Apparent Multiple Catalytic Sites Involved in the Ester Hydrolyses of Juvenile Hormones by the Hemolymph and by an Affinity-Purified Esterase from Manduca sexta Johansson (Lepidoptera: Sphingidae)

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The esterases which metabolize juvenile hormone (JH) in some insects may be important in regulating the hormone titers. The JH ester-hydrolyzing activity (JHE) in the larval hemolymph of the tobacco hornworm (Manduca sexta) was found to be attributed to two forms of esterase with almost equivalent activity based on selectivity and kinetics of inhibition by two 3-substituted thi-1,1,1-trifluoropropan-2-one and a phosphoramidithioate. Neither of the two forms were inhibited by diisopropyl phosphorofluoridate or indo-acebamate. Steady-state kinetics of JH II hydrolysis supported the inhibition studies and showed that the two forms were widely different in their affinity for JH II. The activity of the hemolymph was found to be bound selectively to an affinity column synthesized by the reaction of epoxy-activated Sepharose with 3-[4-(3-mercaptobutylthio)-1,1,1-trifluoropropan-2-one. This column offered a quantitative, one-step purification of JH esterase with a purification factor of ~800 and specific activity of ~570 nmol JH III hydrolyzed min⁻¹ mg protein⁻¹. The purified protein showed only a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a molecular weight of ~65,000. However, the purified enzyme apparently revealed the same two kinetic forms as the native enzyme, which indicates that two sites of the same protein are likely to be involved in JH hydrolysis.

During the early portion of the larval larval stadium of numerous lepidopteran species, it has been demonstrated that a reduction in the titer of juvenile horm-

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4 Abbreviations used: JH, juvenile hormone; JHE, JH esterase; DFP, diisopropyl phosphorofluoridate; EPPAT, O-ethyl-S-phosphorothiophosphate; OTPP, S-acetylthio-1,1,1-trifluoropropan-2-one; BPTPP, 3-(4-butyrophanythio)-1,1,1-trifluoropropan-2-one; MBTPP, 3-(4-mercaptobutylthio)-1,1,1-trifluoropropan-2-one; MBTPP-Sepharose, the resin produced from reacting epoxy-activated Sepharose with MBTPP; PTH, phenylthiourea; RSA, bivariate serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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after referred to as JHE) could disrupt metamorphosis led to the hypothesis that is some species an increase in catabolism, in addition to a decrease in biosynthesis. led to the reduction in JH titre (4-7). The evidence for this hypothesis recently has been reviewed (3).

It is thus important to examine the pharmacokinetics of JH degradation as well as the regulation of the JHE. To this end a detailed study of the kinetics of JHE as well as a method for its purification are requisite. Evidence is mounting that in the lepidopterous species examined, a single enzyme or a closely related group of highly specialized esterases are responsible for the majority of JH catabolism during the early part of the last larval stage (5, 8).

In the tobacco hornworm (Manduca sexta) and the tobacco (Sphenodole), there is some confusion. Earlier studies indicated multiple enzymes involved in JH hydrolysis (10, 11), while later studies indicated a majo involve of a single blood protein (9, 12). This study was undertaken in an attempt to resolve these differences and to lay the foundation for further studies into the regulation and pharmacokinetics of the JH-JHE interaction.

**EXPERIMENTAL PROCEDURES**

**Heliothis preparation.** Larvae of H. armigera and their artificial diets were provided through the courtesy of David A. Schelinsky and Mike J. Johnson. Larvae were obtained as late fourth instar, and they were reared in our laboratory until used. Hemolymph was from larvae having weights and developmental markers indicative of the prewandering peak of JHE activity (5, 13). Hemolymph was collected from the clipped and bron thoracic legs of the larvae into 15 ml centrifuge tubes that contained a few drops of 4-proprinol-ol (PTU) to inhibit tyrosinase. The hemolymph was mixed extending and then centrifuged at 3000 rpm for 10 min at 22°C, and the supernatant (plasma) was kept in aliquots at -20°C until used. JHE activity in the supernatant showed no change for several months under these conditions. The plasma was diluted with sodium phosphate (pH 7.4, 1:2) containing 0.01% PBU, 0.05% Triton X-100, 0.01% sodium arbo- cres, and 1% 2-mercaptoethanol (buffer control). Preliminary experiments showed no effect of the latter components on JHE activity. They were expected to wash out the enzyme during the purification procedure.

**Enzyme assay.** JHE activity was assayed by the method of Hammarnd and Sparks (14). For routine analysis 1 ml of 0.5 mg JH III or JH II was added to 100 μl of diluted plasma and the reaction mixture was incubated for 30 min at 30°C. In all cases the reaction was carried out under conditions that catabolized the time point of the reaction (usually 10 min with 1% plasma in buffer). The reaction was diluted to 10 ml of 104.1 Cm solution or 104.1 Cm solution from New England Nuclear, and the reaction was followed in a Beckman model DU spectrofluorimeter at 340 nm.

**Inhibition.** Five compounds were tested for their inhibitory potential against JHE of M. sexta. They included: 

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibitory Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5-Bromo-2'-(N,N-dimethylamino) benzaldehyde</strong></td>
<td>Significant</td>
</tr>
<tr>
<td><strong>3-(4-Bromophenyl)-1,1-dimethylurea</strong></td>
<td>Significantly</td>
</tr>
<tr>
<td><strong>3,4-Dichloroaniline</strong></td>
<td>Moderate</td>
</tr>
<tr>
<td><strong>2,4-Dichlorophenoxyacetic acid</strong></td>
<td>Minor</td>
</tr>
<tr>
<td><strong>2,4-Dichlorophenoxyacetic acid</strong></td>
<td>Trace</td>
</tr>
</tbody>
</table>

Inhibition curves were fitted to the equation for a single progressive activity (3, 15). These compounds were added to the substrate mixture. Two criteria were used for testing the potency of three compounds. The first was the K_i, the concentration of the inhibitor required to reduce the activity of the enzyme by 50% after 20 min preincubation. The second was the potency of the compound as a partial inhibitor, where the activity of the enzyme was used as a reference.

Control experiments revealed only 1% ethanol. Semi-log plots of percentage inhibition against inhibitor concentration were used to provide the inhibition curves. The second criterion was purely kinetic since it measured the rate of inhibition as a function of the preincubation time at has been described before (15). It should be mentioned that the time scale for the preincubation was variable depending on the concentration of the compounds and inhibitor concentration the time of incubation with the substrate was kept constant. The rate of inhibition for each inhibitor was measured at several times and the average percentage residual activity was plotted against preincubation time on semilog paper as test for primary-direct, the data obtained from semi-log plots, of plots found to be valid for two further kinetic treatment. These kinetic treatment were the first (15, 16) and second (17, 18) inhibition and as will be presented under Results and Discussion.

**Steady-state kinetics of JH I hydrolysis.** Double reciprocal plots for the JH II-hydrolyzing activity of 0.025% and 0.125% of diluted plasma or diluted purified enzyme (>1 mg/liter) was examined using final substrate concentrations ranging from 0.5 × 10^{-5} to 1.0 × 10^{-4} M. In order to estimate the initial velocity accurately, the irreversible velocity was used as a Dixon plot at 0°C carried out in carbonate-treated glass tubes. The same kinetic treatment also performed in
The presence of OTPP at two concentrations (5.47 x 10^{-7} M, 1.48 x 10^{-7} M). The inhibitor was added in 1% ethanol solution to 100 μl of 0.25% diluted plasma, and the reaction was terminated for 10 min at 37°C prior to addition of substrate and analysis of activity as discussed earlier.

Preparation of the affinity column (MTFF-Sepharose). The affinity matrix was prepared by reacting epoxy-activated Sepharose with 3-(4-mercaptobutyloxy)-1,1,1-trifluoro propane-trione (MTFF) (Fig. 1). Sepharose CL-4B was epoxy-activated according to literature procedures (21, 22). Thus, 40 ml of Pharmacia Sepharose CL-4B (washed with 10 vol. of distilled H2O) was added to 28 ml of 1% NaOH containing 52 mg Na2S2O5, and 5.7 ml of 1.4-butanediol diglycol ether (Aldrich). The slurry was stirred at room temperature for about 20 h using an orbital shaker. Then the epoxy-activated resin was washed extensively with water, watermethanol (1:1), and water, and then dried under suction. An aliquot of the resin was assayed for free oxides by adding 1.9 ml of 1.0 M Na2HPO4 (pH 7.0) to 0.3 g of the resin followed by back-titrating of the resultant solution using 0.01 M KOH. Values of [Ox/Resin] were obtained for these preparations. MTFF was synthesized from the reaction of 1,4-dimethoxybuthane (Aldrich Chemical Co.) with equimolar 2-bromo-1,1,1-trifluoromethane (PCI Research Chemical Inc., Gaithersburg, Md.). The details of the synthetic procedure are covered elsewhere (6, 7, 23). To 11.0 g of epoxy-activated resin (6.10 M Na2HPO4, pH 7.0) was added 10 ml of 0.3 M NaHCO3, which was added to give a pH of 8.5-9.0, followed by 30 ml of 40 mM MTFF in methanol. The slurry was stirred for 24 h at room temperature, excess methanethiol was added, and then the slurry was washed in a stirred glass funnel with 20 ml of methanol-water (1:1) and methanol, alternately. Then the gel was washed with 0.1 N NaOH, 1% L-phenylalanine, water, ethanol, water and ethanol, and was stored at 4°C in absolute ethanol containing a crystal of 0.1 M HCl (0.2%) as an antioxidant.

Affinity chromatography on MTFF-Sepharose. Affinity purifications were performed at 2°C using a 1-ml tuberculin syringe. A 100-μl bed of the affinity gel was packed in the column over a thin layer of glass wool and then washed with 1 ml of each of ethanol, watermethanol (1:1), ethanol-water (1:1), ethanol-water (1:3), and water. Then the column was equilibrated with the buffer cocktail. The buffer was monitored to ensure that inhibitors were not leaching from the matrix. Plasma of M. ornata diluted to 30% in the above buffer was pre-equilibrated with 2 x 10^{-7} M DFP for 15 min at 0°C to inhibit general esterases (10, 11). The activity toward 2 x 10^{-8} M JH III was determined before and after adding DFP. The diluted plasma was then pumped onto the column at a flow rate of 3 ml/min using a peristaltic pump, and 2- to 3-ml fractions were collected automatically. A 100-ml sample of diluted plasma took 24 h to pass through the column. All extracts of the plasma taken before loading the column were checked repeatedly for their ability to hydrolyze JH III, and were used as a standard to monitor recovery. The ability of the diluted plasma to hydrolyze JH III was essentially stable for the duration of the experiment. The enzyme activity on JH III and the protein concentrations of the loading fractions were assayed. No detectable JH activity eluted from the column during loading, and the protein concentration in the loading fractions was indistinguishable from that of the original diluted plasma. The column was then washed with the aforementioned buffer for about 20 h at a flow rate of 5 ml/h. Except for the first 2-3 fractions (2 ml each) there was no measurable protein and no JH activity was detected in any of the fractions. The column was then eluted with 1.0 ml of a OTPF solution (2.9 m) in buffer for 2-3 h.

Fig. 1. Outline of the synthesis of MTFF-Sepharose resin for affinity chromatography.
with shaking every 2 h. This procedure allowed the recovery of measurable protein in six fractions (0.36 ml each).

Removal of OTPP inhibited JHE to 20 ml from each of the above fractions was added 15 ml of a bovine serum albumin (BSA, Fraction V) solution at a concentration of 1 mg/ml, and the enzyme was adjusted to 2 ml using the buffer cocktail. No effect of the above BSA concentration on JHE hydrolyzing activity of the plasma was found. This condition gave a 100 dilution of both free and bound inhibitor, which was found to be critical for the reaction of JHE activity. These samples were dialyzed as has been described before (16) for 3 days against 2 liters of the buffer, and samples were withdrawn periodically and tested for JHE activity.

Protein samples Protein concentrations for all studies were determined by a dye binding method [113], as modified by Bio-Rad, using BSA (Fraction V, Sigma) as the protein standard. The method has been adapted to sample 100 ml of samples and 400 ml of 0.5 diluted dye (30% in distilled water). Ten minutes after the addition of the dye the absorbance was measured at 520 nm using a Gilford EEA manual ELISA reader interfaced with an Atari 400 microcomputer (programmed by E. Watanabe, this laboratory).

SOD-polyurethane gel elctrophoresis. Molecular weight determinations and homogeneity of JHE eluted with OTPP were assessed by denaturing polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS). A Hoefer SE600 vertical slab gel unit was employed to conduct discontinuous SDS-PAGE according to Laemmli (26) on 0.75-mm gels (12% acrylamide in separating gel, 5% in stacking gel).

RESULTS and DISCUSSION

Selective inhibition Inducetamidine and DPP were found to be essentially inactive in that at 10 mg the former gave no inhibition of JHE and the latter gave only 10% inhibition. However, EPATP, OTPP, and BPTPP were found to be unusually potent which enabled the evaluation of their concentration-inhibition curves. In all cases, inhibitor concentrations were selected to give 0-50% inhibition after a 10-min preincubation period. The residual enzyme activity was monitored using JH III at a final molar concentration of 5 × 10^{-4} M, and the percentage inhibition was plotted against log inhibitor concentration (Fig. 2). With the three inhibitors used, the inhibition pattern was not a simple curve and in fact there is an apparent indication of the presence of two forms of JHE-hydrolyzing activity with different sensitivity to these inhibitors. These forms are indicated by either a clear plateau in the inhibition curve or an inflection point near 50% inhibition. This behavior is quite different from that of JHE from larvae of the cabbage looper, Trichoplusia ni, where only a steep sigmoid curve was obtained with OTPP and EPATP (15), a characteristic of homogenous enzyme activity. However, other inhibitors indicate the possibility that there are multiple forms of JHE in T. ni as well.

Equivalent activity was assumed for the two forms based on the shape of the inhibition curves, and percentage inhibition from each form was recalculated as follows: All the percentages of inhibition under 50% are expected to be from the sensitive form and should be doubled to be relative to that form since it contributes —50% to the total activity. All the percentages of inhibition above 50% are expected to represent a complete inhibition of the sensitive form in addition to inhibition of a certain fraction of the less sensitive form. Therefore, 50% was subtracted from the percentage of inhibition and the residual was doubled to be relative to the less sensitive form. When the corrected percentage of inhibition was plotted against log inhibitor concentration, apparently straight lines were obtained for each form which resembled the inhibition of two homogenous catalytic units. Regression analysis of these lines gave molar log values for OTPP of 1.2 × 10^{-4} and 3.8 × 10^{-6} for BPTPP of 5.9 × 10^{-4} and 1.2 × 10^{-5}, and for EPATP of 1.1 × 10^{-4} and 1.1 × 10^{-6}, respectively, for the sensitive and less sensitive forms. Solubility of the tested inhibitors seems not to be responsible for the behavior on the inhibition curves since the inhibition point in each curve occurred at concentrations lower than 50% M. However, it was decided to use another line of evidence for the multiplicity of JHE from M. sexta which is the time-course of inhibition.

Time course of inhibition. As has been reported before (15, 16) both OTPP and EPATP inhibit JHE from T. ni in a time-dependent fashion. Therefore, these two compounds were used to evaluate the multiplicity of JHE-hydrolyzing activity in M.
FIG. 2. Plots of percentage inhibition of JH E from larval hemolymph of M. sexta against log molar concentration of OTFP (A), EFPA (B), and 2PFP (C). The inhibition curves showed the behavior of the sensitive and less sensitive forms of JH E with equivalent activity. Resolution of the inhibition for each form resulted in apparent straight lines for the sensitive (C) and less sensitive (B) forms. JH III at a final concentration of 6 X 10^-6 M was added 30 min after the addition of the inhibitor. Each point on the curves was the average of at least four replicates with a standard deviation of less than 5% of the mean.

sects using a time course to measure the rate of inhibition for each inhibitor concentration, a procedure that eliminates the solubility of the tested compound as a reason for the complex behavior observed. In the case of OTFP, four concentrations, ranging from 1.22 x 10^-5 to 1.37 x 10^-4 M, were preincubated with the enzyme for 0-
18 min and sampled at 0.5-min intervals, and the residual activity was then measured by adding JH III as substrate at a final concentration of $5 \times 10^{-5}$ M. In each of the concentrations used, the residual activity decreased gradually until it reached about 50% of the original activity, and then stayed unchanged and independent of the preincubation time. Since the uninhibited residual activity is independent of the concentrations used, it is not likely that this plateau is due to the inhibited enzyme reaching a steady-state condition.

Figure 3 shows an example of the pseudo-first-order plots of JHE inhibition by two inhibitor concentrations using JH III as substrate. Also, with one of these concentrations JH II was used instead. It is interesting that although the proportion of the less sensitive form is different based on the substrate used, the corrected percentage residual activity for the sensitive form was almost identical for the two substrates. Pseudo-first-order plots for the inhibition of JH II and JH III hydrolysis by $1.37 \times 10^{-5}$ M OTPP gave second-order rate constants ($k_2$) of $5.43 \times 10^6$ and $7.73 \times 10^5$ M$^{-1}$ min$^{-1}$, respectively.

An extensive effort has been devoted to studying the mode of inhibition of JHE from T. urticae by OTPP (15,16). In these studies, the compound was shown to act as a slow, tight-binding inhibitor, which seems to be the case with the sensitive form of JHE from M. sexta. If this is the case, one would expect a relationship between $I_0$ and $k_2$, according to the equation (28)

$$k_2 = 0.693/k_0(t).$$

It is worth noting that substitution for the 10-min preincubation $I_0$ value in the above equation resulted in a calculated $k_2$ value of $5.68 \times 10^5$ M$^{-1}$ min$^{-1}$, which is almost identical to the experimental value obtained from the pseudo-first-order kinetics. This reproducibility not only indicates that the reaction of the sensitive form with OTPP is of second order kinetics and demonstrates the characteristics of slow tight binding inhibition (27), but also gives additional supportive evidence for the presence of two forms of JHE in the hemolymph of M. sexta.

In order to test the time course of inhibition of the less sensitive form, higher concentrations of OTPP ($2.2 \times 10^{-5}$, $5.5 \times 10^{-5}$ M) were used to ensure complete inhibition of the sensitive form. Figure 4 shows that the less sensitive form reacts with OTPP in a time-independent manner for preincubation times greater than 2 min. This behavior opens the question of why some trifluoromethylketones behave as pseudoirreversible inhibitors while other act as classical competitive inhibitors of esterases. The dehydrase of the corresponding hydrolyze back to the active keto form as a rate-limiting step; a hypothesis that was suggested by Brodie et al. (28), cannot explain time-dependent and time-independent courses of inhibition by the same compound, OTPP. However, as has been hypothesized before, this difference in the behavior of OTPP is likely to be due to the nature of its binding to the enzyme.

![Figure 3: Pseudo-first-order plots for the inhibition of JHE from larval hemolymph of M. sexta by OTPP at final molar concentrations of $6.64 \times 10^{-5}$ M (C), $1.37 \times 10^{-5}$ M (D), and $6.64 \times 10^{-5}$ M (E) using JH III as a substrate. Inset shows pseudo-first-order plots of the inhibition of the sensitive form by $1.37 \times 10^{-5}$ M OTPP after correction for the less sensitive form. Data point represents the mean of three separate determinations.](image-url)
rather than to its dehydration back to the keto form. OTPF is expected to react with the sensitive form in roughly equimolar amounts, and it behaves as a slow, tight-binding inhibitor (27, 29-31); in contrast, the same compound interacts with the less sensitive form at higher concentrations, a condition required to ensure the classical behavior of competitive inhibition for this type of compound under steady-state conditions.

The data in Fig. 4 also support the reproducibility of the overall percent inhibition based on the assumption that two forms of JHE with equivalent activity are present in the hemolymph of M. sexta. At the concentrations used to generate that figure, the sensitive form is expected to be completely inhibited, which represents 50% of the total activity. However, the regression equation for the inhibition line of the less sensitive form (Fig. 2A).

![Graph showing relationship between percentage residual activity and preincubation time](image)

**Percentage inhibition (less sensitive)**

\[
-270.7 + 42.4 \log(\text{OTFP}) \quad [2]
\]

can be used to calculate the total percentage inhibition as follows:

**Total percentage inhibition**

\[
= \frac{(-270.7 + 42.4 \log(\text{OTFP}))}{2} + 50. \quad [3]
\]

The calculated values from the above equation at 2.19 \times 10^{-7}, 8.76 \times 10^{-7}, and 5.47 \times 10^{-6} M OTPF are, respectively, 50, 61.4, and 78.3, which are in a good agreement with average maximal percentage of inhibition (Fig. 4). The correlation between expected and experimental values is higher \((r^2 = 0.99, \text{Fig. 4 inset})\); better yet, the slope of the line is very close to unity (0.996), which gives additional evidence for the consideration of two forms with equivalent JHE activity in the hemolymph of M. sexta. As expected, EPFAT would be a good candidate to be tested as a time-dependent inhibitor since it acts as a phosphorylation (irreversible) inhibitor of JHE (16, 42).

With the use of EPFAT, different rates of inhibition were obtained, first fast and then slow (Fig. 5). Extending the same argument for the presence of two forms of JH-hydrolyzing activity, it was suggested that the data points fit a straight line in the slow phase of inhibition represent a pseudo-first-order plot for the inhibition of the less sensitive form in a time scale where the sensitive form was completely inhibited. These data points for each inhibitor concentration were subjected to linear regression analysis. The regression equations of the pseudo-first-order plots gave intercepts on the y axis equivalent to percentage residual activities ranging from 46.4 to 51.0, with an average of 49.5.

This percentage represents the proportion of the less sensitive form in the overall JHE activity, and supports the conclusion drawn from the inhibition curves and the time-course inhibition by OTPF. The equations were also used to extrapolate these lines back in order to calculate the expected percentage residual activity of the less sensitive form on the time scale of the fast phase of inhibition. This calculation permitted the resolution of the percentage
residual activity of the sensitive form since the data points in this phase represent the percentage residual activity of both forms. A semi-log plot of the corrected percentage residual activity of the sensitive form plotted against preincubation time (Fig. 6) seems to fit a straight line for each inhibitor concentration. The slopes of these regression lines \( \Delta \text{loge} / \Delta t \) as well as those for the less sensitive form (Fig. 5) were evaluated by the first-order kinetic approach developed by Main and his co-workers (17-19) for the inactivation of enzymes by organophosphates according to the following equation:

\[
\Delta \text{loge} / \Delta t = i / k_i + 1 / k_e, \tag{4}
\]

where \( i \) is the inhibitor concentration, \( k_i \) is the phosphorylation rate constant, and \( k_e \) is the overall bimolecular rate constant which equals \( k_p / K_e \), where \( K_e \) is the equilibrium dissociation constant. The steps in the reaction of the enzyme \( E \) and the inhibitor \( I \) that are controlled by the aforementioned constants can be depicted as:

\[
\text{E} + I \overset{k_i}{\underset{k_e}{\rightleftharpoons}} \text{EI}, \tag{5}
\]

A plot of \( \Delta \text{loge} / \Delta t \) against time gave a straight line for each form (Fig. 7), from which the above kinetic constants were calculated to be \( k_i = 0.65 \times 10^{-4} \text{ min}^{-1} \), \( k_e = 7.05 \times 10^{-2} \text{ min}^{-1} \), and \( K_e = 9.25 \times 10^{-5} \text{ M} \), respectively, for the less sensitive and sensitive forms.

The data for the sensitive form should be taken with caution since the second-order reaction constant is expected to be even higher than the calculated value as there is an upper limit for this rate constant, especially when the diffusion-controlled encounter of the enzyme and inhibitor is the rate-limiting step. Furthermore, the concentrations of EPPAT used are far higher than the \( K_e \) value of the sensitive form, a condition which requires very short preincubation times or simultaneous inhibition techniques in the presence of the
substrate for higher accuracy (23). However, the kinetic constants for the less sensitive form seem more accurate since there is a good agreement between the experimental \( k \) value calculated from the inhibition curve \((1.1 \times 10^{-3} \text{m})\) and that calculated from the \( k \) value and a 10-min preincubation time (9.8 \( \times 10^{-3} \text{m} \)) according to Eq. [1].

(Substrate specificity) The substrate used in most of the inhibition experiments was JHE II since it is the most water soluble of the JH hormones. Since JHE II appears to be more predominant than JHE III in larvae of \( M. scutellata \) (34), it was used for the substrate specificity studies.

The data for the double-reciprocal plot (25) were generated from 12 concentrations of JHE II, with the activity being measured after a 1-min incubation using two plasma dilutions. Figure 8A represents a typical plot from this experiment, which shows a straight line at the lowest seven concentrations and then a downward curvature making a steeper line at the highest five concentrations. This complex behavior does not indicate the presence of multiple forms exclusively since one could observe a similar behavior if there were enzyme activation at high substrate concentrations.

In order to exclude the possibility of substrate activation before discussing the complex behavior of JHE II hydrolysis by JHE II from \( M. scutellata \), two concentrations of OTFP were preincubated with the enzyme for 10 min before adding the same substrate concentrations, and the double-reciprocal plots from these experiments are shown in Fig. 9B. In the presence of the low concentration of OTFP (0.47 \( \times 10^{-5} \text{m} \)), two distinctive lines were also obtained; however, with 4.38 \( \times 10^{-5} \text{m} \) of the compound (i.e., 40 \( \times \) the \( k \) value of the sensitive form) the line became straight, indicating that substrate activation is not the reason behind the complex behavior obtained in the absence of the inhibitor.

Although it is hard and sometimes even impossible to calculate the \( K_s \) value of the
low-affinity form especially if they are two sites of unknown cooperative behavior, the high-affinity form is expected to behave independently of such cooperation. It has a $K_a$ value of $3.07 \times 10^{-8}$ M. More important are the $V_{max}$ calculated from the two lines (18.55 nmol min$^{-1}$ ml$^{-1}$ for the high-affinity form and 38.75 nmol min$^{-1}$ ml$^{-1}$ for the total activity) which indicate that the high-affinity form contributes $48\%$ of the total activity, in excellent agreement with the proportion of the two forms obtained from the inhibition kinetic data.

**Partition of JHE.** To the best of our knowledge only four publications have dealt with the partition of JHE from M. serot (10-13). In those studies, classical partition techniques were used. Although affinity chromatography offers rapid and quantitative procedures for the purification of enzymes, it has never been applied to JHE for any insect species. The fundamental reasons behind the delay in using this powerful and specific technique is usually the absence of a specific reversible inhibitor that can be attached to an insoluble support and used as a ligand for affinity chromatography. The unusually high inhibitory potency and selectivity of trifluoroketones to JHE indicated that these compounds might be good affinity ligands (4, 7, 36). Thus, MHTFP was reacted with epoxy-activated Sepharose (MHTFP-Sepharose) to yield the column used in this study.

Several trials to test for the efficiency and consistency of this column indicated that the JHE activity in $20$ ml of hemolymph ($\approx 400$ mg protein) was retained almost completely upon loading $100$ ml of MHTFP-Sepharose in a 1-ml tuberculin syringe, run at a flow rate of 3-5 ml/h of 1:5 diluted plasma. Interestingly, there was no detectable change in the protein content before and after passing through the column which, in addition to the fact that there was a drastic decrease (98%+) in JHE activity upon passing the column, indicates the efficiency and selectivity of the ligand used. However, the enzyme was bound so tightly to the column that elution was exceptionally difficult. This observation is not surprising since these compounds were found to act as slow, tight-binding inhibitors (15, 16), and sometimes to behave as pseudoreversible inhibitors. Eluting the column for $24$ h with $\approx 100$ ml of the buffer failed to regenerate the enzyme bound to the column. However, only the first 3-5 fractions exhibited measurable protein. Furthermore, OTFP at $10^{-3}$ M in buffer was not able to displace the enzyme upon regular washing. Therefore, it was decided to equilibrate the column with $\approx 9$ vol of OTFP solution for 6-12 h with shaking every 2 h. This procedure was successful in eluting most of the activity in six fractions of 0.50 ml each. The data collected from one of several columns run are presented in Table 1, and the specific activity and the behavior of the purified protein(s) on SDS-PAGE are consistent with the other columns.

Six fractions of OTFP-inhibited enzyme were obtained and dialyzed independently against phosphate buffer for 9 days. The activity of inhibited enzyme in each fraction was regenerated gradually, and peaked after dialysis for 7 days (Fig. 9). Control experiments with $1/2$ plasma preincubated with ethanol or ethanol-OTFP solution at 1 final concentration of $1.0 \times 10^{-3}$ M were dialyzed identically but separately. These experiments indicated, respectively, that the enzyme was stable at least for 7 days under the dialysis conditions and that the inhibited enzyme followed exactly the same patterns of regeneration as the enzyme eluted from the column. The activity 7 days after dialysis was subjected to further evaluation (Table 1). As seen in this table, the specific activity for the six fractions ranged from 441.5 to 702.8 with an average of 572 picomol JH III hydroxylase min$^{-1}$ mg$^{-1}$ protein$^{-1}$. The corresponding purification factors were 604.9-962.5 with an average of 765.9.

In attempting to compare the purification of JHE by the affinity column with the classical purification by Coudron et al. (12) a minor error was found due to their assuming the specific activity of the hemolymph was 0.05 instead of 0.19 nmol min$^{-1}$ mg$^{-1}$ . Thus, an actual purification factor of 442 was obtained in four steps to give 75% yield of apparently homogenous JHE with a specific activity of 84 nmol min$^{-1}$ mg$^{-1}$ which, in comparison with the data ob-
TABLE I
PURIFICATION OF JHE FROM THE HEMOLYMPH OF M. ascens BY AFFINITY CHROMATOGRAPHY

<table>
<thead>
<tr>
<th>Material assayed</th>
<th>Volume (ml)</th>
<th>Mg protein/ml (&lt;SD)</th>
<th>Specific activity (mol min⁻¹ mg⁻¹ ± SD)</th>
<th>Purification factor</th>
<th>Total activity (nmol/min)</th>
<th>Percentage recovery</th>
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<tr>
<td>Hemolymph</td>
<td>19.00</td>
<td>20.14</td>
<td>0.73</td>
<td>278.92</td>
<td>17.06</td>
<td></td>
</tr>
<tr>
<td>OTFP fractions</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>0.95</td>
<td>0.89</td>
<td>589.15</td>
<td>897.1</td>
<td>47.58</td>
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<tr>
<td></td>
<td>(0.004)</td>
<td>(0.001)</td>
<td>(21.37)</td>
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<tr>
<td></td>
<td>0.95</td>
<td>0.92</td>
<td>583.90</td>
<td>606.3</td>
<td>39.25</td>
<td>14.07</td>
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<tr>
<td></td>
<td>(0.002)</td>
<td>(0.001)</td>
<td>(3.88)</td>
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<tr>
<td></td>
<td>0.95</td>
<td>0.76</td>
<td>620.18</td>
<td>843.2</td>
<td>44.90</td>
<td>16.10</td>
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<td></td>
<td>(0.006)</td>
<td>(0.004)</td>
<td>(8.43)</td>
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<tr>
<td></td>
<td>0.95</td>
<td>0.81</td>
<td>762.8</td>
<td>966.8</td>
<td>27.38</td>
<td>9.00</td>
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<tr>
<td></td>
<td>(0.002)</td>
<td>(0.001)</td>
<td>(12.12)</td>
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<tr>
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<td>0.95</td>
<td>0.71</td>
<td>441.51</td>
<td>604.8</td>
<td>29.78</td>
<td>10.68</td>
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<td></td>
<td>(0.001)</td>
<td>(0.001)</td>
<td>(17.61)</td>
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<tr>
<td></td>
<td>0.95</td>
<td>0.64</td>
<td>574.09</td>
<td>787.24</td>
<td>20.57</td>
<td>11.00</td>
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<tr>
<td></td>
<td>(0.005)</td>
<td>(0.005)</td>
<td>(20.61)</td>
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</tr>
</tbody>
</table>

The six fractions obtained from the column showed a single band upon SDS-PAGE (Fig. 10) with a calculated molecular weight of ~60,000. Previous studies showed a molecular weight of ~68,000 (12) using both SDS-gel and gel-permeation chromatography, and 67,000 (10) using sedimentation and diffusion techniques. Comparing our data with those of Coudron et al. (12) was not meant to underestimate the quality of their classical purification approach of...
JHE from *M. sexta* since it was the first study to give homogenous enzyme. However, we offer in this study a more specific, quantitative, and rapid technique. Therefore, we had to compare the affinity column with the classical purification that gave the highest purification factor and specific activity (12) among all the published work.

**Kinetics of purified JHE** Although it has been shown that the hemolymph of *M. sexta* exhibits two separable kinetic forms of JH-hydrolyzing activity, several physicochemical techniques (9, 12, 13) indicated the presence of a single JHE in the hemolymph of the same species. The above information, in addition to the fact that the hemolymph used in the present work showed only one peak of JH-hydrolyzing activity upon gel-permeation chromatography (data not shown), might indicate that the two forms are, in fact, two catalytically active sites on the same protein. This seems likely since only one major band of protein of the purified enzyme was shown on SDS-PAGE gels (Fig. 10 and Ref. (13)). Therefore, it was decided to apply some of the kinetic approaches used with the hemolymph to the purified enzyme. Inhibition of the purified enzyme by EPPAT (Fig. 11) indicates the presence of two forms of JH-hydrolyzing activity toward JH III. Qualitative comparison of Figs. 11 and 2D indicates that the inhibition pattern is the same for both purified crude enzyme except that the proportion of the sensitive form was only 35% of the total activity of the purified enzyme instead of 50% of the activity of the crude enzyme. Quantitative resolution of the inhibition of each form, as explained before for the crude enzyme, resulted in \( I_{50} \) values of 6.0 \( \times 10^{-11} \) and 1.5 \( \times 10^{-10} \) m, respectively, for the sensitive and less sensitive form, as compared with 1.1 \( \times 10^{-10} \) and 1.1 \( \times 10^{-9} \) m for the crude enzyme. The higher \( I_{50} \) value for the less sensitive form (1.8X) and the lower value for the sensitive form (1.8X) from the purified enzyme as compared with the crude enzyme is not surprising since the \( I_{50} \) value of irreversible inhibitors is dependent on the molar equivalence of the available active sites for the inhibitor (27, 38, 39).

A double-reciprocal plot for the activity of the purified enzyme against the same concentrations used with the hemolymph is shown in Fig. 12. This figure shows that the data points fit two lines, as is the case with the crude hemolymph (Fig. 8A), with a \( V_{max} \) of 230.2 for the high-affinity form and a total \( V_{max} \) of 1078.9 nmol min \(^{-1}\) mg protein \(^{-1}\). In support of the inhibition data, this gives a proportion of 50:50 for the two forms. It is now clear that the high-affinity form is the same as the EPPAT-sensitive form, a conclusion that could not be drawn from the kinetic studies on the crude enzyme since both forms contribute equally to the overall activity of the hemolymph enzyme. It is rather interesting that the high-affinity form from the purified enzyme has a \( K_{m} \) value of 2.3 \( \times 10^{-9} \) m, which is almost identical to the \( K_{m} \) of this form from the crude enzyme. The data which fit this high-affinity form from Figs. 8A and 11 were subjected to a weighted least-squares analysis (89) of velocity against substrate concentration. The analysis revealed \( K_{m} \) values of 2.66 \( \pm 0.21 \times 10^{-9} \) and \( 2.40 \pm 0.09 \times 10^{-9} \) m, respectively. The \( V_{max} \) value calculated for the crude enzyme was 38.7 mU nmol \(^{-1}\) ml plasma \(^{-1}\). Recal-
involved studying the steady-state kinetics of JH II hydrolisis. The two sites were cat-
alysically equivalent at substrate satura-
tion and exhibited selectivity toward the inhibitors and specificity toward the sub-
strate. The equivalent activity of these sites was consistent in at least five different, he-
zymolymph pools, and was independent of the number of larvae used in each pool. The purification technique used failed to prove that these two sites were on separate proteins. Only one band was detected upon electrophoresis of the active JHE fractions from the affinity column. This information also fails to indicate that these two sites are on separate proteins. However, there is the possibility that either two JHEs co-
chromatograph on both affinity chroma-
tography and electrophoresis or that one form with a lower turnover number ob-
cures the presence of a second form with a very high turnover number.

The Kᵢ, exhibited by the hemolymph JHE of M. sexta clearly is low enough to re-
move JH from the carrier protein by mass action as previously discussed for T. ni (15). The discovery of two sites on the enzyme with different apparent Kᵢ's in-
dicates that a careful examination of the kinetics of ester hydrolisis in other lepi-
dopterous species will yield Kᵢ's lower than those reported to date (see Ref. (6) for re-
view). However, other biological impli-
cations of this work are not fully clear. The Kᵢ of the JHE from M. sexta certainly is low enough to make it an effective scav-
genger for a substrate existing at very low concentrations. For many enzymes in-
volved in the metabolism of endogenous substrates, the physiological concentration of the substrate approaches the Kᵢ of the enzyme which metabolizes it. However, the concentration of JH by the time JHE reaches its maximum titer in the early larval stadium is far below the substrate concen-
tration of catalytic sites and below the apparent Kᵢ of JHE as well. Thus, when only a very small proportion of the avail-
able JHE molecules will be interacting with even a single substrate, it is difficult to envision a biological role for two non-
equivalent catalytic sites. This observation again raises the possibility that JHE may have other as yet undetermined roles.

CONCLUSION

Three different approaches were used independently to indicate the presence of two catalytic sites involved in JH hydro-
lysis by the hemolymph and by an affinity purified esterase from the larvae of the to-
bacco hornworm. In two of these ap-
proaches, three inhibitors were used either in an empirical approach (inhibition curves) or in a pure kinetic treatment of the inhibition reaction. The third approach

![Graph showing the relationship between JH hydrolisis and substrate concentration.](image-url)