

## PROPERTIES OF ESTERASES FROM PHARATE PUPAL MOULTING FLUID OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*\*

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**Abstract**—1. Three esterolytic fractions (I–III) were partially purified by chromatographic procedures from pharate pupal moulting fluid of the tobacco hornworm, *Manduca sexta* L.

2. Maximum activity toward 1-naphthyl acetate occurred at pH  $\geq 8$  for I and between pH 6 and 7 for II and III.

3. The apparent Michaelis constants for 1-naphthyl acetate were 145,  $\geq 500$  and 46  $\mu\text{M}$  for I, II and III, respectively.

4. I and III were inhibited by diisopropylphosphorofluoridate. II was inactivated by *p*-chloromercuribenzoate.

5. I also hydrolyzed acetylthiocholine and this activity was inhibited by eserine.

6. While all of the fractions hydrolyzed juvenile hormone, III was greater than 10 times more active than the other enzymes.

7. These results suggest that I is a mixture of carboxylesterases and cholinesterases, that II is an arylesterase, and that III contains carboxylesterase and JH esterase enzymes.

### INTRODUCTION

Practically all insect tissues contain enzymes that have activity towards esters of aromatic alcohols (Ahmad, 1970; Katzenellenbogen & Kafatos, 1971; Breigel & Freyvogel, 1973; Gadallah & Marei, 1973; Hipps & Nelson, 1974; Kramer & Childs, 1977; Turunen & Chippendale, 1977; Brick *et al.*, 1979; Tanada *et al.*, 1980). The endogenous function of these enzymes is for the most part unknown. Nevertheless, a thorough study of these enzymes from moulting fluid could add to our understanding of the regulation of the moulting process and reveal several possible control points that might serve as targets for insecticide development. With this in mind, three esterase fractions from pharate pupal moulting fluid of the tobacco hornworm, *Manduca sexta* L., were partially purified and characterized. Two of these (I and III) contain carboxylesterases that have properties similar to the esterases found in silkworm moulting fluid (Katzenellenbogen & Kafatos, 1971). I also hydrolyzes a choline ester and III cleaves juvenile hormone. The third enzyme (II) is probably an arylesterase.

### MATERIALS AND METHODS

#### *Insects and fluid collection*

Eggs of *Manduca sexta* (L.) were a gift from the Metabolism and Radiation Laboratory, Agricultural Research Service, US Department of Agriculture, Fargo, North Dakota. Larvae were reared on a standard diet (Bell & Joachim, 1976) and kept at 28°C and 60% r.h. during a 16:8 hr light:dark photoperiod.

Moulting fluid was taken from pharate pupae when the animals were at the thoracic bar stage (Reinecke *et al.*, 1980). A capillary was inserted between the old and new cuticles at the head capsule and the moulting fluid drawn up; care was taken to avoid contamination with haemolymph. The samples were diluted (1:1 v/v) with 5 mM Tris, pH 8.5 buffer containing  $\sim 10^{-5}$  M phenylthiourea (added to inhibit tyrosinase activity). Samples of moulting fluid were used immediately or frozen. Haemolymph was collected from day-2 pupae by cutting off the proboscis and expressing the fluid into a beaker chilled on dry ice.

#### *Gel filtration chromatography*

Sephacryl S-200 and S-300 gel permeation columns (1.5 cm i.d.  $\times$  100 cm) were equilibrated at 25°C in 0.1 M ammonium bicarbonate, pH 8.5. Crude moulting fluid was first chromatographed at 4°C on Sephacryl S-200 and fractions were analyzed for protein content (280 nm absorbance) and esterase activity (see section on assay methods). Esterase I was subjected to a second chromatography on Sephacryl S-300 after hydroxylapatite and/or anion-exchange chromatography (see below).

#### *Hydroxylapatite chromatography*

Hydroxylapatite, HTP Grade (Bio-Rad Labs) was hydrated in 5 mM sodium phosphate, pH 6.5 buffer, poured into a column (2.5 cm i.d.  $\times$  22 cm) and equilibrated at 27°C with the same buffer. Pooled fractions from gel filtration were prepared for HA chromatography by reducing

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the conductivity to less than  $1 \times 10^3 \mu\text{mho}$  and the pH to 6.8 by ultrafiltration of the sample using an Amicon PM-10 membrane and the equilibrating buffer. Adsorbed protein was eluted at  $4^\circ\text{C}$  with a linear gradient of sodium phosphate from  $2.5 \times 10^3$  to  $25 \times 10^3 \mu\text{mho}$ . The absorbance at 280 nm, conductivity, and 1-naphthyl acetate hydrolytic activity of the fractions were measured. Active fractions were pooled and stored at  $4^\circ\text{C}$ .

#### DEAE chromatography

The enzyme (I) which eluted from the hydroxylapatite column at  $5 \times 10^3 \mu\text{mho}$  was pooled and concentrated by ultrafiltration using 5 mM Tris, 10 mM NaCl, pH 7.0 buffer. A 1.5 cm i.d.  $\times$  70-cm DEAE-Biogel column (Bio-Rad Lab) was equilibrated at  $4^\circ\text{C}$  with Tris buffer (5 mM Tris, 10 mM NaCl, pH 7.0). Elution of adsorbed proteins was achieved by using a linear NaCl gradient formed from equal volumes of the wash buffer and a high salt buffer (5 mM Tris, 200 mM NaCl, pH 8.5). Fractions were tested for 1-naphthyl acetate hydrolytic activity, absorbance at 280 nm and conductivity.

#### Enzyme assays

1-Naphthyl acetate (1-NA, Sigma) hydrolytic activity of chromatographic fractions was determined spectrophotometrically (Katzenellenbogen & Kafatos, 1970). Aliquots of column eluent were added at  $27^\circ\text{C}$  to 1 ml of 0.05 M phosphate buffer pH 6.5 containing  $5 \times 10^{-4}$  M 1-NA and 0.5 mg/ml fast red TR salt. The red colour resulting from the conjugate of the product 1-naphthol and the diazonium compound was measured at 625 nm. 1-NA hydrolytic activity was expressed either as the increase in absorbance at 625 nm in 30 min/50  $\mu\text{l}$  fraction of mg protein or as  $\mu\text{mole}/\text{min}/\text{mg}$  protein. For kinetic experiments a continuous spectrophotometric assay was used with 1-NA ( $3 \times 10^{-5}$  M) in 0.1 M phosphate buffer pH 6.5. The enzyme activity was monitored at 321 nm.

Juvenile hormone hydrolytic activity was measured using JH-I as substrate by the thin-layer chromatographic procedure of Kramer & Childs (1977). The inhibition of JH hydrolytic activity of the hydroxylapatite fractions was tested using diisopropylphosphorofluoridate (DFP, Sigma), *p*-chloromercuribenzoate (PCMB, Sigma) and eserine (Sigma) after preincubation with the inhibitor at  $27^\circ\text{C}$  for 30 min.

Acetylcholine esterase activity was measured using acetylthiocholine as substrate by the method of Ellman *et al.* (1961).

#### Polyacrylamide gel electrophoresis

Samples were subjected to electrophoresis at pH 8.5 in 7% gels to assess purity under non-denaturing conditions (Davis, 1964). 1-NA hydrolytic activity was detected according to the procedure of Katzenellenbogen & Kafatos (1970). Protein was visualized after fixation in 5% (w/v) trichloroacetic acid by staining with 0.25% (w/v) Coomassie Brilliant Blue R-250 dissolved in 45% (v/v) methanol, 9% (v/v) acetic acid solution. Destaining was achieved with several washes of 25% (v/v) methanol:7.5% (v/v) of acetic acid solution.

#### Protein assay

Protein assays were performed using the method of Lowry *et al.* (1951) or by absorbance at 280 nm using the conversion factor of 1.0 absorbance unit equal to 1 mg protein/ml.

## RESULTS

#### Fractionation of esterases

Gel filtration on Sephacryl S-200 was the first step used to separate the 1-naphthyl acetate hydrolyzing esterases present in moulting fluid from pharate pupae of *M. sexta*. A single peak of esterase activity was observed that had an elution volume between that of bovine serum albumin and the void volume (data not shown). The esterase fractions were pooled and subjected to hydroxylapatite (HA) chromatography at pH 6.8. Three peaks of esterase activity were eluted with a sodium phosphate gradient (Fig. 1). These were EI (84% of total activity), fractions 35–58; EII (4%), fractions 125–128; and EIII (12%), fractions 166–171.

The major esterase peak (EI) from the hydroxylapatite column was further purified by chromatography using DEAE-Biogel at pH 7.0. A single peak of esterase activity was eluted at 0.16 M NaCl (data not shown). After pooling the active fractions, EI was subjected to chromatography on Sephacryl S-300 at pH 7.5. Here a single peak of activity was eluted after the void of the column.

The purification of 1-naphthyl acetate esterase fractions is summarized in Table 1. The final purifications

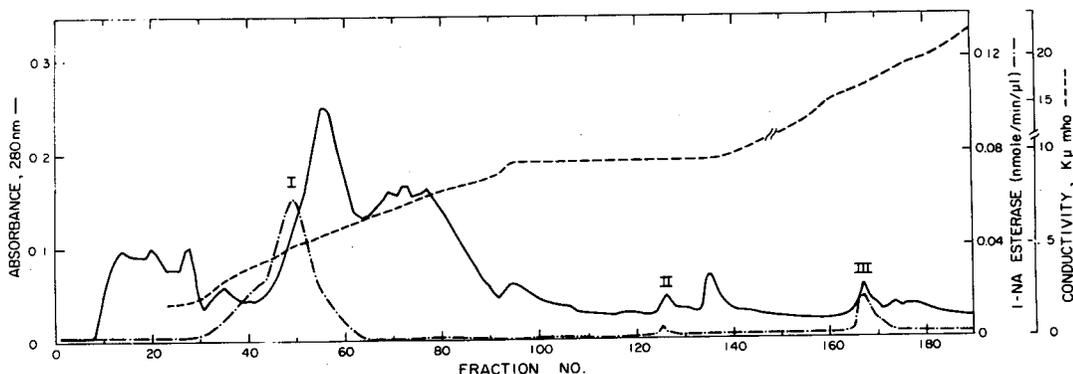


Fig. 1. Hydroxylapatite chromatography of *M. sexta* moulting fluid esterase fraction from Sephacryl S-200 chromatography. Equilibration buffer was 5 mM sodium phosphate, pH 6.8. Protein was eluted at room temperature with a phosphate gradient to 0.3 M. Symbols: absorbance at 280 nm, —; 1-NA hydrolytic activity, - - -; conductivity, ····.

Table 1. Purification of esterase fractions I, II and III from *M. sexta* moulting fluid

Step	Total units	Total protein (mg)	Specific activity	Overall yield (%)	Overall purification
1. Crude moulting fluid	7.46	120	0.06	100	1
2. S-200 chromatography	4.15	17	0.17	56	4
3. Hydroxylapatite chromatography					
I	0.75	2	0.43	10	6.8
II	0.03	0.17	0.18	0.4	2.9
III	0.11	0.12	0.93	1.5	15.0
4. Anion-exchange chromatography					
I	0.25	0.3	0.82	3.7	13.2
5. S-300 chromatography					
I	0.76	0.2	3.78	2.1	60.8

\* Unit =  $\mu\text{mol}/\text{min}$ .

were approx 60-, 3- and 15-fold with overall yields of 2.1, 0.4 and 1.5% for EI, EII and EIII, respectively.

#### Polyacrylamide gel electrophoresis

The heterogeneity of the enzyme fractions was examined by polyacrylamide gel electrophoresis followed by esterase and protein staining procedures. Pharate pupal moulting fluid exhibited five enzymatically active bands. A similar analysis of the purified esterase fractions revealed that EI consisted of at least three enzymes the major one having a relative mobility of 0.35 and that both EII and EIII exhibited only a single esterase band with  $R_m = 0.08$  and 0.32, respectively. Coomassie Blue staining for protein revealed that all esterase fractions were heterogeneous. A similar analysis of *M. sexta* haemolymph showed that 7 or 8 1-NA esterases are present and that 5 of these have mobilities identical to those detected in moulting fluid.

#### Stability and pH optimum

In general the esterases were stable at various stages of purification for one week at pH 8 and 4°C. However, the most purified preparations exhibited a 20–35% decrease in activity after two weeks of storage. Freezing did not inactivate EI, but EII lost 35% and EIII 65% of their activities after freezing. EI was stable during lyophilization while EII and EIII were partially (80%) and totally inactivated, respectively. Although EI was completely stable for several weeks at pH 8, it lost 30% activity after 30 min at pH 5 and 7. EII was most stable at pH 7 while EIII was stable at pH 5, 7 and 8. The pH optimum for EII and EIII was approx 7 while that of EI was  $\geq 8$ .

Table 2. Kinetic parameters for 1-naphthyl acetate hydrolysis by esterase fractions from *M. sexta*

Enzyme	$V_{\max}$ (nmol/min/ml $\times 10^3$ )	$K_m$ ( $\mu\text{M}$ )	$V_{\max}/K_m$ ( $\text{min}^{-1}$ )
I	$32 \pm 7$	$145 \pm 17$	0.221
II	$6 \pm 2$	$\geq 445$	$\leq 0.012$
III	$10 \pm 3$	$46 \pm 11$	0.223

#### Kinetics

Analysis of initial velocity experiments by the Lineweaver–Burk method generated the  $V_{\max}$  and apparent  $K_m$  values listed in Table 2. EIII had the lowest Michaelis constant for 1-naphthyl acetate, followed by EI and then EII. Upward curvature of the Lineweaver–Burk plot was observed with EII. This behaviour was not exhibited by the other enzyme fractions. Apparently EII is susceptible to substrate inhibition or to denaturation caused by the trace amounts of methanol added to the reaction mixture to help solubilize the substrate.

Juvenile hormone hydrolytic activity was also measured for the individual esterase fractions. Conditions were established so that all of the fractions tested exhibited a comparable level of 1-NA hydrolytic activity, 0.3–0.4  $\mu\text{mol}/\text{min}/50 \mu\text{l}$  fluid (Fig. 2). The relative JH hydrolytic activity of EIII (38  $\mu\text{mol}/\text{min}/50 \mu\text{l}$ ) was more than 10-times greater than those of EI or EII ( $< 3 \mu\text{mol}/\text{min}/50 \mu\text{l}$ ) under these conditions. These results suggested that a JH esterase is present in EIII.

The esterases were also tested for acetylcholinesterase activity. Only EI exhibited a low level of activity toward acetylthiocholine (0.5  $\mu\text{mol}/\text{min}/50 \mu\text{l}$ ).

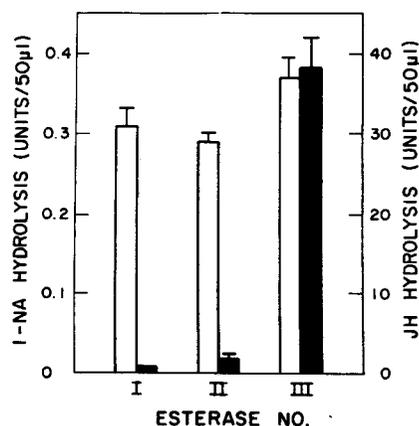


Fig. 2. Relative 1-naphthyl acetate (open bar) and JH I (shaded bar) hydrolytic activity of esterase fractions from *M. sexta* moulting fluid. Units =  $\mu\text{mol}/\text{min}$ .

Other potential substrates tested included two amino acid esters, benzoylarginine ethyl ester and benzoyltyrosine ethyl ester. Neither of those compounds were cleaved by the fractions.

The effects of several potential inhibitors of the 1-naphthyl acetate esterases were also measured. Esterase I was inhibited completely by diisopropylphosphorofluoridate (DFP,  $5 \times 10^{-4}$  M), *p*-chloromercuribenzoate (PCMB,  $\sim 10^{-4}$  M) and partially by eserine (40% at  $5 \times 10^{-5}$  M). Esterase II was inhibited only by PCMB and not by DFP or eserine. The 1-NA hydrolytic and JH hydrolytic activities of EIII were completely and partially (28%) inhibited by DFP, respectively. PCMB had no effect.

Based on the substrate and inhibitor specificities of the esterase fractions, they can be classified as follows: EI appears to be a mixture of carboxylesterases and cholinesterases. EII probably contains an arylesterase, while the EIII fraction is probably a mixture of one or more carboxylesterases and JH esterases.

#### DISCUSSION

We have partially purified three esterolytic enzyme fractions from *M. sexta* pharate pupal moulting fluid by chromatographic procedures. On the basis of electrophoretic mobility, the same or similar enzymes appear to be present in haemolymph and we also have preliminary chromatographic evidence that they occur in scraped cuticle. Hydroxylapatite chromatography was used to separate similar (perhaps identical) enzymes from the latter tissue (data not shown).

For the most part the function of these enzymes in moulting fluid, haemolymph and cuticle is obscure because the natural substrates are unknown. However, some of the esterase fractions can be classified according to established nomenclature which is based on substrate and inhibitor interactions (Dixon & Webb, 1979). Because EII was inhibited by mercurials and was insensitive to organophosphates, it may be an arylesterase or "A-esterase". This is the first report where this type of enzyme has been detected in moulting fluid. Fractions I and III were inactivated by organophosphate, so that these fractions are probably carboxylesterases. EI also hydrolyzed a choline ester and was partially inhibited by eserine. Thus a cholinesterase may be present there. EIII was the most active fraction toward juvenile hormone so it may contain a JH esterase. This is the first report of moulting

fluid esterases degrading acetylcholine and juvenile hormone. The true identity of these enzymes must await their isolation and more detailed biochemical and physiological analyses.

There are general similarities between the esterases from *M. sexta* and those obtained from other invertebrate species. Silkworm moulting fluid and mosquito whole body extract exhibited esterase electrophoretic profiles similar to that of the hornworm (Katzenellenbogen & Kafatos, 1971; Houk *et al.*, 1978). Also the apparent  $K_m$  values of the hornworm fractions I and III for 1-naphthyl acetate were similar to those of other esterases (Table 3). However, the Michaelis constant of *M. sexta* esterase II was much higher than any other value previously reported.

Two esterase fractions were characterized from silkworm moulting fluid (Katzenellenbogen & Kafatos, 1971). Those were termed carboxylesterases because of their reactivity towards 1-naphthyl acetate and sensitivity to inhibitors. Two of the esterase fractions from *M. sexta* behaved in a similar manner. In addition, one was active toward acetylthiocholine and the other toward juvenile hormone. The silkworm esterases were not examined for activity toward the latter two esters. Amino acid ester substrates were not hydrolyzed by esterases from either insect species.

From all of the above it can be seen that multiple forms of esterases which hydrolyze 1-naphthyl acetate occur in several different species and tissues. Each enzyme no doubt performs a special function in the insect's life cycle. Perhaps the moulting fluid esterases metabolize esters found in cuticular lipids, waxes and hormones. Inhibitors of the esterases described here may disrupt moulting and perhaps other morphological events as well.

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Table 3. Comparison of Michaelis constants for 1-naphthyl acetate hydrolysis by esterase fractions

Species	Tissue source	$K_m$ ( $\mu$ M)	Reference
<i>Lymantria dispar</i>	Midgut	One enzyme: 43	Kapin & Ahmad (1980)
<i>Manduca sexta</i>	Haemolymph	Two enzymes: 17 and 190	Sanburg <i>et al.</i> (1975)
	Moulting fluid	Three enzymes: 46, 145 & $\geq 445$	This paper
<i>Periplaneta americana</i>	Midgut and gastric caecum	Six enzymes ranging from 27 to 128	Hipps & Nelson (1974)
<i>Tegenaria atrica</i>	Digestive juice	Six enzymes: 120-260	Mommsen (1978)

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