes 3 and 4 was initially interpreted as a response enabling the specific inactivation of the topical JH III.

In order to clarify the functional roles of the four α-NA esterases of *I. typographus*, we performed experiments to determine the JH activity and the inhibition patterns of these isozymes. Esterase activity was measured using (^{3}H)-JH III as a substrate, after native PAGE separation of whole body homogenate proteins. For all developmental stages tested, the esterase activity on JH III activity comigrated with the proteins of isozyme 2. Furthermore, isozymes 1 and 2 appeared to differ from the two other isozymes in their reaction to the panel of five inhibitors; the most obvious differences among the isozymes were detected with OTFP, PTFP, and DFP. These results might suggest that isozyme 2 hydrolyzes JH III. However, it is possible that the enzyme which hydrolyzes JH III simply comigrates with isozyme 2. This latter hypothesis is supported by the observation that paraoxon partially inhibited the esterase activity on α-NA without inhibiting JHE in the isozyme 2 region of the gel. With respect to isozymes 3 and 4, our results show that these esterases apparently cannot catabolize JH III at a detectable rate. These data caution against the assumption that esterases induced by JH are necessarily involved in the metabolism of JH.

Analysis of JH Metabolism—Technical Considerations

A partition assay was used to generate the data on JH III metabolism presented in the figures and tables of this paper. It has been previously cautioned that a partition assay for either JHE or JH epoxide hydrolase (JHEH) is dangerous to use unless it is already known that a single pathway dominates the metabolic picture (Hammock and Roe, 1985). Thus, chromatographic systems were used to determine the qualitative nature of the JH metabolites of the two coleopteran species and to validate quantitative data obtained with the partition assay. It was first necessary to obtain authentic standards. To this end, JH III acid, JH III diol, and JH III diol acid were produced enzymatically, and these standards were shown to cochromatograph on TLC and

HPLC with chemically produced standards both as free metabolites and as derivatives. Since the chemically produced and enzymatically produced standards were chromatographically indistinguishable, we then used enzymatically produced standards routinely. This procedure allowed us to make the standards in small amounts near the time that the experiments were being run rather than be concerned about storage.

No chromatographic technique can unequivocally identify a metabolite, but rigorous chromatography can be used to support a tentative identification. We used TLC as a matter of general protocol, which offers the advantage of allowing multiple parallel studies to be performed at one time. In every case the only JH III metabolite that we observed always cochromatographed with JH III acid (which was clearly resolved from JH III diol and JH III diol acid on TLC plates). This metabolite reacted with acid, suggesting the presence of an epoxide, and with diazomethane, suggesting the presence of a carboxylic acid. It failed to react with BBA, indicating the absence of 1,2 or 1,3 groups capable of forming a diester with BBA. In both insects the JHE activity as determined by partition assay agreed with TLC determinations, whether the data were collected by a position scanner or by scraping and liquid scintillation counting.

The metabolite standards for JH III are expensive to use because their extinction coefficient is low and is at a wavelength which quenches fluorescent TLC indicators poorly. Thus, colored or UV dense standards have been suggested as a visual indicator of the mobility of compounds in individual TLC lanes and as mass carriers of JH metabolites (Hammock et al., 1985). Here the use of methoprene and methoprene acid proved fortuitous because of their high extinction coefficients, because their maximum absorbance quenched the plate fluorescence well, and because these compounds showed an R_f indistinguishable from JH III and JH III acid, respectively. Thus, while above the location of the radioactivity was determined by a scanner, the methoprene and methoprene acid served as visual indicators of the quality of the TLC chromatograph. However, it is certain that methoprene and methoprene acid could be separated from JH III and its metabolites under other conditions given their structural differences, so they should not be used as standards for other chromatographic systems.

HPLC often offers higher resolution than TLC, and a reversed phase technique separates compounds on a different principle than does TLC. Therefore, additional support for the assignment of the structure of the JH III metabolite was obtained by cochromatography on HPLC using the reverse phase method of Halarnkar and Schooley (1990). HPLC results supported the qualitative assignment of JH acid as the only detectable metabolite of JH III. It was disappointing that recovery of the JH III acid was poor by this technique, so that there was not a good correlation between HPLC data and the quantitative partition and TLC data. However, the recovery was excellent when the JH III acid was first derivatized with diazomethane. Surprisingly, the BBA diester of JH III diol was stable under the reverse phase conditions, and HPLC presented itself as another technique for testing the hypothesis that chemical and enzymatic methods for forming standards for JH III metabolism studies yield the same compounds.

JH Metabolism in Two Coleopteran Species

As discussed above, JHE appears to dominate the metabolism of JH under the conditions of this study. TLC data qualitatively and quantitatively supported the partition data presented in this study. Chemical derivatization and HPLC analysis also supported that JH III acid was the only metabolite detected in these studies. One would expect membrane-bound JHEH to be removed by gel filtration. In *I. typographus*, the JHE activity was similar throughout the life cycle. The highest activity was found in the prepupae with 95.8 pmol / min / mg. In the brown adult, however, the activity decreased to 12.1 pmol / min / mg, or about 85% less activity than in the late pupae. Young adults build up their sexual organs (Vyplel, 1983), and the JH titer is hypothesized to be high, whereas the JHE titer should be low. During rearing of *I. typographus* in logs, we tried to collect as many different days of one stage as possible. However, specific stages with distinguishable JHE peaks were not observed. Several studies have suggested that two enzymes, JHE and JHEH, are responsible for the degradation of JH in homogenates of *T. molitor* (Edwards and Rowlands, 1977; Sparks and Hammock, 1980; Connat, 1983).

Surrogate Substrates and Inhibitors of Esterases

The pupa of *I. typographus* had a very low α-NA esterase activity. This corresponds with the native PAGE findings, where only isozymes 1 and 2 were stained; α-NA activity (60 nmol / min / mg) seems to be contributed mainly by the two slower running isozymes. A similar trend was observed comparing activities for HEXTAT and α-NA. Hydrolysis of both substrates showed the same 66% decrease in the pupal stage and shortly afterwards a five- to sevenfold rise in the late pupae. From these and other data, it can be concluded that HEXTAT and α-NA may be hydrolyzed by the same enzymes.

All of these results emphasize that JHE(s) should be defined as esterase(s) which degrades JH. We should not define JHE based on its ability or inability to degrade surrogate substrates such as α-NA or HEXTAT or by whether it is inhibited or resists inhibition by OTFP, DFP, paraoxon, TX100, and other compounds. Once JHE substrate specificity and inhibitor sensitivity are determined for a species, surrogate substrates and inhibitors can then be useful tools.

For *I. typographus* larvae and adults, the data in Tables 1 and 3 together indicate that the majority of α-NA activity is contributed by isozymes 3 and 4 rather than 1 and 2. These results and those from IEF are also consistent with JHE activity not being associated with isozymes 1, 3, and 4. The three isozymes might catabolize toxic substances which are taken up with the bark. Slama (1969) and Purtich and Nijhout (1974) observed juvenoids in the bark of conifer species, and Brattsten (1988) suggested that these lipophilic substances are hydrolyzed by general esterases. Pupae are not feeding, and they might not have a need for such enzymes.

The results in Table 3 further indicate that it is not valid to extrapolate among species regarding esterase inhibitors. OTFP is a very potent inhibitor of JHE from *T. molitor* but only a moderately potent inhibitor of JHE from *I. typographus*. Table 3 clearly shows that the esterases responsible for the majority of α-NA hydrolysis are highly resistant to OTFP and that these isozymes

contribute little to hydrolysis of JH III. PTFP, with a shorter carbon chain than OTFP, is a slightly less potent JHE inhibitor, as expected. As observed in *M. sexta* (Sanburg et al., 1975), DFP is a poor inhibitor of JHE activity in both *T. molitor* and *I. typographus*. However, it is a potent inhibitor of HEXTAT hydrolysis in *I. typographus*. This observation indicates that HEXTAT is a poor selection as a surrogate JH III substrate in crude homogenates of this species, although it proved to be a good surrogate JH substrate with hemolymph of *M. sexta* and *H. virescens* (McCutchen et al., 1993). In addition, inhibition of JHE by PMSF is known to vary greatly in different species: it is a good inhibitor of JHE from *M. sexta* and *H. virescens* but not a potent inhibitor of JHE in *T. ni*. (Hammock, 1985). We show here that it is a potent inhibitor of the JHE in *T. molitor* and a moderate inhibitor of the *I. typographus* enzyme.

Paraoxon is known to be a potent inhibitor of JHE from a variety of species (Hammock, 1985), but, as in the study of Sparks and Hammock (1980), it had little effect on JHE activity in *T. molitor* in our study. This observation could be exploited for affinity purification of JHE from this species, allowing for inhibition of many general esterases and proteases in crude homogenates by paraoxon (or DFP) before applying them to the esterase affinity column. On the other hand, paraoxon is only a moderate inhibitor of JHE in *I. typographus* and of α-NA hydrolyzing enzymes in both species. This large difference in inhibitory potency with paraoxon supports the hypothesis that those enzymes responsible for the majority of JHE hydrolysis are different from the esterases responsible for most α-NA hydrolysis.

In contrast to observations in *L. decemlineata* (Kramer and de Kort, 1976), concentrations of TX100 did not strongly inhibit any of the esterase activities detected in this study, indicating that these esterases are resistant to denaturation by this surfactant and that it could be used in affinity purification of the esterases. Another possibility is that TX100 inhibited the JHE activity of *L. decemlineata* by sequestering JH. If this is the case, then one can conclude that the JHEs of *I. typographus* and *T. molitor* can extract JH from the resulting detergent micelle more effectively than the JHE of *L. decemlineata*.

Affinity Purification of JHE

JH metabolism has not been well studied in species outside of the lepidoptera. Several species of Coleoptera, including *Ips sp*, are of economic importance. Esterases have been used to monitor populations, but it is also important to determine their role in metamorphosis. Attempts to use genes from lepidopteran larvae to probe for coleopteran JHE have not been successful. Thus, the large and well-studied *T. molitor* is being used for purification efforts in hopes that it will yield antibody and oligonucleotide probes useful with other species.

To verify that the proteins of EST-2 hydrolyze JH III and α-NA as described in Materials and Methods, we purified JHE from *T. molitor* pupae. Previous studies on affinity purification of JHE from *H. virescens* have shown that the apparent affinity of the enzyme for the affinity gel as well as the capacity of the gel are both dependent upon ligand concentration and structure (Shiotsuki et al., 1994). Thus, one would expect relatively lower affinity of JHE for the MTFO gel because it lacks a β-thioether. Conversely trifluoromethyl ketones

with β-thioethers are very powerful inhibitors of the JHE of *T. molitor,* so that gels with ligands containing the thioether should have higher affinity for the esterase than MTFO—while presenting more difficulty in recovering the esterase from the column (Abdel-Aal and Hammock, 1985b; Shiotsuki et al., 1994). If one used a powerful inhibitor like OTFP to remove the JHE from such a column, it would then be difficult to reactivate the eluted enzyme from the inhibitor complex. However, this system would be useful in isolating large amounts of protein for raising antibodies and for peptide digestion studies.

With PTFP as a relatively weak eluting agent, little protein was removed from the MTFO-affinity gel, but it was possible to partially reactivate the enzyme. Only 49-fold purification was achieved even though protein stains of IEF gels indicated a high degree of purity. The low recovery (1.56%) of JHE activity is probably caused by low regeneration of active enzyme from the esterase-PTFP complex. Alternatively, reduced stability after purification might have decreased the specific activity of the purified enzyme. Other affinity gels that are available (e.g., a gel with an aromatic ring moiety (Shiotsuki et al., 1994) which resembles α-NA) might have yielded a better recovery. Enough JHE from *T. molitor* was purified to demonstrate that the JHE activity of the purified protein had the same pI of 4.34 (estimated) as the JHE activity of *I. typographus* and of *T. molitor* homogenates.

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