Characterization of Affinity-purified Juvenile Hormone Esterase from *Trichoplusia ni*  

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Juvenile hormone (JH) esterase was purified greater than 1000-fold in one step from hemolymph and whole larval homogenates from the last larval instar of *Trichoplusia ni* to give a single diffuse band that migrates at \( M_r \approx 64,000 \) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purification was based on an affinity chromatography procedure that employs trifluoromethyl ketone ligands. Isoelectric focusing of the purified preparations resulted in multiple bands that coincided to all significant hydrolysis of juvenile hormone detected in this manner. Kinetic experiments using optically pure enantiomers of JH II as substrates showed the two main electromorphs of JH esterase from the hemolymph to have apparently identical kinetic parameters as well as a similar capability to distinguish between substrates that differ in the orientation of the oxygen-moiety of JH. However, the enzyme could hydrolyze esters lacking the JH structure. The proteins were shown to be monomers and to have asparagine-linked oligosaccharides, most likely of hybrid structure. Immunochemical and other evidence showed that the affinity-purified proteins were responsible for all significant JH esterase activity during periods of rapid esterolysis *in vivo*.

The juvenile hormones are a series of farnesoate derivatives found in insects that play an important role in developmental events such as metamorphosis and oogenesis. In species of the lepidopteran order, JH exerts its influence at extremely low levels. For instance, in larvae of the moth *Trichoplusia ni* it has been shown that JH must be reduced from 250 pg/g of tissue to even lower levels for the larva to develop into the pupal form of its metamorphic life cycle (1). This reduction apparently is brought about by reduced biosynthesis of JH by a gland located immediately behind the brain and by increased degradation caused by the appearance of a hydrolyzing enzyme(s) termed juvenile hormone esterase (Fig. 1). This enzyme's activity appears, and then rapidly disappears in the blood of the insect at two stages in the last instar prior to pupation, and this seems to be a phenomenon general to all lepidopteran species examined (2, 3). JH esterase is of interest due to its critical role in insect development. The dramatic, sustained increase in JH esterase activity in the hemolymph of last instar larvae is among the first known biological events in the series of changes that lead to pupation, and has been shown to be necessary for proper development (4). Understanding of the regulation of JH esterase will allow a better understanding of the events leading to insect metamorphosis and will explain the different regulatory systems for the two different times of peak activity on the 2nd and 4th days of the last larval instar (5). Because of the apparent difference in regulation, it is important to determine whether the same protein(s) is responsible for the activity at these times. The isolation of the enzyme may provide a biotechnological avenue to the selective means of control of insect pests. Also, as the terpenoid-derived JH performs analogous functions in insects as the related steroids do in vertebrates, its regulation may provide a model for the regulation of the less accessible steroid-modifying enzymes that are under developmental control in vertebrates.

Isolation of JH esterase from *T. ni* as well as other insects has been sought by a number of laboratories in recent years (6–10). Thus far, classical means of purification of this enzyme from different species have not yielded sufficient quantities to be useful in extensive characterization or for the production of antibodies (6–10). This situation is mainly due to the low abundance of the enzyme in hard-to-obtain hemolymph even during peak activity. Due to this problem, an affinity purification procedure based on putative transition state mimics was adapted for use on JH esterolytic activities present in hemolymph and whole-body homogenates of *T. ni* (11–14). The enzyme was then characterized from both pre-(2nd day)- and postwandering (4th day) stages of development of the last larval instar.

**MATERIALS AND METHODS**

**RESULTS**

Affinity Chromatography of JH Esterase—Both column and batch loading procedures were done as described in the Min-
JH esterase was due to the slow tight binding of the 3-substituted, thio-
period of time (see insets, Figs. 3 and 5). This delay apparently
the affinity matrix with the inhibitor OTFP took an extended
when added to the biological material after the loading pro-
cedure. Elution of a significant fraction of the protein from
the affinity matrix with the inhibitor OTFP took an extended
period of time (see insets, Figs. 3 and 5). This delay apparently
was due to the slow tight binding of the 3-substituted, thio-
trifluoropropanoic moiety to JH esterase (22). This phenom-
enon also occurred in the recovery of activity of JH esterase
from the eluting inhibitor where extensive dialysis (about 2
weeks) was required for full recovery of the enzyme’s activity.

Electrophoretic Analysis—The purified preparations from
the differently prepared starting materials from two different
developmental stages in the fifth instar of T. ni yielded single
diffuse bands on SDS-PAGE (Fig. 6). However, with similar
protein loading as shown in Fig. 6, the enzyme from the
preupal homogenate gave a more diffuse band, indicating the
possible presence of a higher molecular weight component(s).
SDS-PAGE with very low protein loading followed by West-
ern blotting or silver staining revealed at least two closely
associated bands upon examination of the affinity-purified
JH esterase from the homogenate but not that from hemo-
lymph. Analysis of the same preparations by isoelectric focus-
ing showed that JH esterase from both preparations focused
at PI 5.5 and 5.4. Gel filtration on Sephacryl S-200 of both
starting materials showed the proteins to be monomers with
the activities eluting in one large peak at Mr = 65,000 (data
not shown).

Characterization as a Glycoprotein—JH esterase was shown
to be a glycoprotein when a chemical determination was used
(21) (data not shown). The protein band representing JH
esterase derivatized with dansyl hydrazine after oxidation
with periodate fluoresced slightly less than the same amount
of the positive control, ovalbumin. The carbohydrate com-
ponent was further defined with lectin blotting and endoglyco-
sidase digestions. A mannos or N-acetylglucosamine content
was shown by the affinity of the lectin concanavalin A for the
proteins bound to nitrocellulose filters (Fig. 8A). Also of note
from this experiment was the glycosylation of numerous pro-

tiens in both the hemolymph and homogenate used as starting
materials for the purification. Successful digestion of JH
esterase with peptide:N-acetylglucosaminidase F as monitored by
loss of lectin affinity showed a minor change in the proteins’
mobility on SDS-PAGE (Fig. 8B). When the glycosidase-
digested preparation was analyzed on isoelectric focusing gels
and compared to the native proteins, the two major and one
minor bands remained. However, their positions relative to
each other were altered and they displayed slightly more acidic
PI values (Fig. 8D). The carbohydrate moiety of JH esterase
proved refractory to hydrolysis by the endoglycosidase specific
for “high mannose” oligosaccharides, endo H, despite prior
heat denaturation in the presence of 0.05% SDS (Fig. 8C).
This was ascertained in the same manner as for the peptide:
N-acetylglucosaminidase F digestion. Confirmation of an active gly-
osidase during the incubation was achieved by observation of
loss of lectin affinity and change of Mr on SDS-PAGE by
ovalbumin present in the same preparation.

Kinetic Studies—Lineweaver-Burk analysis of the enzyme’s
velocity as a function of the concentration of JH II enantiom-
ers revealed a kinetic similarity of the two main isoelectric
forms of JH esterase from the hemolymph, as summarized in
Table I. It also showed a capacity of the enzyme to distinguish
substrate molecules that differ only in the orientation of the

**Fig. 1. Structures of major juvenile hormone homologs and
the reaction mediated by JH esterase.** JH 0, R=R'=R"=ethyl;
JH I, R=R'=ethyl, R"=methyl; JH II, R=ethyl, R'=R"=methyl; JH
III, R=R'=R"=methyl.

**Fig. 6. SDS-PAGE of JH esterase.** Both preparations of puri-
fied enzyme along with the crude starting materials were electropho-
resed through a 10% polyacrylamide gel in the presence of SDS and
stained with silver stain. Lane 1, molecular weight markers, 0.5 µg
each of phosphorylase b, bovine serum albumin, ovalbumin, and
carbonic anhydrase; lane 2, JH esterase purified from hemolymph,
1.2 µg; lane 3, JH esterase purified from whole-body homogenate, 1.5
µg; lane 4, whole-body homogenate, 20 µg; lane 5, hemolymph, 15 µg.
Characterization of JH Esterase from T. ni

Fig. 7. Isoelectric focusing of JH esterase. JH esterase purified by affinity chromatography was focused on a horizontal slab gel with a pH 4.0-6.5 gradient. A, JH hydrolytic activity in the crude starting materials. Volumes representing 40 μg each of homogenate (●) and hemolymph (×) protein were applied to each lane and focused across 100 mm. Activities and pH (■) were generated by assaying buffer or double-distilled H2O in which gel slices had incubated overnight. Of the applied activities, 52 and 38% were recovered from the hemolymph and homogenate, respectively. The silver-stained proteins were compared to JH hydrolytic activity in the crude starting materials run at the same time. B, purified enzyme from both preparations stained with silver stain. Lane 1, JH esterase purified from hemolymph, 4 μg; lane 2, JH esterase derived from a whole-body homogenate, 4 μg. No other bands or activities were evident on a wide-range pH gradient gel (pH 3.5-9.0) for either starting material. Bands marked with an arrow were shown to be artifacts of sample loading in an experiment involving loading the samples at the opposite end. C, high-resolution isoelectric focusing of affinity-purified JH esterase. After dialysis to remove the eluting inhibitor, JH esterase purified from hemolymph was subjected to isoelectric focusing on a 1 × 245-mm gel with a pH gradient of 4.0-6.5 for 2 h at 4°C. Activity profile was generated as above from gel slices (2 mm) that were eluted overnight in buffer. For an accurate assessment of the positions of the protein, an esterase activity stain with 1-naphthyl acetate was done at room temperature on an adjacent lane loaded with protein having an activity of 1.5 nmol of JH III/min. The three areas of JH esterase activity corresponded to the bands detected with silver stain and 1-naphthyl acetate. The detected peaks accounted for 95% of the applied activity.

Fig. 8. Glycoprotein characterization. Lectin blotting and endoglycosidase digestions were used to examine the carbohydrate content of purified JH esterase. Blots were done on proteins transferred to a nitrocellulose filter from an SDS-PAGE gel. They were probed with biotin-labeled concanavalin A, and then visualized by subsequent incubation in a solution of avidin conjugated to horseradish peroxidase followed by development with its substrate, 4-chloro-1-naphthol. JH esterase purified from the hemolymph was digested with the specific endoglycosidases peptide:N-acetylglucosidase F and endo H. The digestions were monitored by SDS-PAGE and lectin blotting as above. In addition, the preparation digested with peptide:N-acetylglucosidase F was subjected to isoelectric focusing on two gels of different pH gradients. A, lectin blot of both preparations of purified JH esterase and starting materials. The SDS-PAGE gel was loaded with: lane 1, hemolymph, 40 μg; lane 2, prepupal homogenate prepared at pH 4.6, 40 μg; lane 3, 2 μg each of phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme; lane 4, enzyme purified from hemolymph, 5 μg; lane 5, enzyme purified from the prepupal homogenate, 5 μg. Lane 3 shows the staining of the positive control, ovalbumin. B, SDS-PAGE (a) and lectin blot (b) of a peptide:N-acetylglucosidase F digestion of JH esterase purified from the hemolymph. Lane 1, standards, 500 ng each; lane 2, undigested JH esterase, 500 ng; lane 3, JH esterase, 500 ng, digested with peptide:N-acetylglucosidase F, 25 milliunits. C, SDS-PAGE (a) and lectin blot (b) of JH esterase and ovalbumin incubated with endo H. Lane 1, JH esterase and ovalbumin, 500 ng each incubated without endo H; lane 2, JH esterase and ovalbumin, 500 ng each incubated without endo H; lane 3, standards, 500 ng each. D, isoelectric focusing with wide and narrow pH gradients of JH esterase purified from hemolymph and digested with peptide:N-acetylglucosidase F. a, narrow pH gradient (4.0-6.5) and b, wide pH gradient (3.5-9.0). Lane 1, peptide:N-acetylglucosidase F, 50 milliunits; lane 2, JH esterase, 1 μg; digested with peptide:N-acetylglucosidase F, 50 milliunits; lane 3, undigested JH esterase, 1 μg.
procedure of the steady-state velocities between JH was performed (Fig. 3).

Affinity as measured by the Protein concentrations used were 10 times higher and incubation times 4-12 times longer for colorimetric versus JH substrates. A high specificity was achieved (Fig. 4). Serum was capable of precipitating over 98% of the JH esterase activity. The purified preparations together with the crude starting materials and molecular weight markers were transferred to a nitrocellulose filter from an SDS gel that was loaded as noted below. The filter was then divided and probed with immune and preimmune sera treated as described under "Materials and Methods" to eliminate nonspecificity. Visualization of the bound antibodies was accomplished with goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase and stained with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium. All blots were processed identically and simultaneously. A, preimmune sera (both treated and nontreated) gave identical results; B, nontreated immune sera; C, treated immune sera. The lanes were loaded as follows: lane 1, molecular weight standards; lane 2, 150 ng of JH esterase from the prepupal homogenate; lane 3, 96 ng of JH esterase from hemolymph; lane 4, 12.5 μg of prepupal homogenate; lane 5, 12.5 μg of hemolymph. B, immunoprecipitation of JH esterase activity from crude starting materials. Solutions of diluted (1:100) immune and preimmune sera combined in a manner so as to have an identical concentration of serum for each point were incubated with diluted hemolymph (920 μg/ml, 2.5 nmol/min/ml) (∗) and a prepupal homogenate prepared at pH 4.6 then raised to pH 7.4 (920 μg/ml, 3.7 nmol/min/ml) (∗) as described under "Materials and Methods." Antigen-antibody complexes were precipitated by centrifugation after a 3-h incubation at 30 °C with immunobeads. Each point is expressed as a percent of control activity which lacked sera and which remained steady throughout the experiment. The data are the average of three different experiments of which the standard error of the mean is indicated in all cases when the error is larger than the datum point. Several experiments result in the precipitation of 93 and 80% of the hydrolytic activity in larval homogenates prepared at pH 7.4 with and without centrifugation at 14,000 × g, respectively. The hydrolytic activity refractory to precipitation appears to be due to hydrolysis of the 10,11-epoxide of JH III.

### Table I

Kinetic parameters (±S.E.) of the main two forms of JH esterase from the hemolymph resolved by isoelectric focusing

<table>
<thead>
<tr>
<th>Isoform (pI)</th>
<th>JH II (enantiomer)</th>
<th>kcat</th>
<th>Km</th>
<th>kcat/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4</td>
<td>10R,11S</td>
<td>2.2 ± 0.39</td>
<td>352 ± 62</td>
<td>6.3 × 10⁶</td>
</tr>
<tr>
<td></td>
<td>10S,11R</td>
<td>1.0 ± 0.14</td>
<td>156 ± 22</td>
<td>6.4 × 10⁶</td>
</tr>
<tr>
<td>5.5</td>
<td>10R,11S</td>
<td>2.3 ± 0.84</td>
<td>324 ± 12</td>
<td>7.1 × 10⁶</td>
</tr>
<tr>
<td></td>
<td>10S,11R</td>
<td>1.2 ± 0.22</td>
<td>139 ± 28</td>
<td>8.6 × 10⁶</td>
</tr>
</tbody>
</table>

### Table II

Relative steady-state rates of hydrolysis by JH esterase upon alternative substrates

Data were obtained as noted under "Materials and Methods" and were normalized to the steady-state rate of hydrolysis of JH esterase toward JH III. Rates were determined simultaneously on the same batch of enzyme for JH III and the colorimetric substrates. However, the protein concentrations used were 10 times higher and incubation times 4-12 times longer for colorimetric versus JH substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% of rate toward JH III</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH I</td>
<td>145</td>
</tr>
<tr>
<td>JH II</td>
<td>131</td>
</tr>
<tr>
<td>JH III</td>
<td>100</td>
</tr>
<tr>
<td>4-Nitrophenyl acetate</td>
<td>110</td>
</tr>
<tr>
<td>4-Nitrophenyl methylene carbonate</td>
<td>7.0</td>
</tr>
<tr>
<td>1-Naphthyl acetate</td>
<td>19.0</td>
</tr>
<tr>
<td>1-Naphthyl methylene carbonate</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Discussion

Compounds with trifluoromethyl ketone moieties have been shown to inhibit a number of esterases (11, 12, 24). These compounds have been used to purify JH esterases from several species but their application to the enzymes of T. ni proved difficult due to the innate differences of proteins from organisms from different families as well as our use of a more complex starting material. The purification procedures described in the Miniprint Supplement are the result of numerous experiments designed to optimize yield and purity. Trifluoromethyl ketone inhibitors are thought to mimic the transition state of ester hydrolysis by forming a hemiketal link with the putative serine at the enzyme’s active site. Two observations made during the optimization of the purification process indicated linearity confirmed by r² values of >0.98.

**Fig. 9. Examination of antisera.** Western blotting and immunoprecipitation of JH hydrolytic activity from crude starting materials were used to examine rabbit polyclonal antibodies raised against purified JH esterase from hemolymph. The purified preparations together with the crude starting materials and molecular weight markers were transferred to a nitrocellulose filter from an SDS gel that was loaded as noted below. The filter was then divided and probed with immune and preimmune sera treated as described under "Materials and Methods" to eliminate nonspecificity. Visualization of the bound antibodies was accomplished with goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase and stained with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium. All blots were processed identically and simultaneously. A, preimmune sera (both treated and nontreated) gave identical results; B, nontreated immune sera; C, treated immune sera. The lanes were loaded as follows: lane 1, molecular weight standards; lane 2, 150 ng of JH esterase from the prepupal homogenate; lane 3, 96 ng of JH esterase from hemolymph; lane 4, 12.5 μg of prepupal homogenate; lane 5, 12.5 μg of hemolymph. B, immunoprecipitation of JH esterase activity from crude starting materials. Solutions of diluted (1:100) immune and preimmune sera combined in a manner so as to have an identical concentration of serum for each point were incubated with diluted hemolymph (920 μg/ml, 2.5 nmol/min/ml (†) and a prepupal homogenate prepared at pH 4.6 then raised to pH 7.4 (920 μg/ml, 3.7 nmol/min/ml (Δ) as described under "Materials and Methods." Antigen-antibody complexes were precipitated by centrifugation after a 3-h incubation at 30 °C with immunobeads. Each point is expressed as a percent of control activity which lacked sera and which remained steady throughout the experiment. The data are the average of three different experiments of which the standard error of the mean is indicated in all cases when the error is larger than the datum point. Several experiments result in the precipitation of 93 and 80% of the hydrolytic activity in larval homogenates prepared at pH 7.4 with and without centrifugation at 14,000 × g, respectively. The hydrolytic activity refractory to precipitation appears to be due to hydrolysis of the 10,11-epoxide of JH III.
procedure support this hypothesis. The results of one optimization experiment show that an intact active site is required for retention of JH esterase on the trifluoromethyl ketone ligand (Fig. 4, Miniprint Supplement). This experiment involved monitoring JH esterase activity in whole-body homogenates of prepupae prepared at pH 7.4 during incubations with two different organophosphate inhibitors and subsequent batch loading of the affinity matrix. One inhibitor, DFP, is an effective general esterase inhibitor but relatively ineffective against JH esterase, whereas O-ethyl, S-phenylphosphoroamidodithioate reduces JH hydrolytic activity to low levels. Both of these organophosphate inhibitors are thought to phosphorylate serines in the active site in an irreversible manner. Examination of the proteins eluted off the affinity matrix with OTFP clearly indicated that the loss of JH esterase activity in the homogenate correlated with elimination of the JH esterase band at 64 kDa. A second observation suggesting a quasi-covalent mechanism of binding is the fact that elution with the inhibitor OTFP is by far the most effective way to remove JH esterase from the affinity matrix. Experimentation with nonspecific eluting procedures under strongly denaturing conditions produced less protein in the eluate. Also supporting a transition state mechanism of binding is the fact that OTFP, which contains the S-substituted thiotrifluoropropasone moiety is a slow, tight-binding inhibitor of JH esterase, a characteristic of many transition state inhibitors (23).

The observation that catalytic activity was required for a protein's retention on the affinity matrix allowed a strategy of purification based on elimination of contaminating proteins' activity rather than their presence. A comparison of the results shown in Figs. 4 and 6 demonstrates that a combination of higher concentrations of DFP and a careful preparation of the homogenate aimed at taking advantage of the stability of JH esterase resulted in an apparently homogeneous band at 64 kDa. As shown in Fig. 4, a variety of proteins present in the homogenate bound to the affinity matrix and were eluted by OTFP; however, pretreatment with 100 μM DFP reduced the number and intensity of lower molecular weight bands. An acidic extraction, pretreatment with a higher concentration of DFP, and a freeze-thaw step completely eliminated the lower molecular weight bands (shown in Fig. 6). Extensive washing of the matrix also aided the purity of the preparation. Another potentially selective procedure was the use of different ligands on the matrix. However, this proved ineffective in the purification of JH esterase with four different affinity matrices that were synthesized with dithiol reagents and differed in the alkyl chain length. The matrices having carbon atom lengths of 4, 5, and 6 in the dithiol reagent gave similar results. The matrix synthesized with a 10-carbon atom length of the dithiol reagent proved ineffective in purifying JH esterase as it bound the enzyme only in dilute solutions.

The fractional yield displayed by these procedures for JH esterase gives rise to concerns as to whether the proteins isolated are those solely responsible for JH hydrolysis in vivo. Five lines of evidence support our contention that the purified and characterized proteins are responsible for all biologically significant JH esterolysis in vivo during periods of high JH esterase activity. Isoelectric focusing of the starting materials yielded peaks of activity only at the positions of the purified proteins (Fig. 7). Essentially all of the JH esterolitic activity was precipitated in the starting materials and a pH 7.4 homogenate of prepupae with a specific antiserum raised against affinity-purified JH esterase (Fig. 9D). The affinity matrix was able to retain virtually all the JH esterolytic activity, as seen in Figs. 3 and 5. The 64-kDa band representing JH esterase in Fig. 4 was the only one not inhibited by DFP that failed to bind to the affinity matrix upon inhibition of JH esterolysis in a homogenate of prepupae prepared at pH 7.4. In addition, a single peak of JH hydrolytic activity associated with the molecular weight of the purified proteins was eluted by gel permeation chromatography of diluted hemolymph. Similar results were obtained upon gel permeation chromatography of the homogenate of prepupae except that low levels of activity representing 6.3% of the total were observed in a very broad peak ranging across volumes associated with $M_\text{r} = 200,000–80,000$ (data not shown).

We tested the hypothesis of whether the multiple forms of JH esterase seen on isoelectric focusing were due to differences in extent of glycosylation among the forms. Experimentation confirmed the presence of carbohydrate but it did not support it as the sole cause of the multiple forms in the hemolymph. This was indicated first by the maintenance of the multiple bands on isoelectric focusing gels after deglycosylation with peptide:N-acetylglycosidase F (Fig. 8, B and D), an endoglycosidase of broad specificity which hydrolyzes most asparagine-linked glycans at the glycosylamine linkage (25). However, the altered arrangement of the bands in Fig. 8D did imply a difference in extent of glycosylation among the forms of JH esterase. Furthermore, the shift to a more acidic pI seen upon digestion with peptide:N-acetylglycosidase F indicated the lack of acidic functionalities on the cleaved oligosaccharide(s) and the appearance of a free aspartate residue(s) on the protein. This observation suggested that the oligosaccharide chain(s) of the enzyme is of the uncharged high mannose or "hybrid" types. However, when the esterase along with a positive control of ovalbumin is denatured with heat and detergent and subjected to a prolonged incubation with the endoglycosidase specific for high mannose structures, endo H, only ovalbumin was seen to lose affinity for lectins and change $M_\text{r}$ on SDS-PAGE. Thus, it appears that the carbohydrate moiety of JH esterase is either of a hybrid construction or is of a high mannose-type structure linked in a manner not susceptible to hydrolysis by endo H even after denaturation. This modification of the enzyme may help to explain the rapid disappearance in vivo of an extremely stable enzyme from its peak activity to a negligible level within a period of 12 h (2). It is known in vertebrate systems that the oligosaccharide moieties of serum proteins can be a major determinant of their half-lives in the blood (26). In addition, the glycosylation of JH esterase may be responsible for the common antigenic determinant(s) detected by antibodies in nontreated immune sera. This was suggested by the similarity of the lectin blot in Fig. 8A to the Western blot in Fig. 9B that employed untreated immune sera in the unlikely event that a proteinaceous antigenic determinant(s) would be common to so many proteins.

So far only juvenile hormones of the 10R,11S configuration of the epoxide moiety have been isolated from insects. Thus a distinct preference for this enantiomer was expected, assuming a distinction could be made by the two main isomers of JH esterase. Table I shows that a faster catalytic rate exists for the 10R,11S-enantiomer, whereas a higher apparent affinity exists for the 10S,11R-isomer. However, no statistically significant difference in preference by either form of JH esterase for the two substrates is found, as judged by the parameter $K_{\text{cat}}/K_m$. The high $K_{\text{cat}}/K_m$ ratios are nearing the theoretical diffusion controlled limit of $10^6$ s$^{-1}$ M$^{-1}$, indicating the enzyme to be highly efficient in its activity with JH II (27). The relatively high rate of hydrolysis of nitrophenyl acetate compared to the JH homologs shown in Table I was
initially surprising for an enzyme thought to be highly specific. However, the specificity of JH esterase for JH is due to a very low $K_m$ rather than to the somewhat low turnover of the stable conjugated ester.

The data support the picture that JH esterase activity in T. ni is due to a group of highly similar proteins. It is unclear whether the cause of the multiple forms is due to different gene products or to differential processing of the same gene. As can be seen in Fig. 7, there are distinct differences between the two affinity-purified preparations in the pattern of bands seen on the isoelectric focusing gel. Differences can also be observed on SDS-PAGE (Figs. 6 and 9). It is likely that these differences are due in part to the presence of intracellular species of JH esterase in prepupal homogenates. However, it is apparent that the proteins present in the hemolymph on the 2nd day of the last instar are identical to some of the proteins present on the 4th day. A high similarity of the remaining proteins present in the homogenate was indicated by the ability of the antisera raised against the enzyme purified from day 2 to precipitate all the activity in the homogenate from day 4 (Fig. 9).

The characterization described in this paper complements the extensive characterization of JH esterase conducted with crude preparations. Inhibitor studies, pH optima, and stability have all been published elsewhere (28–31). However, it must be pointed out that certain discrepancies exist between this and previous work as to the presence of multiple forms and the molecular weight (6, 10, 32). The isolation of JH esterase from T. ni will allow experimentation to be conducted on its regulation.

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REFERENCES

10. Rudnicks, M., and Jones, D. Insect Biochem. 17, 373-382
30. Sparks, T. C., and Hammock, B. D. (1979) Insect Biochem. 9, 411-421

Continued on next page.
Characterization of JH Esterase from *T. ni*

Terry R. Bolling and Bruce D. Hammock

MATERIALS AND METHODS

Chemicals and solutions. All chemicals were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Stock solutions of Tris (100, 200, 400, and 800 mM) and NaCN (200 mM) were prepared in 0.1 M NaOH and stored at -20°C. The pH of the stock solutions was adjusted to 7.4 before use. In some experiments, NaCN was added to the assay mixture to inhibit JH esterase activity. The pH of the assay mixture was adjusted to 7.4 before use. In some experiments, NaCN was added to the assay mixture to inhibit JH esterase activity.

Results. The enzyme was purified from the *T. ni* hemolymph by ammonium sulfate precipitation, followed by chromatography on DEAE-Sepharose CL-6B, and finally electrophoresis on agarose gel. The enzyme was purified to homogeneity, as judged by SDS-PAGE analysis of the native enzyme in the absence of detergent.

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Purification of JH esterase from *T. ni*. The enzyme was purified by ammonium sulfate precipitation, followed by chromatography on DEAE-Sepharose CL-6B. The enzyme was purified to homogeneity, as judged by SDS-PAGE analysis of the native enzyme in the absence of detergent.

Electrophoresis and protein determinations. SDS-PAGE was conducted in 10% gels with the discontinuous buffer system of Laemmli (1970) and stained using Coomassie Blue or silver stains with standard procedures. Immunoblotting and Western blotting were conducted for 3 hours at 100 V. The gels were exposed to X-ray film at -80°C before autoradiography using a phosphorimager (Storm 840, Molecular Dynamics). Staining was done with silver stains after removal of the gels by soaking the gel in a solution of 2% SDS and 5% glycine in 250 mmol/l of Tris, pH 7.8, for 5 minutes. Staining of the gels was performed by overnight soaking in a solution of 2% SDS and 5% glycine in 250 mmol/l of Tris, pH 7.8, for 5 minutes. The gels were then stained for 5 minutes in a solution of 2% SDS and 5% glycine in 250 mmol/l of Tris, pH 7.8, before autoradiography.

Enzyme assays and kinetic experiments. Activity of JH esterase was measured by a modified method of Bradford (1976). Enzymatic activity was assayed by incubating mixtures containing JH esterase and substrate at pH 7.4. The reaction was stopped by the addition of 0.5 M Tris-HCl (pH 8.0) to a final concentration of 0.1 M. The absorbance was measured at 25°C with a spectrophotometer. The reaction was stopped by the addition of 0.5 M Tris-HCl (pH 8.0) to a final concentration of 0.1 M. The absorbance was measured at 25°C with a spectrophotometer.

Fig. 3. Affinity chromatography of JH esterase from *T. ni*. After purification, the enzyme was centrifuged at 13,000 g for 10 minutes to remove any debris. The supernatant was dialyzed against 0.1 M NaCl at pH 7.4 for 2 hours to remove any remaining impurities. The enzyme was then applied to a column of DEAE-Sepharose CL-6B. After washing with buffer A, the column was eluted with buffer B containing 2 M NaCl. The eluate was monitored at 220 nm and the activity was measured using a spectrophotometer at 220 nm.

Fig. 4. Purification of JH esterase from *T. ni*. The enzyme was purified by ammonium sulfate precipitation, followed by chromatography on DEAE-Sepharose CL-6B. After washing with buffer A, the column was eluted with buffer B containing 2 M NaCl. The eluate was monitored at 220 nm and the activity was measured using a spectrophotometer at 220 nm.

Fig. 5. Purification of JH esterase from *T. ni*. The enzyme was purified by ammonium sulfate precipitation, followed by chromatography on DEAE-Sepharose CL-6B. After washing with buffer A, the column was eluted with buffer B containing 2 M NaCl. The eluate was monitored at 220 nm and the activity was measured using a spectrophotometer at 220 nm.
Characterization of JH Esterase from T. ni

**Fig. 4.** Effect of inhibitors on killing of JH esterase to the affinity matrix. A homogenate of T. ni larval hemolymph containing 1 mg of esterase was divided into 20 ml aliquots to which were added 20 ml of either 0.1 M phosphate buffer (pH 7.4) containing 0.5% Triton X-100 or the affinity matrix was used and the mixture was incubated at 30°C for 30 minutes. The matrix was boiled by centrifugation, washed, and then the supernatant was assayed to determine the relative amount of esterase activity. The homogenate was incubated with 50 ml of 1 M sodium chloride, pH 7.4, and the supernatant was assayed to determine the relative amount of esterase activity. The homogenate was incubated with 50 ml of 1 M sodium chloride, pH 7.4, and the supernatant was assayed to determine the relative amount of esterase activity.

**Fig. 5.** Affinity chromatography of JH esterase from a whole larval homogenate using a batch loading procedure. A homogenate of prepupae (1600 mg protein) was diluted to 50 ml in buffer containing 50 mM Tris-HCl, pH 7.4, and 1 M sodium chloride, pH 7.4, and the matrix was incubated with 100 ml of the affinity matrix at 4°C. The matrix was collected by centrifugation and washed with buffer as noted in the supplement. No activity was detected during the washing procedure. Insert shows the subsequent elution of JH esterase protein from the affinity matrix with a specific activity of 1.61 nmol/min. After a 1540-fold purification with 10% recovery of the original material.

**Cell filtration.** Gel filtration experiments were carried out employing a column 2.5 cm × 95 cm of Sephacryl S-200 (Pharmacia). The prepupal homogenate was prepared as described above. The homogenate was incubated with 1 M sodium chloride, pH 7.4, and the supernatant was assayed to determine the relative amount of esterase activity. The homogenate was dialyzed against 1 M sodium chloride, pH 7.4, and the supernatant was assayed to determine the relative amount of esterase activity. The homogenate was dialyzed against 1 M sodium chloride, pH 7.4, and the supernatant was assayed to determine the relative amount of esterase activity. The homogenate was dialyzed against 1 M sodium chloride, pH 7.4, and the supernatant was assayed to determine the relative amount of esterase activity.

**Immunological procedures.** Rabbit polyclonal antibodies were raised against JH esterase purified from the hemolymph. JH esterase (100 µl) was mixed with Freund's complete adjuvant (1:1) and injected subcutaneously at multiple sites on the back of a New Zealand White rabbit. The rabbit was bled and the collected sera was frozen after collection. To eliminate the non-specificity of the immune sera, hemolymph containing JH esterase was incubated with JH esterase purified from the homogenate. JH esterase was then dialyzed against 1 M sodium chloride, pH 7.4, and the supernatant was assayed to determine the relative amount of esterase activity. The homogenate was dialyzed against 1 M sodium chloride, pH 7.4, and the supernatant was assayed to determine the relative amount of esterase activity. The homogenate was dialyzed against 1 M sodium chloride, pH 7.4, and the supernatant was assayed to determine the relative amount of esterase activity.

Western blotting was conducted along lines stated by Towbin et al. (17). Protein extracts derived from SDS-PAGE were electroblotted from nitrocellulose filter and probed with a 1:1000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad) according to the manufacturer's recommendations. Diluted preimmune and immune sera were combined in a manner such that a constant amount of serum was present for each experiment.

**Discopyrotein characterization.** Detection of the incorporation of JH esterase by chemical means was done according to a previously published procedure (22). The SDS-PAGE gel was stained with Coomassie Blue R-250. The gel was then destained with 10% acetic acid and incubated in 50 ml of 1 M sodium chloride, pH 7.4, and the supernatant was assayed to determine the relative amount of esterase activity. The gel was then destained with 10% acetic acid and incubated in 50 ml of 1 M sodium chloride, pH 7.4, and the supernatant was assayed to determine the relative amount of esterase activity. The gel was then destained with 10% acetic acid and incubated in 50 ml of 1 M sodium chloride, pH 7.4, and the supernatant was assayed to determine the relative amount of esterase activity.