Characterization of Affinity-purified Juvenile Hormone Esterase from the Plasma of the Tobacco Hornworm, *Manduca sexta*

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Juvenile hormone (JH) esterase found primarily in the hemolymph and tissues of insects is a low abundance protein involved in the ester hydrolysis of insect juvenile hormones, JHs. The enzyme was purified from the larval plasma of wild-type *Manduca sexta* using an affinity column prepared by binding the ligand, 3-[4-(mercapto)butylthio]-1,1,1-trifluoropropan-2-one (MBTFP), to epoxy-activated Sepharose. The purification was greater than 700-fold with a 72% recovery, and the purified enzyme appeared as a single protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoelectrophoresis, reverse phase high performance liquid chromatography, and amino acid sequence analysis. The molecular weight was 66,000. The plasma JH esterase in wild-type, black, and white strains of *M. sexta* was similar when analyzed by immunotitration, wide range (pH 3.5–9.0) isoelectric focusing, and inhibition with MBTFP and 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP). Inhibition studies revealed a sensitive and insensitive form (Iapp = 10^{-9} and 10^{-8} M, respectively) in these three biotypes. Narrow range isoelectric focusing (pH 4.0–7.0) indicated the presence of two major isoelectric forms with pI values of 6.0 and 5.5, but their inhibition kinetics with OTFP and O,O-diisopropyl phosphorofluoridate were identical.

Juvenile hormones (JHs) are sesquiterpenes and are known to regulate development and reproduction in insects (reviewed by de Kort and Granger, 1981). A number of studies have shown that catabolism of JH by specific esterases, in addition to a decrease in JH biosynthesis, is important in regulating JH titer at critical times of insect development, thus allowing for normal metamorphosis from a larva to the adult moth or butterfly (reviewed by Hammock, 1985; Roe and Venkatesh, 1990). In the hemolymph of most insects studied, ester hydrolysis of JH to the metabolite JH acid, by JH esterase(s) is the primary pathway of metabolism (Roe, 1985, 1986). During the fifth larval instar (the end of larval development) of *Manduca sexta*, there are two peaks of JH esterase activity. The first initiates wandering behavior, where the larva moves to the soil to undergo metamorphosis. The second peak regulates the transformation from the larva to the adult moth. The purification and characterization of this important regulatory enzyme is an essential step to furthering our knowledge of the regulation of insect metamorphosis as well as developing molecular approaches to regulate the enzyme activity as a means of selective insect control.

A major focus in JH esterase research has been the cabbage looper, *Trichoplusia ni*, and the tobacco hornworm, *M. sexta*. JH esterase was purified from the hemolymph of *T. ni* (Yuhas et al., 1983; Rudnicka and Jones, 1987; Wozniak et al., 1987) and *M. sexta* (Coudron et al., 1981) using classical approaches. From structure-activity and kinetic studies with polarized trifluoromethyl ketones, however, the affinity column, Sepharose-S(CH3)2SOCH2COOF, was discovered that made possible the rapid purification of JH esterase from hemolymph and other tissues (Abdel-Aal and Hammock, 1985, 1986; Abdel-Aal et al., 1988; Hanzlik and Hammock, 1987; Roe and Venkatesh, 1990). From these and other studies on *M. sexta* JH esterase, conflicting reports were made on the number of hemolymph JH esterases (Sanburg et al., 1975; Coudron et al., 1981; Sparks et al., 1983; Wing et al., 1984, Abdel-Aal and Hammock, 1985, 1986; Sparks et al., 1989) and multiple catalytic sites (Abdel-Aal and Hammock, 1985). There are also no published data available on the immunogenicity, amino acid composition, and sequence information on the JH esterase for this important insect model.

**EXPERIMENTAL PROCEDURES**

*Animals—*Wild-type *M. sexta* larvae from the original Yamamoto colony (Yamamoto, 1968) were reared according to Bell and Joachim (1970) at 27 ± 1 °C under a 16-h:8-h light-dark cycle. Fourth instar larvae exhibiting head capsule slippage were separated from the main colony and those undergoing ecysis between 2 and 6 h after lights were designated as day 0, last instar larvae. Gates were selected as described previously (Venkatesh and Roe, 1988). Only gate I larvae were used in all the experiments that follow. A black mutant colony (Safranek and Riddiford, 1975) was obtained from Dr. L. M. Riddiford, Dept. of Zoology, Washington State University, Pullman, WA, and reared as described above. A white mutant of *M. sexta* occurred spontaneously from a batch of field-collected larvae in March of 1988 at North Carolina State University and was also reared as above.

*Plasma Preparation and Enzyme Assays—*Hemolymph from fifth instar day three (gate I) larvae was collected via a clipped horn and anal prolegs into a culture tube (10 X 75 mm) held at 4 °C, containing a few crystals of phenylthiourea. The hemolymph was centrifuged for 5 min at 1000 g, and the resulting plasma was filtered through glass wool and stored at −85 °C until used. Freeze-thawing had no detectable effect on the esterase activity. JH esterase activity was assayed by the partition method described by Hammock and Roe (1985). The substrate, H-labeled JH III (12 Ci/mmol, tritiated at C-10, Du Pont-New England Nuclear) was mixed with the unlabeled JH III (Calbiochem) to obtain a final concentration of 5 μM when 1 μl of plasma was diluted in standard sodium phosphate buffer (pH 7.4, 1/25 M) containing 0.1% BSA, 0.1% Triton X-100, and 0.2 M KCl.
Juvenile Hormone Esterase of *M. sexta*

0.01% phenylthiourea, 10% sucrose). The reaction mixture was incubated for 5–20 min at 30 ± 0.5 °C. The initial dilution and incubation time for the assay was within the linear portion of the reaction. α-Naphthyl acetate esterase activity was assayed according to Spearks et al. (1979) at a final substrate concentration of 250 μM. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, 1977) using bovine serum albumin (BSA Fraction V, Sigma) as a standard.

The affinity ligand, 3-[4-mercaptobutylthio]-1,1,1-trifluoro- 
propan-2-one (MBTFP), and the eluting agent, 3-octylthio-1,1,1-tri-
fluoropropan-2-one (OTFP), were tested for their inhibitory potency against plasma JH esterase of wild, black, and white mutant strains of *M. sexta*. The Kᵢ value (the molar concentration of the inhibitor required to reduce the enzyme activity by 50%) was calculated after adding 1 μl of an ethanol inhibitor solution to 100 μl of 0.5% plasma in standard sodium phosphate buffer. The reaction mixture was preincubated for 10 min at 30 °C before the addition of substrate. Control experiments received 1 μl of ethanol only. The rate of inhibition for each inhibitor concentration was measured three times, and the average percent inhibition was plotted against the log inhibitor concentration.

**Purification of JH Esterase**—The affinity ligand, MBTFP, was prepared by reacting 1,4-dimercaptobutane (Aldrich Chemical Co.) with equimolar 3-bromo-1,1,1-trifluoroacetone (Chem Services, West Chester, PA). The resulting thiol, MBTFP, was in turn reacted in ethanol with epoxy-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) containing 1.2 μeq of epoxide per g of gel. A 500-μl bed of affinity gel was packed into a 1 ml tuberculin syringe over a thin layer of glass wool, and the column was washed with 50 ml each of ethanol, ethanol:water (3:1, 2:1, 1:1, 1:3), and water. The column was then equilibrated with the purification buffer (sodium phosphate, F = 0.2 M, pH 7.4, 0.01% phenylthiourea, 0.005% Triton X-100, 0.02% sodium azide, 5% sucrose, 0.001 M 2-mercaptoethanol). The plasma diluted to 20% with purification buffer was preincubated for 10 min at 30 °C with 2 × 10⁻⁴ M O,O-diisopropyl phosphorofluoridate (DFP) to inhibit general esterases and proteases; JH esterase activity toward 5 μM JH III was unchanged before and after the addition of diluted plasma using LKB Ampholine (Bio-Rad) precast, 11.0 cm gel plates were then washed three times in BBS and then incubated with p-nitrophenyl acetate (pNP) at pH 8.3 (borate-buffered saline, BBS) was added to a 96-well ELISA column plate and incubated at room temperature for 2 h. The resulting JH esterase containing an approximately equal amount of JH esterase activity present in 5 μl of plasma was also run as described above except that supernatant was then preincubated overnight at 30 °C with 0.1% Triton X-100, 0.02% sodium azide, 5% sucrose, 0.001 M 2-mercaptoethanol.

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**Enzyme-linked immunosorbent assay (ELISA)**—The purified enzyme was coated with 0.1 μg/ml mouse MAb JH-1 (a gift from Dr. J. W. Bidlingmyer, University of California, Berkeley) and incubated at room temperature for 3 h on a shaker. The plates were then washed three times in PBS and after adding to each well 100 μl of goat-anti-mouse IgG conjugated to alkaline phosphatase, the plates were incubated overnight at 4 °C. The plates were washed extensively in BBS and then incubated with p-nitrophenyl phosphate (1 mg/ml) in 0.1% diethanolamine buffer (pH 9.5) for 0.5 h. The absorbance was read at 405 nm.

**RESULTS AND DISCUSSION**

**Purification of JH Esterase**—The purification of JH esterase from the plasma of last instar day three (gaste) larvae of *M. sexta* was accomplished using the affinity matrix MBTFP-Sepharose. A number of other affinity ligands and linkages to *M. sexta* were also examined, but MBTFP-Sepharose produced the optimum column. When the plasma was passed through this affinity column, there was a 90% decrease in the JH esterase activity without any detectable loss of protein in the column effluent. This indicates the highly selective nature
of the ligand used for preparing the affinity matrix. From 100 ml of plasma containing 2100 mg of protein (specific activity of 0.71 nmol of JH III min⁻¹ mg⁻¹ of protein), 1.95 mg of purified JH esterase was recovered having a specific activity of 554 nmol of JH III min⁻¹ mg⁻¹ of protein, respectively. Plasma and purified JH esterase was from the hemolymph of last instar day 3 (gate I) wild-type M. sexta larvae.

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Interactions will be evaluated in future studies by interspecies comparisons determined by Edman degradation and two residues Val-9 and Val-10 of M. sexta matches with the cDNA deduced amino acid sequence of H. virescens obtained from cDNA clone. The amino acid sequence data of H. virescens are from Hanzlik et al. (1989).

The protein sequence of plasma JH esterase of H. virescens obtained from cDNA clones was found to be homologous to the NH₂ terminus of the major form found in purified JH esterase with one exception at position 10. Interestingly, only 1 residue, Val-9 of M. sexta, matches with the NH₂-terminal amino acid sequence of the major form of H. virescens determined by Edman degradation and two residues Val-9 and Val-10 of M. sexta matches with the cDNA deduced protein sequence (Fig. 3). The lack of homology is surprising since JH esterase from both species has a Kₘ for JH in the range of 10⁻⁷ M. This indicates that either the NH₂ terminus is not important in in vitro catalytic activity or a great deal of latitude may exist in this region relative to its function. Questions about the functional significance of the primary structure cannot be addressed fully until the active site has been identified and the three-dimensional structure of JH esterase is described. The differences noted between these two species may also be important in interactions of JH esterase with in vivo elements. We have noted that the protein function for other M. sexta proteins varies significantly between species even in closely related insect species. In vivo interactions will be evaluated in future studies by interspecies transformations of JH esterase.

Immunotitration of JH Esterase—The plasma JH esterase activity of wild, black, and white strains of last instar day three (gate I) larvae of M. sexta was compared with that of purified JH esterase from wild-type larvae of the same developmental age by immunotitration (Fig. 4). Plasma JH esterase and the purified, reactivated JH esterase containing equal amounts of enzyme activity were used against different concentrations of mouse polyclonal, anti-JH esterase serum. The results presented in Fig. 4 show that the immunotitration pattern for the three different strains was similar, indicating that the JH esterase activity is due to the same enzyme(s) in all these different strains. It appears that the quantity of immune serum required to immunoprecipitate 50% of the purified enzyme activity was less than that required for plasma (Fig. 4). This was further demonstrated by using an ELISA to test the cross-immunoreactivity of our immune serum using an equal amount of plasma protein and purified JH esterase (Fig. 5). The concentration of JH esterase in last instar day three plasma is roughly 0.1% of the total protein. Therefore, 0.5 μg of plasma protein (the highest concentration tested in Fig. 5) contains 0.5 ng of JH esterase. This amount of JH esterase is below the detection limit of the immune serum used in the present study (see Fig. 5). At a concentration of up to 0.5 μg of plasma protein versus an equal amount of purified JH esterase, the anti-JH esterase serum showed no cross-reactivity with plasma protein. As the protein concentration of the plasma increased beyond 0.5 μg, the ELISA cross-reactivity became evident (data not shown). An examination of Western blots of plasma revealed the presence of a

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**Table II**

| Amino acid | Mole % | Residues/molecule |
|------------|--------|-------------------|
| Alanine    | 12.2   | 74                |
| Arginine   | 5.6    | 35                |
| Aspartic acid | 10.7  | 67                |
| Cysteine   | ND²    | ND                |
| Glutamic acid | 10.7  | 67                |
| Glycine    | 6.9    | 56                |
| Histidine  | 1.8    | 11                |
| Isoleucine | 4.2    | 26                |
| Leucine    | 7.4    | 47                |
| Lysine     | 4.0    | 21                |
| Methionine | 1.4    | 9                 |
| Phenylalanine | 5.1   | 30                |
| Proline    | 7.0    | 42                |
| Serine     | 7.5    | 47                |
| Threonine  | 5.3    | 32                |
| Tryptophan | ND²    | ND                |
| Tyrosine   | 3.2    | 19                |
| Valine     | 5.1    | 30                |
| Total      | 100.0  | 613               |

¹ Aspartic and glutamic acid values also include asparagine and glutamine, respectively.
² ND, not determined.

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**Figure 3.** Comparison of the NH₂-terminal sequences of JH esterase from M. sexta and H. virescens. The HPLC-purified JH esterase from last instar day 3 (gate I) wild-type M. sexta larvae was subjected to automated Edman degradation on an Applied Biosystems Model 470A gas-phase microsequencer. A, amino acid sequence of M. sexta JH esterase. B and C, amino acid sequences of M. sexta and H. virescens, respectively, obtained from Edman degradation. D, amino acid sequence of H. virescens obtained from cDNA clone. The amino acid sequence data of H. virescens are from Hanzlik et al. (1989).

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**Figure 4.** Immunoprecipitation of JH esterase. Immunoprecipitation of purified JH esterase (JHE) from wild-type larvae and the plasma JH esterase from wild, black, and white strains of M. sexta. The JH esterase was from last instar day 3 (gate I) larvae. Different concentrations of immune serum in sodium phosphate buffer (I = 0.2 M, pH 7.2) were incubated at 4°C overnight with JH esterase having equal enzyme activity. Following centrifugation at 10,000 x g for 2 h, the supernatants were assayed for JH esterase activity. Values are the mean of three independent determinations with a standard deviation less than 5% of the mean.

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**Figure 5.** ELISA of JH esterase. ELISAs were performed using purified JH esterase (JHE) and plasma from day 3 fifth instar (gate I) wild-type larvae. Preimmune serum against purified enzyme served as the control. Values are the mean of three independent determinations with a standard deviation less than 5% of the mean.
number of cross-reacting proteins (data not shown). In a similar study, Hanzlík and Hammock (1987) reported that the antibodies developed against affinity-purified JH esterase from the plasma of *T. ni* also cross-reacted with other proteins in the plasma. These authors attributed this cross-reaction to similarities in glycosylation of JH esterase with glycosylation of other serum proteins. Interestingly, Wozniak et al. (1987) reported that the polyclonal antibodies developed to JH esterase purified from the plasma of *T. ni* by classical techniques was highly specific to JH esterase and showed no cross-reactivity to other plasma proteins. The reasons for the above discrepancy are not clear. It is possible that the affinity purified enzyme used in the present study and that used by Hanzlík and Hammock (1987) was contaminated with other plasma proteins in low abundance and high antigenicity. However, this appears unlikely in the present study, because Western blotting of purified JH esterase detected only a single band, and no other cross-reacting proteins were evident. Wozniak et al. (1987) also found that in *T. ni*, the quantity of anti-JH esterase serum needed for immunoprecipitating 50% of the JH esterase activity in the fresh hemolymph was less than that required in the case of purified JH esterase; this was attributed to a possible loss of enzymatic activity during purification.

In our studies, an examination of the plasma before and after immunoprecipitation showed no differences in the a-naphthyl acetate esterase activity (data not shown). This indicates that the anti-JH esterase serum was specific to JH esterase and not to other esterases in the plasma. The anti-JH esterase serum of *M. sexta* showed no cross-reactivity with the JH esterase of *T. ni* and the Colorado potato beetle, *Leptinotarsa decemlineata* when examined by immunotitration (data not shown). Wozniak and Jones (1987) also reported that the anti-JH esterase serum of *T. ni* showed no cross-reactivity toward *M. sexta* and very little cross-reactivity toward JH esterase from other lepidopterans. This is not surprising considering the differences at the amino terminus (Fig. 3) discussed earlier. Therefore, the function of the protein is conserved with respect to its action on JH, but the protein structure appears to be quite variable between species.

**Multiple Forms of Plasma JH Esterase**—The plasma JH esterase activity from the fifth instar day 3 larvae of black, white, and wild strains were compared for their sensitivity to inhibition by the affinity ligand, MBTFP, and the eluting agent, OTFP. The inhibitor concentrations (10⁻⁹ to 10⁻⁶ M) were selected to give 0 to 95% inhibition after a 10-min incubation time. The residual enzyme activity was monitored using JH III at a final substrate concentration of 5 μM, and the percent inhibition was plotted against log inhibitor concentration. The inhibition curve for MBTFP for all the three strains was a simple sigmoid curve, and the *I₅₀* values were determined to be 6.48 × 10⁻⁹ M, 2.25 × 10⁻⁸ M, and 8.13 × 10⁻⁸ M for wild, black, and white strains, respectively (Fig. 6). The inhibition curves for OTFP in these same strains are shown in Fig. 7. Unlike MBTFP, OTFP was found to be a highly potent inhibitor of JH esterase activity. The inhibition curves were similar, but the inhibition pattern is complex, indicating the presence of two forms of JH-hydrolyzing activity. These forms are indicated by an inflection point near 50% inhibition. This inhibition pattern was different from that of MBTFP, where only a sigmoid curve was obtained indicating homogeneous enzyme activity (Fig. 6). The OTFP inhibition pattern, however, indicates the possibility that there are multiple forms of JH esterase in the plasma of *M. sexta* (Fig. 7). The percent inhibition for each strain was recalculated by assuming equivalent activity for both forms, such that the percent inhibition under 50% is expected to be from the less sensitive form and that above 50% is from the sensitive form. The corrected percentage inhibition thus calculated when plotted against log inhibitor concentration yielded straight lines resembling the inhibition of two homogeneous catalytic sites (Fig. 7). The regression analysis for these lines yielded *I₅₀* values in the range of 10⁻⁹ M and 10⁻⁸ M for the sensitive and less sensitive forms, respectively, for all three strains, and is similar to that reported earlier by Abdel-Aal and Hammock (1985) for a different wild-type strain of *M. sexta*. The similarity of inhibition curves obtained for all three strains suggests that the JH esterase activity is due to the same enzyme(s) and that the catalytic activity is not altered by strain differences. As shown in this study, OTFP is a stronger inhibitor of plasma JH esterase activity than is MBTFP.
The purified enzyme had the same isoelectric point of 6.0. By differences in the molecular weight of JH esterase activity isoelectric forms were resolved at pH values 6.0 and 5.5. These were found roughly in the same proportion (Fig. 8B). The two isoelectric focusing, two peaks of activity were resolved which from wild, black, and white strains and purified enzyme (Fig. 8A). The major peak of activity in all three strains and adult development; in the plasma of last instar day 3 wild, white, and black strains of M. sexta, JH esterase is glycosylated as in T. ni (Hanzlik and Hammock, 1987), and JH esterase does not bind to ampholytes (Jones et al., 1986).

To determine whether the isoelectric forms have different chemical reactivities, their sensitivity to inhibition by OTFP and DFP was determined. DFP at $10^{-4}$ M gave no inhibition of either of the JH esterase activities (data not shown). This confirms the hypothesis (Hammock, 1985; Roe and Venkatess, 1990) that a specific plasma JH esterase is responsible for JH metabolism and is insensitive to inhibition by the general esterase inhibitor, DFP. The OTPF inhibition curves for both isofoms were identical (data not shown). Jesudason and Roe2 have also shown multiple catalytic function in individual isoelectric forms.

This study has shown that although JH esterase is functionally similar between lepidopteran families, it appears to differ significantly immunologically and in the NH2-terminal amino acid sequence. The JH esterase activity in M. sexta is due to a group of highly similar proteins distinguishable only by high resolution isoelectric focusing and contains multiple catalytic sites.

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