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JUVENILE HORMONE ESTERASES IN TWO HELIOTHINES: KINETIC, BIOCHEMICAL AND IMMUNOGENIC CHARACTERIZATION

YEHIA A. I. ABDEL-AAL,* TERRY N. HANZLIK,† BRUCE D. HAMMOCK,† LAWRENCE G. HARSHMAN† and GLENN PRESTWICH‡

*Department of Plant Protection, College of Agriculture, Assiut University, Assiut, A.R. Egypt, †Departments of Entomology and Environmental Toxicology, University of California, Davis, CA 95616, USA (Tel.: 916-752-7519) and ‡Department of Chemistry, State University of New York, Stony Brook, NY 11794, USA

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Abstract—1. Juvenile hormone hydrolyzing activity in larval hemolymph of the tobacco budworm, *Heliothis virescens*, and the corn earworm, *H. zea*, was characterized by several biochemical approaches.

2. Evidence for enzyme differences between species came from using several alkylthiotrifluoropropanones as selective inhibitors.

3. The enzyme purified by affinity chromatography from *H. zea* showed two bands on SDS-PAGE and two activity peaks on narrow range (pH 4–6.5) isoelectric focusing (IEF), while purified enzyme from *H. virescens* showed only one band on SDS-PAGE and one peak of activity on IEF.

4. ELISA tests using polyclonal antisera elicited by the purified enzyme from each species showed the enzyme from *H. virescens* and *H. zea* to be antigenically distinct.

5. A kinetic analysis of enzyme from both species showed that the slow tight binding kinetics, often observed with inhibition by transition state analogs, were both compound and enzyme dependent.

INTRODUCTION

Ester hydrolysis appears to be the major route of juvenile hormone (JH) metabolism in the lepidopteran larvae examined (Hammock, 1985). At least three lines of evidence have shown that metabolism by JH esterases (JHEs) is a well regulated function that is closely related to the cascade of events leading to pupation. First, in the last stadium of *Trichoplusia ni*, there is a significant negative correlation between JH-titer (Jones, 1985) and JHE activity (Sparks *et al.*, 1979). Second, when the esterase activity was thoroughly inhibited, JH-like effects, i.e. extension of the feeding phase and delay of pupation, were observed (Sparks and Hammock, 1980; Hammock *et al.*, 1984; Prestwich *et al.*, 1984). Third, when the purified JHE from *Manduca sexta* was injected into larvae of *T. ni* or *M. sexta* during a sensitive period, apparent anti-JH effects were obtained (unpublished data, this laboratory). In this respect, JHE appears to have not only an important biological function but also a mechanism for its own regulation (Jones *et al.*, 1981). If this were the case one might expect the enzyme to be highly conserved, especially between closely related species.

Comparative studies of homologous enzymes can provide valuable taxonomic data. Hardwick (1970) has constructed a phylogenetic analysis of the Heliothinae and was the first (Hardwick, 1965) to establish the corn earworm complex as a separate genus (*Helicoverpa*). Molecular characters may assist in the taxonomy of groups which have considerable morphological resemblance. Variation in juvenile hormone esterase may be useful for the often difficult classification of the Heliothinae.

Address correspondence to B. D. Hammock.

The JHEs from *H. virescens* and *H. zea* were studied with respect to their interaction with substrates, their sensitivity to inhibition by alkylthiotrifluoropropanones, their M_r as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), their pI as determined by isoelectric focusing (IEF) and their immunogenic cross-reactivity. Alkylthiotrifluoropropanones are putative transition state mimics for esterases. These inhibitors and the availability of JHEs from two related species permitted experiments to be run to evaluate if the slow tight binding phenomenon commonly associated with transition state mimics is dependent upon the enzyme and/or the inhibitor used.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Aldrich Chemical Co., unless otherwise stated. Unlabeled racemic [2*E*,6*E*] JH III and JH II were purchased from Calbiochem. The corresponding radiolabeled [10-^{3}H] substrates at a specific activity of about 11 Ci/mmol were from New England Nuclear. Both labeled (58 Ci/mmol) and unlabeled optically pure 10*R*,11*S* and 10*S*, 11*R* enantiomers of [2*E*,6*E*] JH II were synthesized by Dr Glenn D. Prestwich and his group (Prestwich and Wawrzenczyk, 1985). Alkylthiotrifluoropropanones were synthesized according to a published procedure (Hammock *et al.*, 1984). The buffer used in all procedures was sodium phosphate, pH 7.4, $I = 0.2$, containing 5% sucrose, 0.01% phenylthiourea, 0.02% sodium azide, 0.05% Triton X-100 and 1 mM 2-mercaptoethanol.

Hemolymph preparation

Larvae of *H. virescens* and *H. zea* along with their artificial diets were provided through the courtesy of Janet Stucky (Dow Chemical Co, Walnut Creek, CA) and Mark Schulder (Shell Development Co, Modesto, CA). Hemolymph was taken from prewandering last stadium larvae

having weights and developmental markers indicative of high JH esterase (JHE) activity. Hemolymph was collected from the clipped thoracic legs of the larvae into 15-ml centrifuge tubes containing a few crystals of phenylthiourea to inhibit tyrosinases. The hemolymph was mixed extensively and then centrifuged at 10,000 *g* for 10 min at 1°C and the supernatant (plasma) was kept in aliquots at −70°C until used. JHE activity showed no change for several months under these conditions. Dilutions of the plasma for kinetic studies and purification of JHE were done in the phosphate buffer.

Enzyme assay

JHE analyses were performed using plasma or purified enzymes from last instar larvae of *H. zea* and *H. virescens*. JH substrates were added in 1 μl ethanol solution to 100 μl of buffer containing an enzyme activity that gave a linear hydrolysis for the course of the incubation time (5–15 min). A final substrate concentration of 5×10^{-6} M, unless otherwise stated, containing *ca* 25,000 dpm/assay was used. At the end of the incubation time the enzyme reaction was halted by immersing the reaction tubes in a dry ice-acetone (−78°C) bath. A radiometric partition assay procedure was then used as described previously (Hammock and Roe, 1985).

Candidate inhibitors were added to the enzyme solution, prior to the addition of the substrate, in 1 μl ethanol and pre-incubated for 10 min at 30°C. Control experiments received ethanol only and the assay procedure was executed as mentioned above.

Purification of JHEs

The previously synthesized MBTFP-Sepharose resin (Abdel-Aal and Hammock, 1985a, 1986) based on the attachment of 3-(4′-mercapto)butylthio-1,1,1-trifluoropropan-2-one (MBTFP) to epoxy-activated Sepharose CL-6B was used as an affinity column for the purification of JHEs from *H. zea* and *H. virescens*. First, a 100 μl bed of the affinity gel was packed in a 1 ml tuberculin syringe over a thin layer of glass wool and then washed with 10 ml each of ethanol, ethanol:water (3:1), (1:1), (1:3) and water. The column was then equilibrated with buffer at 2°C. Plasma from the above two species was diluted to 20% (v/v) in buffer and incubated with diisopropyl fluorophosphate (DFP) at a final concentration of 0.1 mM for 10 min at 30°C. Under the conditions used DFP (Sigma) did not inhibit JHE activity, however, it is known that at this concentration alpha-naphthyl acetate hydrolyzing activities were completely abolished. Another advantage of the DFP is to inhibit contaminating proteases that might be present due to leakage of gut contents during hemolymph collection. The treated plasma was then pumped onto the affinity column at a flow rate of 5 ml/hr. Under these conditions the column appeared to retain at least 95% of the original JHE activity. The ability of the diluted plasma to hydrolyse JH III was essentially stable for the duration of the purification, and no change in the concentration of the protein within the sensitivity limit of the dye-binding assay (Bradford, 1976) was detected between the original plasma and the eluted fractions. The column was then washed using the buffer cocktail at a flow rate of 10 ml/hr. The lack of enzyme activity in any wash fraction indicated that the enzyme was tightly bound to the ligand on the affinity gel. 3-Octylthio-1,1,1-trifluoropropan-2-one (OTFP) at 1 mM in buffer cocktail was successfully used as an eluting agent. For the measurement of purification and recovery, a sample of the eluted protein was dialyzed as previously described (Abdel-Aal and Hammock, 1985a) and maximum activity was attained after 8 or 9 days of dialysis, respectively, for JHE from *H. zea* and *H. virescens*.

Electrophoresis and protein determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted in a 12.5% gel with the discontinuous buffer system of Laemmli (1970) and stained with Coomassie Blue or silver stain procedures. Isoelectric focusing (IEF) was conducted for 2 hr on precast 1 mm horizontal slab gels (LKB) with a pH gradient of 4.0–6.5 after a prefocusing period of 45 min. Activity and pH profiles of these gels were generated by overnight elution of 2.5 mm slices in 0.5 ml of buffer or distilled water, respectively. Isoelectric points (pIs) were estimated by comparing peaks of activity with the pH profile determined at 5°C.

Protein concentrations for all studies were determined by a dye binding method (Bradford, 1976) as modified by Bio-Rad, using fraction V of BSA (Sigma) as the protein standard. The detailed procedure was reported previously (Abdel-Aal and Hammock, 1985a).

Immunization of rabbits and ELISA procedure

Initially one rabbit each was immunized with a total of 50 μg of purified JHE from either *H. virescens* or *H. zea* suspended in 0.1 M phosphate buffer pH 7.4 and mixed 1:1 with Freund's Complete Adjuvant (CalBiochem). For the initial immunization rabbits were shaved and about 40 injections of 25–50 μl each were made on the back (Vaitukaitis, 1981). One month later the animals were boosted with an additional 25 μg of the appropriate antigen and bled 10 days later. Two more boosts followed at one month intervals and the animals exsanguinated after the second boost. Blood was collected in tubes, allowed to clot and centrifuged at 800 *g* for 10 min. The clear serum was removed carefully, sodium azide added to give a final concentration of 0.02% and the serum was stored at −20°C.

Ninety-six well titer plates (Nunc, Vangard International) were coated with 0.2 ml of a 2.0 μg/ml solution of purified JHE in 0.5 M carbonate buffer, pH 9.8, overnight at 4°C. The following day, several dilutions of the antisera were made in phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween). The plate was washed with PBS-Tween three times and 0.2 ml of dilute antisera was added per well. After a 2 hr incubation at room temperature, the plates were again washed three times with PBS-Tween and goat anti-rabbit IgG conjugated to alkaline phosphatase (Miles), diluted 1/2500 with PBS-Tween, was added to each well. The plates were incubated for two more hours at room temperature and then washed three times with PBS-Tween. *p*-Nitrophenyl phosphate (1 mg/ml) in diethanolamine buffer, pH 9.8 was then added and incubated at room temperature for 30 min. Absorbances were read in a Titertek Multiskan (Flow Laboratories) at 405 nm (Voller *et al.*, 1976; Wie and Hammock, 1982).

RESULTS AND DISCUSSION

Kinetics of JH III hydrolysis

The kinetics of hydrolysis of JH III by JH esterase (JHE) from the plasma of *H. virescens* and *H. zea* were examined under steady state conditions using 6 or 7 substrate concentrations ranging from 1.89×10^{-8} to 6.34×10^{-7} M. Double reciprocal plots (Lineweaver and Burk, 1934) of the above data revealed a straight line relationship (Fig. 1) with a correlation coefficient of 0.99. Lineweaver–Burk regression lines resulted in K_m values of 0.93×10^{-7} and 1.97×10^{-7} M and maximum specific activity of 1.2 and 4.0 nmol/min·mg protein respectively for *H. virescens* and *H. zea*. It is worth noting that K_m values obtained from separate experiments and a

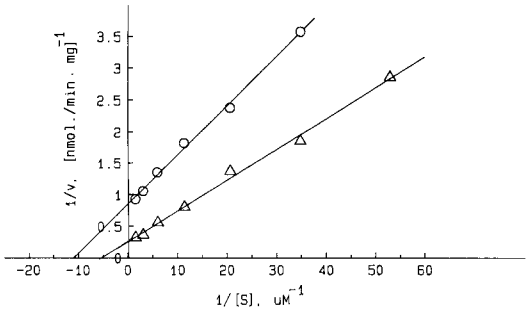

Fig. 1. Double reciprocal plots of substrate concentration against initial velocity for JH III hydrolysis by the hemolymph JH esterases of *H. virescens* (○) and *H. zea* (△). The activity at each substrate concentration was the mean of at least four replicates with an average SD of less than 5% of the mean.

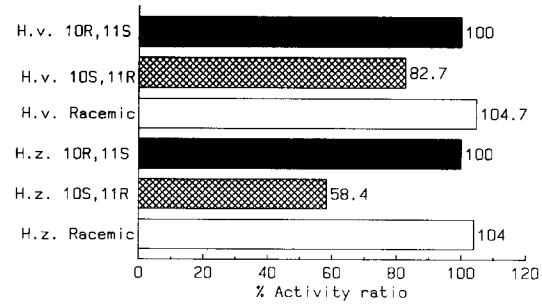

Fig. 2. Selectivity spectrum of the hydrolysis of racemic JH II and its optically pure 10*R*,11*S* and 10*S*,11*R* enantiomers by hemolymph JH esterases from *H. virescens* (H.V.) and *H. zea* (H.Z.). The per cent activity ratio was calculated from the specific activity measured at a final substrate concentration of 5 μM using 10*R*,11*S* as a reference. The specific activity was calculated from the dependence of JH hydrolysis on both protein and time of incubation.

different kinetic treatment (Abdel-Aal and Hammock, in preparation) were, respectively, 1.03×10^{-7} and 1.25×10^{-7} M. These consistent values for K_m indicate that the substrate concentrations used for steady state kinetics were within an acceptable range to follow the Michaelis–Menten equation. The K_m value for JHE from *H. virescens* obtained in the present study is at least three times lower than that reported by Wing *et al.* (1984). When measuring kinetic constants, a range of substrate concentrations must fit in the middle region of the rectangular hyperbola of the substrate–activity curve on one hand and the initial velocity should be measured at the shortest possible incubation time on the other hand. These practical problems, in addition to the basic assumptions required for steady state kinetics, are likely to be responsible for the inconsistency of K_m values obtained by different researchers.

Enantiomeric selectivity toward JH II

The enzymes from the two species appear to differ mainly in their turnover number rather than in their affinity toward JH III. This has also been shown with electrophoretically homogeneous, affinity-purified enzymes. Therefore, the enzymes from the two species were tested for their ability to distinguish between JH II enantiomers which differ only in the absolute configuration of the [10,11]-epoxide functionality. The resulting data were compared with data generated with the racemic mixture. The specific activities measured from protein-dependent, as well as time-dependent, hydrolyses of these substrates under saturation conditions (final concentration of 5 μM) were averaged and calculated as per cent activity ratios using 10*R*,11*S* as a reference (Fig. 2). Interestingly, the 10*R*,11*S*, which is believed to be the natural enantiomer (Meyer *et al.*, 1971; Schooley, 1977) is apparently hydrolyzed faster than its 10*S*,11*R* counterpart. In general, the same selectivity spectrum toward JH II enantiomers for JHEs from the two species was obtained not only with the crude enzymes (Fig. 2) but also with the affinity-purified ones (data not shown). The enantiomeric selectivity as well as the high apparent affinity (low K_m value) support the application of the term "JH specific esterase" to this enzyme (see Hammock, 1985 for review). This selectivity for the orientation of the epoxide moiety, in addition to the structure–activity relationships observed with inhibitors (discussed below), indicate that much of the JH molecule is involved in recognition by the enzyme.

Inhibition of JHEs by alkylthiotrifluoropropanones

Seven 3-alkylthio-1,1,1-trifluoroproan-2-ones were synthesized and tested for their inhibitory potency against JHEs from the two species. The enzyme dilutions were chosen so that the activity based on per min · ml plasma was the same for the enzyme from the two species and within the linear range of activity incubation time. Two series of inhibitor concentrations were used and the range of concentrations that gave percentages of inhibition from 15 to 85% were used in the regression analysis for the molar I_{50} values (Table 1). The activity of the compounds increases as the number of carbon atoms increases and reach the maximum potency at C_8 and C_9, respectively, for JHE from *H. zea* and *H. virescens*. The activity then decreases for longer alkyl substituents. Since the change in the inhibitory potency per methylene group is not the same for the enzyme from the two species and decreases as a function of the total number of carbon atoms, the interaction between the enzyme and the inhibitor which involves this part of the molecule is not likely to be a non-descript partition. However, the complementariness seems to be a linearly decreasing function of non-overlapping volumes of the inhibitor and a receptor cavity at or near the enzyme active sites. If this is the case, one expects that this cavity is of different size and/or configuration in the enzyme from the two

Table 1. Inhibitory potency of alkylthiotrifluoropropanones on *Heliothis* JH-esterase
General formula: $C_nH_{(2n+1)}SCH_2C(O)CF_3$

	Molar I_{50}		Selectivity ratio
N	*H. virescens*	*H. zea*	(v/z)
4	3.6×10^{-6}	1.1×10^{-7}	33
5	1.1×10^{-7}	9.8×10^{-9}	11.2
8	5.4×10^{-9}	5.2×10^{-9}	1.04
9	2.7×10^{-9}	7.1×10^{-9}	0.4
10	3.3×10^{-9}	7.6×10^{-9}	0.4
11	9.0×10^{-9}	1.1×10^{-8}	0.8
12	2.4×10^{-8}	3.9×10^{-8}	0.6

species. A similar structure–activity relationship was obtained for JHE from *T. ni* (Hammock *et al.*, 1984; Abdel-Aal and Hammock, 1985b). The fact that the enzyme from three different species showed similar structure–activity relationships on the one hand, and a decrease in the selectivity ratio from 33 to 1.04 in moving from butyl to octyl (Fig. 3) on the other hand, indicates that the cavity is likely to be part of the enzyme catalytic site. The decrease in selectivity ratio as a function of the alkyl size might then be due to a change in the mechanism of inhibition from a classical reversible type to a slow tight binding type. At a certain point, the steric configuration of the molecule, in addition to the electronic properties of the polarized ketone, enables the compound to have the binding energies of a transition state analog. When this point is reached the compound will probably act as a stoichiometric inhibitor and eventually will not differentiate between the two enzymes. This also assumes that catalytic rate constants of the two enzyme systems are not substantially different.

The concept of developing "transition state mimics" has been addressed by many workers and reviewed by Wolfenden (1976). He defines transition state mimics as compounds which result from an experimental approach to exploit the tight binding of an enzyme and its substrate in the transition state. If we extend this definition of transition state inhibitors to include mimics of transient or metastable intermediates, it seems clear that all of the compounds illustrated in Figs 3 and 4 fit the definition in that each compound is expected to mimic a tetrahedral, transient intermediate in the hydrolysis of JH by forming a hemiacetal at the enzyme catalytic site.

The slow tight binding phenomenon is often observed with potent transition state mimics. Certainly, not all tight binding inhibitors are transition state mimics, but a more debatable point is if transition state mimics must show slow tight binding kinetics (Williams and Morrison, 1979; Morrison, 1982; Cha, 1975; Abdel-Aal *et al.*, 1984; Abdel-Aal and Hammock, 1985c). The causes of the slow tight binding phenomenon also are debatable.

In hopes of illuminating some of the above points, the time dependence of inhibition of the enzymes from the two species by butylthio-1,1,1-trifluoropropan-2-one and its pentyl and nonyl analogs was examined (Fig. 4). All of the tested compounds possess the polarized ketone and the sulfide bond as essential recognition elements that conspire to ease the inhibitor into the transition state and then stabilize the enzyme inhibitor complex. However, the size of the alkyl group and the enzyme source appear to be of critical importance in defining the time dependent pattern of inhibition. For example, at the concentrations used the nonyl, in contrast to the butyl, analog showed complete time dependent inhibition of the enzymes from the two species. It is of interest to note that although the pentyl analog showed classical type inhibition similar to that of the butyl analog against the JHE from *H. virescens*, it acts as a slow equilibrating inhibitor against the JHE from *H. zea* with a time dependent inhibition that ranged from 0 to about 90 sec. At longer pre-

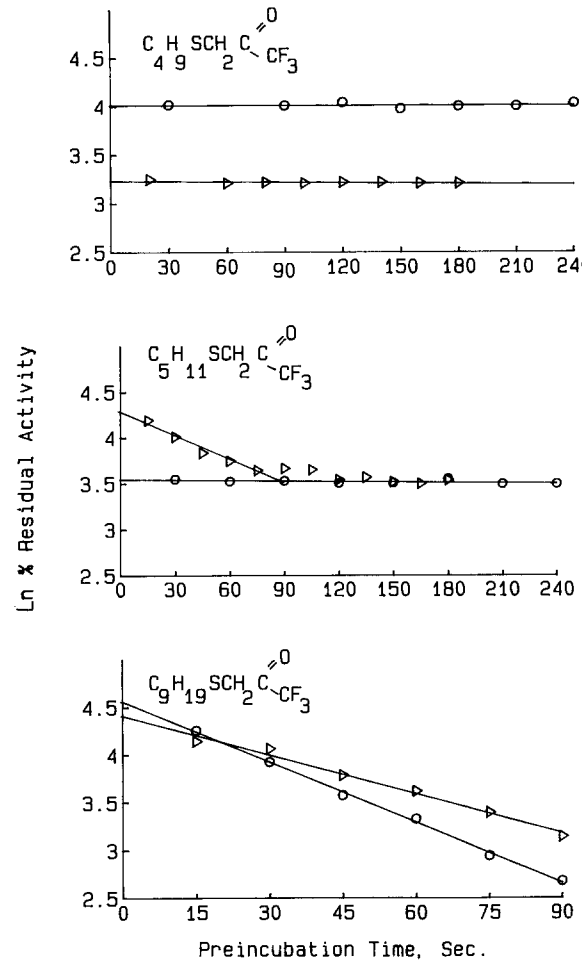

Fig. 4. The time dependence of inhibition of JH esterase from *H. virescens* (○) by 10^{-7} M 3-nonylthio-1,1,1-trifluoropropan-2-one and 2×10^{-6} and 10^{-5} M, respectively, of the 3-butyl and 3-pentyl analogs. The corresponding concentrations when JH esterases from *H. zea* (△) were used were, respectively, 10^{-7}, 1.25×10^{-7}, and 2.5×10^{-6} M. The percentage residual activity was from duplicate determinations. Preincubation with the inhibitor and incubation with 5 μM JH III were done in phosphate buffer, pH 7.4 at 30°C.

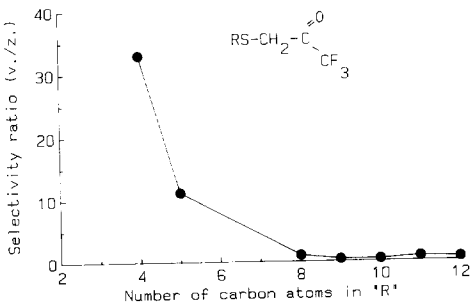

Fig. 3. Selective inhibition by alkylthiotrifluoropropanones of JH esterases from *H. virescens* (V.) and *H. zea* (Z.) as a function of the number of carbon atoms in the alkyl chain. The selectivity was calculated from the ratio (v/z) of the molar I_{50} values. This figure is based on the data reported in Table 2. The molar I_{50} was from 10 min preincubation experiments with the use of 5 μM JH III as substrate.

incubation times a steady state concentration of the enzyme inhibitor complex was reached.

These data fail to support the argument of Brodbeck *et al.* (1979) that the time dependence of inhibition by trifluoroketones is dependent only on the conversion of the hydrated carbonyl back into the trigonal form in aqueous solution. It is obvious that slow tight binding kinetics will be easier to observe with more potent compounds, and the data in Fig. 4 illustrate this point. The pentyl analog is over 10 times more potent as an inhibitor with the *H. zea* enzyme and time dependence is observed with the enzyme from this species but not the enzyme from *H. virescens*. These data clearly demonstrate that observation of the slow tight binding phenomenon is a function of the inhibitor, the enzyme and the experimental conditions. The data also demonstrate a continuum between classical and slow tight binding inhibition.

Thus, the phenomenon of slow tight binding inhibition is likely to be commonly observed with many reversible inhibitors if their potency were such that the time dependence could be observed experimentally. For example, it is very likely that the pentyl analog would exhibit time dependent inhibition with the enzyme from *H. virescens* if very short preincubation times were used. The cause of the phenomenon is also likely to be different with different enzyme inhibitor systems. In this case such inhibition might be observed when interaction of the enzyme with the hydrophobic chain of the inhibitor is sufficiently strong to hold the *gem*-diol of the inhibitor near the catalytic site for sufficient time for the inhibitor and the enzyme to form the slowly reversible hemiketal of the catalytic site.

Affinity purification of JHEs

Based on our studies on the inhibition by alkylthiotrifluoropropanones of JHE (Hammock *et al.*, 1982, 1984; Abdel-Aal *et al.*, 1984; Abdel-Aal and Hammock, 1985a, b, c) we were able to devise a biospecific ligand and an eluting agent, based on 3-(4′-mercapto)-butylthio-1,1,1-trifluoropropan-2-one and 3-octylthio-1,1,1-trifluoropropan-2-one, respectively, for the affinity purification of JHEs. This affinity system has been successfully used for the purification of JHEs from several lepidopteran larvae (Abdel-Aal and Hammock, 1985a, 1986; Hanzlik and Hammock, 1987). Table 2 shows the data from the one step purification using our affinity system. A purification factor of 700–1000-fold appeared to be enough to obtain an electrophoretically homogeneous enzyme based on SDS-PAGE (Fig. 5). As seen in this figure, the purified protein from *H. zea* comprises two bands with similar mol. wts. Those two bands were equally radiolabeled (^{3}H) with the active site directed irreversible inhibitor, *O,O*-diethyl *p*-nitrophenyl phosphate. On the other hand, JHE from *H. virescens* appeared as a single band on SDS-PAGE.

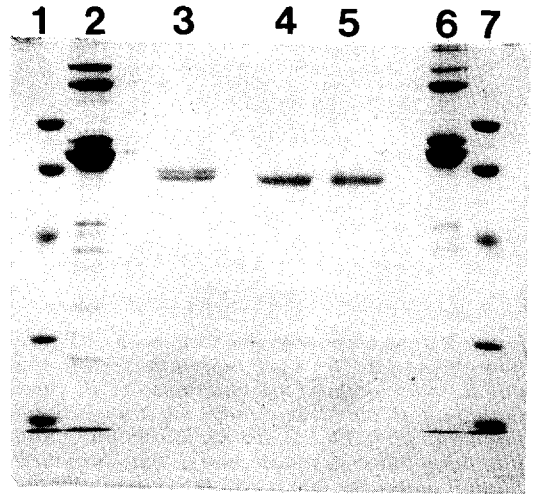

Fig. 5. Coomassie Blue-stained 12.5% SDS-polyacrylamide gel of affinity purified JH esterases from *H. zea* (lane 3) and *H. virescens* (lanes 4 and 5). Plasma from the above species (lanes 2 and 6, respectively) was overloaded at 20 μg per lane to illustrate the low abundance of the enzyme. In lanes 1 and 7 are the protein standards: from top to bottom phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500. A lectin blot illustrates the presence of sugar residues on the upper of the two bands from *H. zea* but no reaction was noted with the lower band and the major band of *H. virescens*.

When the enzymes from the two species were subjected to isoelectric focusing with a 4–6.5 pH gradient, the activities of JHE present in the hemolymph had identical isoelectric points (pIs) to the activities of the affinity-purified enzymes from each species. However, the activity of JHE from hemolymph and affinity-purified preparation of *H. zea* appeared at two pI values clearly distinct from the single pI value of the crude and affinity-purified activities from *H. virescens* (Fig. 6). This observation

Table 2. Affinity purification of JH-esterases from two heliothines

Species	Substrate*	Hemolymph JHE Specific†	Hemolymph JHE Total‡	Purified JHE Specific†	Purified JHE Total‡	Recovery (%)	Purification
H. virescens	A	1.79	1864	2012	966	52	1124
	B	1.27		915			720
	C	1.05		735			700
	D	1.33		906			681
H. zea	A	5.21	3310	4001	1840	56	768
	B	2.98		2984			1001
	C	1.74		1946			1118
	D	3.10		3518			1135

*A = JH III (Racemic); B = [10*R*,11*S*] JH II; C = [10*S*,11*R*] JH II; D = JH II (Racemic).
†Specific activity on nmol substrate hydrol./min.mg.
‡Total activity in nmol substrate hydrol./min.

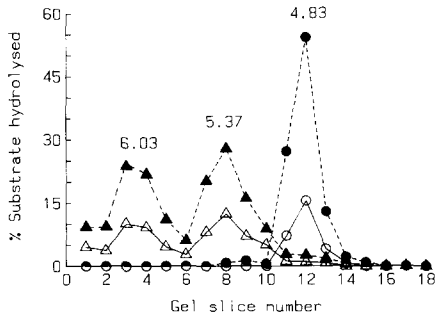

Fig. 6. Narrow range isoelectric focusing of JHE activity toward 5 μM JH III. Plasma (solid symbols) and affinity purified (open symbols) JH esterases from *H. virescens* (circles) and *H. zea* (triangles) were run side by side on an Ampholine gel (pH 4–6.5) and the activity was measured after slicing and eluting the focused proteins from the gels. Numbers above the peaks indicate experimentally determined pIs. The pattern shown in this figure was always repeatable in three separate measurements using different plasma pools and purified enzyme from different affinity gels. No additional peaks of activity were obtained from wide range gels.

corresponds to the presence of two protein bands for *H. zea* and a single band for *H. virescens* when the purified enzymes were analyzed by SDS-PAGE indicating multiple forms of JHE from *H. zea* as compared with *H. virescens*. These electrophoretic differences observed for JHE from the two species are consistent with the work of Sluss *et al.* (1978) who conducted an electrophoretic survey using *H. zea* and *H. virescens*. They recorded frequency or presence/absence of alleles at 19 loci and found distinct differences between the species.

The JHEs from *H. zea* corresponding to the two activity peaks were eluted from the IEF gel and subjected to inhibition by OTFP and BTFP (Fig. 7). As seen in this figure, the two electromorphs showed an identical pattern of inhibition by each using OTFP and BTFP. The two catalytic sites of JHEs from *H. zea* appear to be similar, although present on distinct proteins. However, presumably multiple and kinetically separate sites were found in *Manduca sexta* although apparently present on the same proteins (Abdel-Aal and Hammock, 1985a). Multiple kinetically distinct enzymes were reported in *Trichoplusia ni*, although only in crude preparations (Jones *et al.*, 1986; Rudnicka and Jones, 1987).

It is known that in some proteins there are exposed peptide segments usually without ordered secondary structure, free to move about in solution, and probably non-essential for the biological activities of the proteins concerned (Tsou, 1986). These segments would probably be more sensitive to biochemical cleavage. Post-translational modification could give rise to different electromorphs from same gene product. This could be supported by finding that JHE from *T. ni* was differentially glycosylated (Hanzlik and Hammock, 1987). When hemolymph proteins and affinity purified JHEs from *H. virescens* and *H. zea* were examined by using lectin blots, glycosylation of many hemolymph proteins from both species were observed. However, only the higher mol. wt protein of the purified JHEs from *H. zea* was shown to react positively under the conditions used (data not shown). Whether or not the two SDS bands of *H. zea* JHEs are responsible for the two activity peaks on IEF is not clear and awaits further investigation. In this regard we believe that JHEs from *H. zea* would be a good model to study post-translational modification of proteins.

Immunogenic reactivity of JHEs

The purified enzyme from both species were used to raise polyclonal antisera which in turn, were employed to develop an enzyme linked immunosorbent assay (ELISA) for each antigen. The results of the ELISA assays are shown in Fig. 8. The antibody raised against the JHEs of *H. zea* showed high reactivity to its own antigen, but low cross reactivity when the enzyme from *H. virescens* was tested as a coating antigen. The same pattern was true for the

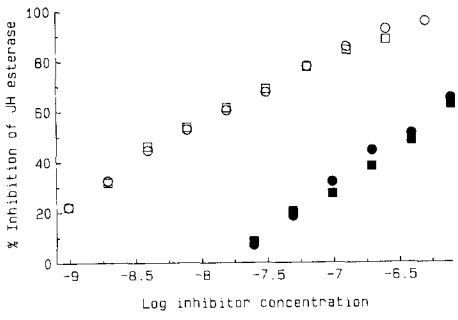

Fig. 7. Inhibition of the two electromorphs of JH esterases purified from *H. zea*. The focused gel (pH 4–6.5) was sliced and eluted in phosphate buffer, pH 7.4, at 4°C overnight. The activity was monitored in each elution separately and the activity at the top of each peak was subjected to inhibition by OTFP (open symbols) and BTFP (solid symbols). The molar I_{50} values determined by regression analysis were, respectively, 7.2×10^{-9} and 3.1×10^{-7} M for the activity corresponding to a pI value of 6.03 (circles) and 7.1×10^{-9} and 3.9×10^{-7} M for the activity corresponding to a pI value of 5.37 (squares).

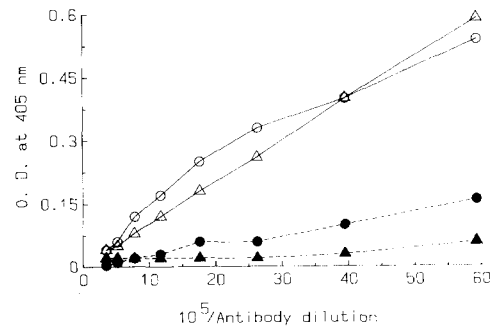

Fig. 8. Binding of an increasing amount of rabbit anti-sera raised against affinity purified JH esterases from *H. virescens* (circles) and *H. zea* (triangles) to a constant concentration (2 μg/ml) of JH esterases used to coat the ELISA plate. Subsequently, goat anti-rabbit Ig conjugated with alkaline phosphatase was added, and the enzymatic activity was measured using *p*-nitrophenyl phosphate as a substrate. Open symbols show reactivity of the antibody towards its own antigen and solid symbols show cross reactivity.

antibody raised against JHE from *H. virescens* indicating that the enzymes from the two species contain some common antigenic determinants, but that an assay based on the antibodies raised against the two respective JHEs can distinguish enzymes from the alternate species.

The immunological, inhibition, electrophoretic and kinetic results reported in this paper indicate differences between the JHEs of *H. zea* and *H. virescens*. There is a strong correlation between immunological differences and amino acid substitution in proteins (Benjamin *et al.*, 1984). The affinity purified enzymes from both species also showed substantial differences in their response to the effect of organic cosolvents (Croston *et al.*, 1987; Abdel-Aal *et al.*, in preparation). JHE appears to be sufficiently variable to be useful in delineating relationships in the Heliothinae. The data support the recent movement by the Entomological Society of America to use Hardwick's classification (Hardwick, 1965) which defines a separate genus for the corn earworm complex (*Helicoverpa*). The differences are surprising in light of the critical role of JHE in lepidopteran larvae. There is a consistent pattern of JHE titers in most lepidoptera studied (Jones *et al.*, 1982), which may indicate that control of JHE expression is more important than differences in the nature of the enzyme.

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