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Purification and Characterization of Hemolymph Juvenile Hormone Esterase from the Cricket, *Gryllus assimilis*

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Abstract: Juvenile hormone esterase (JHE) from the serum of the cricket, *Gryllus assimilis*, was purified to homogeneity in a four-step procedure involving polyethylene glycol precipitation, hydrophobic interaction FPLC, and ion exchange FPLC. This procedure could be completed in 4 days and resulted in a greater than 900-fold purification with greater than 30% recovery. The purified enzyme exhibited a single band on a silver-stained SDS PAGE gel and had an apparent subunit molecular mass of 52 kDa. The native subunit molecular mass, determined by gel permeation FPLC, was 98 kDa, indicating that JHE from *Gryllus assimilis* is a dimer of two identical or similar subunits. The turnover number of the purified enzyme (1.41 s^{-1}), $K_{M(JH-III)}$ (84 ± 12 nM) of nearly-purified enzyme, and k_{cat}/K_M (1.67 × 10^7 s^{-1} M^{-1}) were similar to values reported for other well-established lepidopteran and dipteran JHEs. JHE from *Gryllus assimilis* was strongly inhibited by the JHE transition-state analogue OTFP (octylthio-1,1,1-trifluoro-2-propanone; I_{50} = 10^{-7} M) and by DFP (diisopropyl fluorophosphate; I_{50} = 10^{-7} M). The shapes of the inhibition profiles suggest the existence of multiple binding sites for these inhibitors or multiple JHEs that differ in inhibition. Isoelectric focusing separated the purified protein into 4 isoforms with pIs ranging from 4.7–4.9. N-terminal amino acid sequences (11–20 amino acids) of the isoforms differed from each other in 1–4 positions, suggesting that the isoforms are products of the same or similar genes. Homogeneously purified JHE hydrolyzed α-napthyl esters, did not exhibit any detectable acetylcholinesterase, acid phosphatase, or aminopeptidase activity, and exhibited only very weak alkaline phosphatase activity. JHE exhibited a low (11 µM) K_M for long-chain α-naphthyl esters, indicating that JHE may have physiological roles other than the hydrolysis of JH-III. Purification of JHE represents a key step in our attempts to identify the molecular causes of genetically-based variation in JHE activity in *G. assimilis*. This represents the first homogeneous purification of JHE from a hemimetabolous insect.

Keywords: juvenile hormone esterase, JHE, esterase, juvenile hormone, JH, JH III

INTRODUCTION

Although a tremendous amount of information is available on the biochemistry, physiology, and molecular biology of insect hormones (Kerkut and Gilbert, 1985; Gupta, 1990; Riddiford, 1996; de Kort and Granger, 1996), relatively little is known about evolutionary-genetic aspects of insect endocrinology. The hydrolytic enzyme, juvenile hormone esterase (JHE), in species of *Gryllus* crickets has recently emerged as one the most intensively studied models with respect to the evolutionary genetics of endocrine variation (Zera and Tiebel, 1989; Zera and Zhang, 1995; Gu and Zera, 1996; Zera *et al.*, 1996; Zera and Huang, 1999). JHE hydrolyzes the key insect developmental and reproductive hormone, juvenile hormone, and partially regulates its titer (Hammock, 1985; Roe and Venkatesh, 1990). JHE also has been implicated in the regulation of ecologi-

cally important polymorphisms such as wing-polymorphism in *Gryllus* crickets (Zera and Tiebel, 1989; Zera and Denno, 1997; Zera and Huang, 1999) and dispersal/reproduction polymorphism in the Colorado potato beetle, *Leptinotarsa decemlineata* (Vermunt *et al.*, 1997).

Using artificial selection on crickets derived from natural populations, we have obtained replicate genetic stocks of *Gryllus assimilis* that differ in hemolymph JHE activity (Zera and Zhang, 1995; Zera *et al.*, 1996; Figure 1). One of our major goals is to identify the molecular, biochemical, and physiological bases of JHE activity differences between selected lines to gain insight into the nature of genetically-based variation in JHE that exists in natural populations. Genetically-based differences in JHE activity could result from any number of causes such as (1) variation in cis or trans acting regulators of JHE transcription, (2) variation in degradation, (3) differential expression of multiple JHE isozymes, or (4) variation in the JHE structural locus itself resulting in allozymes that differ in kinetic or stability properties. An important step in distinguishing among these various possibilities is obtaining and characterizing homogeneously purified enzyme. Here we report on a rapid and efficient purification of JHE from *Gryllus assimilis* using classical chromatographic procedures, and kinetic and physical characterization of homogeneously purified enzyme. This is the first characterization of a homogeneously purified JHE from a hemimetabolous insect.

MATERIALS AND METHODS
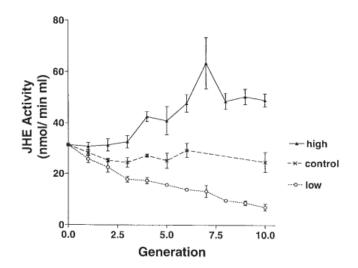
Chemicals

Unless otherwise stated, all chemicals, buffer components, molecular weight standards, and non-radiolabelled substrates for JHE were purchased from Sigma Chemical Company (St. Louis, MO) or Fischer Scientific (Rockville, MD). Radiolabelled racemic $[10^{-3}H]$-juvenile hormone-III (12 Ci/mmol; 444 GBq/mmol) and unlabeled racemic juvenile hormone-III (JH-III), used in the standard JHE assay, were purchased from New England Nuclear (Boston, MA) and Sigma Chemical Co., respectively. Narrow range (pH 4.0–

6.5) LKB Ampholine PAGplate precast isoelectric focusing gels (5% polyacrylamide, 2.2% ampholites) were purchased from Pharmacia (Piscataway, NJ). IEF standards were purchased from Bio Rad (Richmond, CA) and PVDF protein electrophoretic blotting membrane (Trans-Blot, 0.2 μm) was purchased from Bio-Rad. The JHE inhibitor, OTFP (octylthio1,1,1-trifluoro-2-propanone), was a generous gift of Dr. Bruce Hammock, Department of Entomology, University of California, Davis, California.

Standard JHE and Protein Assays

JHE activity was measured using the standard radiochemical assay of Hammock and Sparks (1977) as modified for *Gryllus* species (Zera *et al.*, 1992). Protein concentration was measured using the bicinchoninic acid assay (Stoscheck, 1990) with bovine serum albumin (fraction V) as the standard.

Figure 1. Change in grand mean hemolymph JHE activity in *Gryllus assimilis* during 10 generations of artificial selection. Each symbol represents the average of the means of 3 lines selected for increased JHE activity, decreased JHE activity, or unselected controls. Bars (SEM) were calculated from the variance in line means. For details of the selection study, see Zera and Zhang (1995) and Zera *et al.* (1996).

Insects, Hemolymph Collection, and Hemolymph Storage

Gryllus assimilis used in this study were a mixture of crickets derived from lines selected for elevated JHE activity or from unselected controls. A description of the selection experiment and the various lines are given in Zera and Zhang (1995) and Zera *et al.* (1996; also see Figure 1). All crickets were reared at 28° C under a 16 light:8 dark photo-regime and were fed the standard dry diet (Zera and Huang, 1999). JHE was purified from frozen serum collected from day 4–7, last-stadium crickets. JHE activity is maximal during this stage in development (Zera and Zhang, 1995). Hemolymph was collected from cuts in the cerci, legs, and abdomen and was allowed to clot at room temperature. Hemolymph was then centrifuged at 14,000*g* for 15 min, serum was removed, phenylthiourea (PTU) was added (0.01% w/v), and the serum was stored frozen at –80° C for up to several months prior to use in purification. Previous studies showed no loss in JHE activity in hemolymph frozen and stored under these conditions.

Purification of JHE

JHE activity was assayed during various stages of purification using the standard radiochemical assay described above. Unless otherwise stated, all procedures were performed on ice or in a cold room at 4–6° C. The procedure described here was used routinely for 3–12 mL of frozen serum.

Serum was thawed and diluted fivefold with 50 mM potassium phosphate buffer, pH 8.0. A 50% aqueous polyethylene glycol (PEG; 8,000 MW) solution was added slowly over a 30-min period to the diluted serum such that the PEG concentration was 11% (e.g., 17 mL added to 60 mL of diluted serum). The solution was stirred for 1 h and centrifuged for 10 min at 3,000*g*. The supernatant, which contained > 95% of the JHE activity, was loaded onto a DEAE anion exchange column (8.8 × 1.6 cm; 22 mL bed volume) that had been equilibrated with 50 mM phosphate buffer pH 8.0 at room temperature. The flow rate was 30–40 mL h^{-1}. All procedures involving this particular chromatographic step were performed at room temperature. The column was washed with 44 mL 30 mM MOPS [3-(N-morpholino)propanes

ulfonic acid], pH 8.0, and enzyme was eluted using a 0–1.0 M NaCl linear gradient in 40 mL wash buffer. The flow rate was 20 mL h^{-1}. Fractions of 2 mL volume were collected. Typically, 80–85% of the activity applied to the column eluted in a single peak in 4 fractions that were combined.

The next step in the purification procedure involved hydrophobic interaction chromatography, in which JHE was bound to a Phenyl Superose column under high salt conditions and eluted with a decreasing salt gradient. This step required that JHE be dissolved in a buffer containing 2M NaCl. Since it was known from previous studies that JHE lost 75% activity when stored in 2 M NaCl for 2 days on ice, salt was added to the DEAE eluant from the previous step just prior to application to the Phenyl Superose column. Solid NaCl was added to the solution on ice over a 20-min period until the NaCl concentration was 2.0 M. The volume of this solution was reduced to 2 mL by centrifugation using a Centricon-30 microconcentrator (Amicon). The JHE solution was loaded onto a Phenyl Superose HR 5/5 column (1 mL; Pharmacia) attached to an LKB 5000 Fast Protein Liquid Chromatographic (FPLC) unit at a flow rate of 0.25 mL min^{-1}. The column had been equilibrated with 2 M NaCl in 30 mM MOPS, pH 8.0. The column was washed with 6 mL of equilibration buffer and JHE was eluted with a 2.0–0 M NaCl gradient over 20 mL. Fractions of 0.5 mL volume were collected. Typically, 50–80% of JHE applied to the column eluted as a single peak in 12 fractions between 0.5 and 0.3 M NaCl. These fractions were pooled and dialyzed overnight against 1 L 30 mM MOPS buffer, pH 8.0. The dialyzed solution was concentrated to 2 mL using a Centricon 30 microconcentrator and applied to a Mono Q HR 5/5 anion exchange FPLC column (1 mL; Pharmacia) that had been equilibrated with 30 mM MOPS, pH 8.0. The flow rate was 0.5 mL h^{-1}. The column was washed with 10 mL of equilibration buffer and JHE was eluted with a 0–1.0 M NaCl gradient in 30 mM MOPS, pH 8.0 over 67 mL. Typically, JHE activity eluted as a single peak in fractions 8–15 of the gradient. In every run, three fractions (0.152–0.148 M NaCl) were pooled starting with fraction 8 or 9 of the gradient. These fractions contained 40–80% of JHE activity applied to this column.

SDS-PAGE

Enzyme purity was assessed by electrophoresis on SDS PAGE gels (10% total acrylamide in the resolving gel) followed by silver staining (Garfin, 1990).

General Esterase Stain

A non-specific esterase stain was used to visualize JHE in native PAGE and IEF gels. The stain contained 3 mL of a 1% α-naphthyl acetate solution in 50% aqueous acetone, 50 mg fast blue BB salt and 50 mL 0.2 M Tris-HCl, pH 7.0 (Murphy *et al.*, 1996).

Isoelectric Focusing (IEF)

Isoelectric focusing was performed using an EC 1001 isothermally controlled electrophoresis unit and narrow pH (4.0–6.5) LKB Ampholine PAG-plate precast gels (5% polyacrylamide, 2.2% ampholites; 11 cm in length). Pooled post Mono Q fractions (5–15 μL) were loaded onto a paper wick that had been placed 1 cm from the cathodal buffer strip. The cathodal and anodal buffers were 0.1 M NaOH and 0.5 M H_3PO_4, respectively. Gels (8 cm wide) were run for 2 h at 2,000 V, 15 mA, and 12–14 W. After focusing, JHE isoforms were visualized with the general esterase stain employing α-naphthyl acetate (Murphy *et al.*, 1996) or with a general protein (Coomassie Blue) stain. General (α-naphthyl) esterase stains can be used to visualize JHE isoforms on gels after the Phenyl-Superose step since all general esterase activity is due to JHE after this step (see Results). Isoelectric points of JHE isoforms were estimated from their position on the gel relative to IEF standards stained with Coomassie Blue.

Apparent Subunit and Native Molecular Masses

Apparent subunit molecular mass of homogeneously purified JHE was estimated on an SDS PAGE gel that had been silver stained. The relative mobility of JHE and the following protein standards with known molecular masses (Sigma M 2789) were analyzed by linear regression: myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, bovine albumin 66 kDa, ovalbumin 45 kDa, carbonic anhydrase 29 kDa, and soybean trypsin inhibitor 20 kDa. Apparent native molecular mass of JHE was determined by gel permeation FPLC using a High-Prep 16/60 Sephacryl S-300 column equilibrated with 30 mM MOPS buffer (pH 8.0) containing 0.15 M NaCl. The following protein standards were used: blue dextran (2,000 kDa; void volume), thyroglobulin 669 kDa, β -amylase 200 kDa, bovine serum albumin 66 kDa, and carbonic anhydrase 29 kDa.

N-Terminal Amino Acid Sequence of Isoforms

Pooled, post-Mono Q fractions were concentrated using a Centricon 30 unit such that the protein concentration was 0.4 μg/μL. Thirty-two micrograms of protein in 80 μL of solution were loaded onto a stack of 4 paper wicks on a narrow range IEF gel and run as described above. After IEF, the gel was separated from its plastic backing and soaked in transfer buffer (see below). The gel was then added to a Bio-Rad Mini Trans-Blot electrophoretic transfer cell containing a PVDF membrane. The transfer buffer contained 90% 50 mM 3[cyclohexylamino]-1-propane-sulfonic acid (CAPS), pH 11.0, 10% methanol, and 0.1% SDS. Protein transfer was performed at 250 mA (~65V) for 1.5 h with stirring in a 4–6° C cold room. The membrane was lightly stained with 0.025% Coomassie Blue R-250 dissolved in 40% aqueous methanol and destained with 50% aqueous methanol. The two major and 2 minor isoforms (see Results) were cut out and individually sequenced on an Applied Biosystems Procise Sequencer at the Protein Structure Core Facility, Department of Biochemistry, University of Nebraska Medical Center.

Estimation of the Michaelis Constant for JH-III

The $K_{M(JH-III)}$ of purified JHE was estimated essentially as described previously for the unpurified JHE of the congener *Gryllus rubens* (Zera *et al.*, 1992). Velocities were measured in triplicate at each of six JH-III concentrations ranging from 500 to 50 nM. These con-

centrations bracketed the $K_{M(JH-III)}$ estimated for the un-purified JHE from *Gryllus assimilis* obtained in an earlier study (Zera and Zeisset, 1996). To conform to the restrictions of steady-state kinetics, no more than 10–15% of the JH-III was hydrolyzed during the assays. The Michaelis constant was estimated directly from the untransformed velocity measurements using the program Enzfitter (Leatherbarrow, 1987). Only a limited amount of homogeneously purified JHE was obtained in the present study, most of which was required for N-terminal amino-acid sequencing and production of a polyclonal antibody. Consequently, the $K_{M(JH-III)}$ estimate and inhibition studies (see below) were performed using enzyme that was a highly purified (344-fold, post-mono-Q fraction), but that was not homogeneously pure.

Inhibition by OTFP and DFP

Inhibition of JHE from *Gryllus assimilis* by OTFP and DFP (diisopropyl fluorophosphate) was determined essentially as described by Zera and Zeisset (1996) for the unpurified enzyme. Briefly, both inhibitor and enzyme were incubated at 30° C for 10 min (OTFP is a slow-binding transition-state analogue; Abdel-Aal and Hammock, 1985), after which JH-III was added and JHE activity was measured using the standard assay.

Hydrolysis of Non-Juvenile Hormone Substrates

Esterase assays employing α-naphthyl or p-nitrophenyl esters were performed in 0.05 M MOPS buffer, pH 7.5, using 5 mM substrate concentration (Gu and Zera, 1994). Acid and alkaline phosphatase activities were measured using 5 mM p-nitrophenyl phosphate in 50 mM sodium-acetate (pH 5.5) or 0.1M Tris [Tris(hydro xymethyl)aminomethane] (pH 9.0) buffers. Acetylcholinesterase activity was measured using 50 mM acetyl-thiocholine iodide in 0.05 M MOPS, pH 7.5, according to Ellman *et al.* (1961) and aminopeptidase activity was measured using 5 mM p-nitroanilide substrates in 0.1M MES [2-(N-morpholino)ethanesulfonic acid] buffer, pH 6.0 according to Sarath *et al.* (1989). All assays were performed at 30° C using one and the same pool

of homogeneously purified JHE. Controls involved assays without JHE. In all cases, assay time and enzyme concentrations were used such that velocities were measured during the linear portion of the progress curve (initial rate measurements). Michaelis constants (KM) and maximal velocities (VMAX) were measured for α naphthyl acetate and α-naphthyl nonanoate using 6–7 substrate concentrations in the range of 13– 250 μM. Kinetic constants and their standard errors were estimated using the program Enzfitter (Leatherbarrow, 1987).

RESULTS

Purification

The protocol used to purify JHE resulted in greater than 900-fold purification with greater than 30% recovery of enzyme activity (Table 1). This protocol could be completed within 3 days and resulted in homogeneously-purified JHE as judged by a single band on a silver-stained SDS PAGE gel (Figure 2A and B). In each purification step, JHE activity eluted as a single peak. Although the initial steps in the purification protocol, PEG precipitation and DEAE anion exchange, resulted in only slight (2.2-fold) purification with 66% recovery, they were employed since they removed > 70% total protein. Hydrophobic interaction chromatography (Phenyl Superose) was the step that resulted in the greatest increase in purity (289-fold increase in specific activity with a 72% recovery of enzyme activity). JHE fractions that eluted from the Phenyl-Superose column contained only a few low molecular mass protein impurities that were removed by Mono Q anion exchange FPLC (Figure 2A and B).
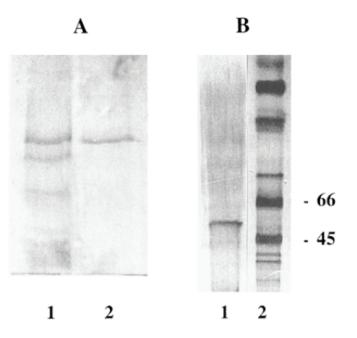
When crude serum from *Gryllus assimilis* was subjected to native PAGE or narrow or broad-range IEF, and gels were stained using the non-specific esterase substrate, α-naphthyl acetate, esterase bands were seen at many positions on the gel. However, after the Phenyl Superose step, all α-naphthyl esterase activity visualized on either narrow-range IEF or native PAGE occurred as 3–4 closely-spaced bands (e.g., Figure 3). When an adjacent lane of either a native PAGE or an IEF gel was

TABLE 1. Purification of JHE From the Hemolymph of Last-Stadium *G. assimilis*

Step	Volume (mL)	Protein (mg)	Total activity (nmol JH-III/min)	Specific activity (nmol JH-III/min/mg)	% Original activity	Purification factor
Diluted plasma[a]	60	428	731	1.7	—	—
PEG precipitation	77	265	700	2.6	95	1.5
DEAE ion-exchange	9.5	125	480	3.8	66	2.2
Phenyl Superose FPLC	7.0	0.31	346	1116	48	656
Mono Q FPLC	3.0	0.15	245	1633	33	960

[a]12 mL of plasma plus 48 mL of 0.1 M phosphate buffer, pH 7.4.

cut into 0.5-cm slices (prior to α-naphthyl esterase stain-ing), and the slices were homogenized in buffer and as-sayed for JHE activity, virtually all (>85%) JHE activ-ity was found in slices adjacent to the stained portion of the gel that exhibited the α-naphthyl esterase bands. Fur-thermore, complete inhibition of JHE by OTFP in post Phenyl Superose samples resulted in complete loss of all α-napthyl esterase activity (data not shown). These results, together with the ability of homogeneously pu-rified JHE from *Gryllus assimilis* to hydrolyze α-naph-thyl esters (see below), indicate that all α-naphthyl ester-ase activity after the post Phenyl Superose step is due to JHE, and that nonspecific esterase stains employing α-naphthyl esters can be used to visualize JHE in PAGE or IEF gels after this purification step.

Stability of JHE During Different Stages of Purification

Prior to purification, JHE is very stable. It lost no activity for 3 months in undiluted serum stored frozen
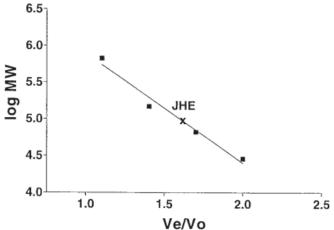

Figure 2. A: A silver-stained SDS PAGE gel of eluants of the Phenyl Superose (lane 1) or Mono-Q (lane 2) columns.

  B: A silver-stained SDS PAGE gel of homogeneously pu-rified JHE (post Mono-Q; lane 1) and M_r standards (lane 2). Numbers along the side of the gel designate the bovine serum albumin (66 kDa) and ovalbumin (45 kDa) M_r standards. Sub-unit M_r of JHE from *Gryllus assimilis* was estimated as 52 kDa by linear regression (see Methods, Results, and Table 5).

Figure 3. Estimation of native M_r of JHE from *Gryllus assi-milis* by gel permeation FPLC. M_r standards used were thyro-globulin (MW = 668 kDa), β-amylase (MW = 200 kDa), bo-vine serum albumin (MW = 66 kDa), and carbonic anhydrase (MW = 29 kDa). M_r of JHE was estimated by linear regres-sion as 98 kDa (see Results and Table 5). V_e is the elution vol-ume of the standards or JHE while V_o (void volume) is the elution volume of blue dextran (2,000 kDa).

at –80° C, or for 3 weeks when diluted 1/ 30 with 0.05 M phosphate buffer, pH 7.1, and kept on ice. Solutions of JHE also were very stable after each of the purification stages (no loss in activity after several weeks when pooled fractions were kept on ice). The main exception was the final Mono Q step. Pooled, post-Mono Q fractions, kept on ice, lost 50% JHE activity within 5 days. Myoglobin (0.5 mg/mL) was added to post Mono Q solutions that were to be subjected to IEF within one week. Addition of this protein resulted in no loss of JHE activity during this time. Furthermore, myoglobin cleanly separated from JHE on narrow range IEF (myoglobin migrates towards the cathode while JHE migrates towards the anode), and hence could be easily removed when pure JHE was required for amino-acid sequence determination of JHE isoforms. Since JHE in solution with myoglobin lost > 75% of its activity when kept for 1 month at 0° C, JHE was stabilized by the addition of 1 mg/ mL BSA when maintenance of enzyme activity was required for longer than 1 week. No loss in JHE activity occurred over 1 month when solutions contained BSA and were kept on ice. As mentioned previously, JHE is very unstable in high-salt solutions (e.g., 2M NaCl; see Methods).

Apparent Subunit and Native Molecular Masses (M_r)

In three separate SDS PAGE gels using JHE from two different purification runs, JHE from *Gryllus assimilis* migrated as a single band between the bovine serum albumin (66 kDa) and the ovalbumin (45 kDa) standards. Typical silver-stained SDS PAGE gels are illustrated in Figure 2A and B. The apparent subunit molecular mass of JHE was estimated as 52 kDa by linear regression. JHE activity also eluted as a single broad peak (78–110 kDa) from the Sephacryl S-300 gel permeation FPLC column. The estimated native apparent molecular mass was 92 kDa (Figure 3). The subunit and native M_rs are consistent with JHE from *Gryllus assimilis* having a dimeric quaternary structure.

Isoelectric Focusing

Initial IEF of purified JHE from several different purification runs on wide range (pH 3.5–9.1) gels always resulted in bands with pIs between 4.0 and 5.5 (data not shown). Hence all subsequent IEF runs were performed on narrow range (pH 4.0–6.0) gels. Homogeneously purified JHE from various runs consistently exhibited two major and 1–2 minor isoforms. The most common pattern is illustrated in Figure 4, in which isoforms with intermediate pIs were the most strongly staining when visualized with a non-specific esterase stain (α-naphthyl acetate). Similar patterns were observed when IEF gels were Coomassie stained or when proteins from IEF were transferred onto a PVDF membrane and Coomassie stained. The most cathodal (acidic) JHE isoform focused at the same position as the phycocyanin (pI = 4.7) standard, while the least acidic isoform focused midway between the phycocyanin and α lactoglobulin B (pI = 5.1) standards. Thus, the pIs of the JHE isoforms range from 4.7 to 4.9. The wavy nature of the pH gradient and closeness of the JHE isoforms precluded estimation of the pIs of individual isoforms.

Figure 4. A narrow-range (pH 4.0–6.5) IEF gel illustrating the two major and two minor isoforms of homogeneously-purified (post Mono Q) JHE from *G. assimilis*. Bands were visualized using a non-specific esterase stain containing α-naphthyl acetate (see Methods). The same general pattern was obtained when the gel was stained for general protein using Coomassie Blue. The top of the gel (+) is the anode (acidic pole). N-terminal amino acid sequences for these isoforms are given in Table 2. See Results and Table 5 for pIs.

Amino Acid Sequence of Isoforms

N-terminal amino acid sequences (11–20 amino acids) were obtained for each of the two major and two minor JHE isoforms from *Gryllus assimilis* (Table 2). All sequences were very similar, consistent with these isoforms being products of the same or very similar genes. For example, amino acids were identical in 15 of 18–19 sites for three of the JHE isoforms (JHE-2, JHE-3, JHE-4). Seven of 10 amino-acid sites were identical between JHE-1 and each of the other three JHE isoforms. Conversely, the amino acid sequences of the two major isoforms differed from each other in 3 of 19 positions while the major and minor isoforms differed in 1–4 positions. Searches of the SwissProt database did not identify any proteins with high similarity to any of these sequences.

Michaelis Constant for JH-III, k_{cat}, and Inhibition Constants for OTFP and DFP

A Hanes plot of JH-III hydrolysis at various JH-III concentrations is presented in Figure 5A. Data showed a good fit to a straight line ($r^2 = 0.97$), consistent with a simple Michaelis-Menten mechanism for JH-III hydrolysis by JHE from *G. assimilis*. The $K_{M(JH-III)}$ for JH-III estimated by non-linear regression of the untransformed velocity data, using the program Enzfitter, was 84 ± 12 nM.

As mentioned previously (see Materials and Methods), the JHE preparation used to estimate the $K_{M(JH-III)}$, while highly purified, was not homogeneously pure. Thus, the turnover number (k_{cat}) of JHE for JH-III could not be estimated in this analysis. However, the specific activity measurement of homogeneously purified JHE (Table 1), could be used to quantify k_{cat}, because this specific activity is essentially equal to V_{max}. This was verified by substituting $K_{M(JH-III)} = 84$ nM (see above), and $[S] = 5,000$ nM (the substrate concentration used to measure specific activity of the homogeneously purified enzyme) into the Michaelis Menten equation. Under these conditions, $v > 98\%$ V_{max}; that is, the specific activity is essentially equal to V_{max}. Using the specific activity of homogeneously purified JHE (1,633 nmol JH-III/min/mg protein; Table 1), together with the subunit molecular mass of JHE given above (52 kDa), we calculated k_{cat} of JHE for JH-III as 1.41 s⁻¹ and k_{cat} / K_M as 1.7×10^7 s⁻¹ M⁻¹ (Table 3).

Inhibition Profiles

Inhibition profiles of JHE by OTFP and DFP are given in Figure 5B and C. Both of these compounds were strong inhibitors of JHE, with 50% inhibition occurring at approximately 10^{-7} M, and 40% inhibition occurring at 10^{-8} M (DFP) or 10^{-11} M (OTFP). The in-

TABLE 2. N-Terminal Amino Acid Sequence of JHE Isoforms From *G. assimilis* and Comparison With Amino Acid Sequences for JHEs From Other Insects

Species	Sequence				Reference
G. assimilis[a]			(K)		This study
JHE-1 (minor)	AEAPD	EVEKA	xGx		
JHE-2	AEAAP	EVEVA	QKRMR	AAAV	
JHE-3	AEADP	EVEVA	QxRMR	GAVVP	
JHE-4 (minor)	AEAAP	EVEVA	QKRMR	AAVVP	
			(L)		
L. dispar	RDRYH	PLLRG	RKGLA	ARGAE	Valaitis (1992)
H. virescens	WQETN	SRSVL	AHLDS	GIIRG	Hanzlik et al. (1989)
T. ni	LPSLS	ADAEA	PSPLS	LKAD	Jones et al. (1993)
M. sexta	RIPST	EEVVV	RTESG		Venkatesh et al. (1990)
Choristoneura fumiferana	ERLRG	ARCAR	RRAGC	AARAR	Feng et al. (1999)
D. melanogaster	LATVD	QLTVC	PPSVG	CLKGT	Campbell et al. (2001)
L. decemlineata	TRKNF	RVYNL	GHVPV	KAEAK	Vermunt et al. (1997)
Tenebrio molitor	FNTLS	PWDKE	VIYNW	KA	Thomas et al. (2000)

[a]JHE-1 is the most acidic and JHE-4 is the least acidic of the isoforms (see Figure 4). A lowercase "x" denotes an unreadable amino acid. A letter enclosed within parentheses above or below the sequence indicates ambiguity in the identity of the amino acid at that position and the most likely alternative.

hibition profiles were biphasic rather than sigmoidal suggesting the existence of multiple JHE isozymes or allozymes that differ in affinity for OTFP. Alternatively, multiple binding sites might exist on the same JHE molecule that differ in affinity for OTFP.

Substrate Specificity

Homogeneously purified JHE from *Gryllus assimilis* hydrolyzed a number of α-naphthyl and p-nitrophenyl esters (Table 4). The K_M for α-naphthyl acetate was 201 ± 26 μM and for α-naphthyl nonanoate was 11 ± 0.1 μM. The V_{max}/K_M ratio for α-naphthyl nonanoate was 17 times higher than the corresponding ratio for α-naphthyl acetate (Table 4) indicating a greater specificity for long-chain aliphatic esters. JHE exhibited no detectable acetylcholinesterase, acid phosphatase, aminopeptidase activity, and only very weak alkaline phosphatase activity.

DISCUSSION

JHE Purification

JHE from several holometabolous insects has been purified to homogeneity or near homogeneity using both affinity chromatography employing JHE transition-state analogues (Abdel-Aal and Hammock, 1985; Hanzlik and Hammock, 1987; Venkatesh *et al.*, 1990; Nussbaumer *et al.*, 2000; Thomas *et al.*, 2000) and classical techniques (Coudron *et al.*, 1981; Rudnicka and Jones, 1987; Valaitis, 1991, 1992; Vermunt *et al.*, 1997; Campbell *et al.*, 1998; Nussbaumer *et al.*, 2000). Typically, the affinity protocol has resulted in higher yields (> 50%; Venkatesh *et al.*, 1990; Abdel-Aal and Hammock, 1986) than classical approaches (< 25% yield; Rudnicka and Jones, 1987; Valaitis, 1992; Vermunt *et al.*, 1997; Campbell *et al.*, 1998). Specific activities (nmol JH acid/min mg protein) of purified JHEs ranged from below 100 (*Manduca sexta*, Coudron *et al.*, 1981), to 400–800 (*M. sexta, Bombyx mori*, Abdel-Aal and Hammock, 1985, 1986; Venkatesh *et al.*, 1990) to near or over 2,000 (*Helicoverpa virescens, H. zea*, Abdel-Aal and Hammock, 1986; *Lymantria dispar*, Valaitis, 1992; *Tricoplusia ni*, Rudnicka and Jones, 1987; *Leptinotarsa decemlineata*, Vermunt *et al.*, 1997).
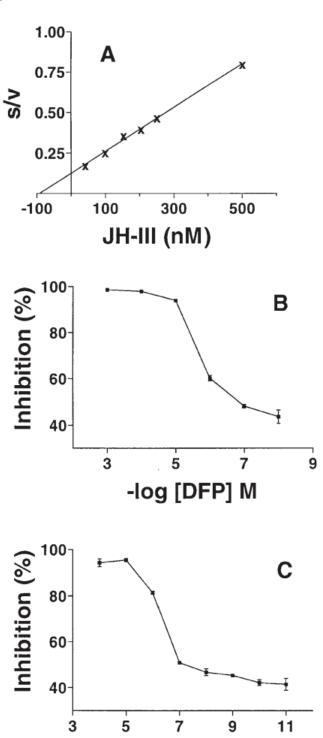

Figure 5. **A**: Hanes plot of JHE activity as a function of JH-III concentration. **B**: Inhibition of JHE by DFP. C: Inhibition of JHE by OTFP. Each point represents the average of three assays. In the Hanes plot, s = substrate concentration and v = velocity obtained at that substrate concentration. The intersection of the regression line on the x axis = $-K_M$.

TABLE 3. Kinetic Constants for Purified JHE from *G. assimilis* and for a Representative Sample of Purified, Well-Established JHEs From Other Insects*

Species	Substrate	K_M (nM)	k_{cat} (s^{-1})	k_{cat}/k_M (10^6 M s^{-1})	Reference
G. assimilis	JH-III	84	1.4	17	This study
D. melanogaster	JH-III	89	0.6	6.8	Campbell et al. (1998)
Helicoverpa zea	JH-III	125, 197[a]	4.3	39	Abdel-Aal and Hammock (1988)
H. virescens	JH-III	93, 103[a]	2.2	22	Abdel-Aal and Hammock (1988)
M. sexta	JH-III	24	1.2	49	Abdel-Aal and Hammock (1985)
T. ni	10S, 11R JH-III	156, 139[b]	1.0, 1.2	6.4, 8.6	Hanzlik and Hammock (1987)

[a] Values obtained in separate determinations.

[b] Values obtained on separate isozymes.

* Characterizations were performed on JHEs purified to homogeneity, except for the JHE from *D. melanogaster*, which was purified to near homogeneity (see Campbell et al., 1998). For the *G. assimilis* JHE, k_{cat} was estimated on homogeneously-purified JHE, while $K_{M(JH-III)}$ was estimated on enzyme purified to near homogeneity (see Results and Discussion).

Previous attempts by us to purify JHE from *Gryllus assimilis* or the congener *G. rubens* (Zera *et al.*, 1992) using trifluoroketones as affinity ligands were unsuccessful due to poor binding of JHE to the affinity resin, even after overnight incubation. Therefore, we turned to more traditional non-affinity procedures. The protocol outlined in Table 1 proved to be very successful in obtaining homogeneously purified enzyme of high specific activity, and high k_{cat}/K_M for JH-III. The yield, while lower than from procedures employing trifluoroketone affinity ligands (e.g., Abdel-Aal and Hammock, 1986; Venkatesh *et al.*, 1990), was higher than other protocols employing classical methods (Rudnicka and Jones, 1987; Valaitis, 1992; Vermunt *et al.*, 1997; Campbell *et al.*, 1998). In some runs, a few protein impurities were present after the mono-Q exchange step. However, these could be removed by isoelectric focusing. As will be discussed below, the enzyme purified from *Gryllus assimilis* exhibited kinetic and physical properties very similar to well-established JHEs isolated from other insects, thus strongly indicating that the purified protein from *Gryllus assimilis* is also a JHE.

Characteristics of JHE From Gryllus Assimilis and Comparison With JHEs From Other Species

Subunit and native molecular masses. All lepidopteran and dipteran JHEs reported thus far are monomers with M_rs of 50–68 kDa (Table 5). By contrast, JHEs recently characterized from two coleopterans exist partially or completely as dimers with subunit molecular masses of 57–71 kDa. The JHE from *G. assimilis*, the first hemimetabolous JHE characterized with respect to quaterary structure, is similar to the coleopteran JHEs in having a dimeric quaternary structure (Table 5). Surprisingly, the native M_r of JHE from the congener *G. rubens* [188 kDa; Zera *et al.*, 1992) is approximately twice the native M_r value of 97 kDa obtained for JHE from *Gryllus assimilis* in the present study (Table 5). There are several possible explanations for the differences in native M_r for JHEs from these two closely related species. First, the higher M_r for JHE from *G. rubens*, which was quantified in diluted plasma, might be due to enzyme aggregation, as JHE From *G. assimilis*

TABLE 4. Activity of JHE From *G. assimilis* Towards Various Esters and Peptides*

Type of activity (substrate)	Mean activity ± SEM (µmol hydrolyzed/min/mL)
α-Naphthyl/p-nitrophenyl esterase	
α-naphthyl acetate	318 ± 4.6 (6)
α-naphthyl butyrate	200 ± 4.9 (6)
α-naphthyl nonanoate	466 ± 7.5 (6)
p-nitrophenyl acetate	742 ± 9.3 (3)
Acid/alkaline phosphatase	
p-nitrophenyl phosphate (pH 5.5)	None detected (3)
p-nitrophenyl phosphate (pH 9.0)	8.3 ± 2.2 (3)
Acetylcholinesterase	
Acetylthiocholine	None detected (3)
Aminopeptidase	
L-leucine p-nitroanilide	None detected (n = 3)
L-phenylalanine p-nitroanilide	None detected (n = 3)
L-glycine p-nitroanilide	None detected (n = 3)

*All activities were measured on one and the same solution of homogeneously-purified JHE. Numbers in parentheses denote the number of replicate assays. Michaelis constants and maximal velocities for JHE for α-naphthyl acetate and nonanoate are given in Results.

TABLE 5. Physical Characteristics of Homogeneously Purified JHE From *G. assimilis* Compared With Other Insects

Species	M_r subunit (kDa)	M_r native (kDa)	Quaternary structure	pI of major isoforms	Reference
Hemimetabolus					
Orthoptera					
G. assimilis	52	98	Dimer	4.7–4.9	This study
Holometabolous					
Coleoptera					
L. decemlineata	57	120	Dimer	5.5, 5.6	Vermunt et al. (1997)
T. molitor	71, 150[a]	71, 150[a]	Monomer/dimer	4.9, 6.7	Thomas et al. (2000)
Diptera					
D. melanogaster	66	54	Monomer	5.4	Campbell et al. (1998)
Lepidoptera					
H. virescens	61	61	Monomer	4.8	Hanzlik et al. (1989)
T. ni	64	64	Monomer	5.3, 5.5	Hanzlik and Hammock (1987), Jones et al. (1993), Rudnicka and Jones (1987)
M. sexta	62–66	62–68	Monomer	5.5, 6.1	Coudron et al. (1981), Venkatesh et al. (1990), Abdel-Aal and Hammock (1985)
L. dispar	62[b]	62	Monomer	No data	Valaitis (1991, 1992)
L. dispar	50[b]	No data	No data	5.1, 5.3	Nussbaumer et al. (2000)

[a] It is uncertain whether the 150 kDa enzyme is a dimer or monomer (Thomas *et al.*, 2000).

[b] Molecular mass and amino-acid sequence data indicate that these two enzymes are not the same protein (Nussbaumer *et al.*, 2000).

has been reported to occur for the JHE from *T. molitor* (Thomas *et al.*, 2000). Alternatively, the quaternary structure may differ between JHEs of these two species. Recent studies suggest that JHEs of different quaternary structure may exist even within the same species (Thomas *et al.*, 2000).

JHE Isoforms

The existence of multiple acidic (pIs = 4.7–4.9) JHE isoforms in *Gryllus assimilis* is typical of most insect JHEs, which occur as several isoforms with pIs ranging from 4.1-6.0 (Table 5). A previous study of the wing polymorphic congener, *G. rubens*, identified JHE isoforms with pIs of 4.1 and 5.2 (Zera *et al.*, 1992). The basis for the isoform diversity has not been identified for JHE from *Gryllus assimilis* or any other insect. JHEs from *T. ni* and *M. sexta* are glycosylated (Venkatesh *et al.*, 1990; Hanzlik and Hammock, 1987; Jones *et al.*, 1993). However, it is unknown whether isoform diversity results from variation in the site of glycosylation, or type of sugar(s) attached, as opposed to some other cause such as variation in primary amino acid sequence. No amino acid differences have been identified between 47 sites compared between various fragments of the two major JHE isoforms of *T. ni* including the 12 amino acid N-terminus (Jones *et al.*, 1993). No direct information is currently available as to whether the JHE from *Gryllus assimilis* is glycosylated. Although available data are limited, N-terminal amino acid sequences suggest that JHE isoforms in *Gryllus assimilis* may exhibit a greater degree of sequence divergence than do JHE isoforms from other insects. For example, the major JHE isoforms in *Gryllus assimilis* differed in 3 out of 19 residues and differences between one of the major and minor isoforms was even greater (see Results). The N-terminal amino-acid sequence of JHE from the hemimetabolous *Gryllus assimilis* also is not similar to N-terminal sequences of other JHEs reported thus far (Table 2). However, this is not unexpected. Thus far, N-terminal amino acid sequences for JHEs have only been reported from holometabolous insects, which are phylogenetically distant from the hemimetabolous *G. assimilis*. Furthermore, N-terminal amino-acid sequences of JHEs from species of the same holometabolous order (e.g., Lepidoptera) show little sequence similarity to each other (Table 2).

An important issue for both the evolutionary genetics of JHE as well as JHE regulation is the number of loci that encode the enzyme. In *H. virescens*, JHE is encoded by a single autosomal locus with no closely related paralogs (Harshman *et al.*, 1994). JHE also exists as a single copy gene in *T. ni* (Venkataraman *et al.*, 1994). The existence of JHE isoforms in *Gryllus assimilis*, which differ by several unclustered amino acid substitutions within the 20 amino-acid N-terminal portion of the enzyme (Table 2), raises the possibility that JHE in *Gryllus assimilis* may be encoded by multiple divergent loci. Alternatively, JHE could be encoded by a single highly variable locus that contains alleles that differ at multiple sites. Ultimately, molecular-genetic analyses, which are in progress, will be required to determine JHE copy number. A related issue concerns the functional significance of the JHE isoform diversity. Thus far, only a limited number of characterizations of individual isozymes have been undertaken, and these have failed to identify differences between isoforms in either kinetics or inhibition (Hanzlik and Hammock, 1987; Venkatesh *et al.*, 1990).

Kinetics and inhibition. Like other JHEs (Table 3), the enzyme from *Gryllus assimilis* exhibited a very low (84 nM) K_M for JH-III, coupled with a high k_{cat}/K_M (1.7×10^7) that approaches the diffusion controlled limit for an enzyme-catalyzed reaction (see table 3.4 of Fersht, 1977). These two kinetic features are diagnostic for JHE (Hammock, 1985), and make this enzyme ideally suited for scavenging juvenile hormone at low concentrations. Interestingly, the k_{cat} for JHE from *Gryllus assimilis* and other insects (0.6–4 s^{-1}; Table 3) is several orders of magnitude lower than k_{cat} for other efficient enzymes (see table 3.4 of Fersht, 1977). However, the K_M for JHEs is also several orders of magnitude lower than these enzymes (Table 3; see table 3.4 of Fersht, 1977), resulting in a high apparent second-order rate constant (k_{cat}/KM). The K_M of purified JHE from *Gryllus assimilis* was close to the K_M measured on unpurified enzyme of this species (106–178 nM; Zera and Zeisset, 1996), and unpurified enzyme from the congener, *G. rubens* (47–81 nM; Zera *et al.*, 1992). This good correspondence indicates that purification does not appear to alter the K_M of JHE from these species, and that there appears to be

no strong modulators of the Michaelis constant of JHE activity in the hemolymph.

JHE from *Gryllus assimilis* was also similar to JHEs from other insects in being strongly inhibited by the transition state analogue OTFP (Abdel-Aal and Hammock, 1985; Venkatesh *et al.*, 1990; Zera and Zeisset, 1992). Forty percent of JHE activity in *Gryllus assimilis* was inhibited at 10^{-11} M OTFP. The biphasic inhibition profile for OTFP, observed in the present study (Figure 5), was observed in a previous inhibition study of JHE from *Gryllus assimilis* using unpurified enzyme (Zera and Zeisset, 1996) and for the JHE of the lepidopteran *M. sexta* (Abdel-Aal and Hammock, 1985; Venkatesh *et al.*, 1990). This pattern raises the intriguing possibility that JHE isozymes or allozymes might exhibit functionally important differences in OTFP inhibition in *G. assimilis*. Alternatively, the same JHE molecule might exhibit multiple binding sites that differ in affinity for OTFP, as has been reported for the JHE from *M. sexta* (Abdel-Aal and Hammock, 1985; Venkatesh *et al.*, 1990).

Substrate selectivity. JHEs from various insect species differ markedly in their rates of hydrolysis of nonjuvenile hormone substrates. For example, the enzymes from *M. sexta* and *L. decemlineata* do not hydrolyze α-naphthyl acetate, while JHEs from *T. ni* and *Drosophila melanogaster* hydrolyze this substrate (Vermunt *et al.*, 1997; Campbell *et al.*, 1998). Furthermore, the JHE from *T. ni.* hydrolyzes p-nitrophenyl acetate and juvenile hormones at comparable rates (Hanzlik and Hammock, 1987). JHE from *Gryllus assimilis* was similar to *T. ni* in that it hydrolyzed both α-naphthyl and p-nitophenyl esters. A direct comparison between JHE activity on JH-III and non-JH substrates is not possible in the present study because assays employing JH-III and non-JH-III substrates were performed using different enzyme preparations.

JHE from *Gryllus assimilis* exhibited a low K_M (11 μM) for α-naphthyl nonanoate (Table 4), suggesting the potential of this enzyme to hydrolyze long-chain aliphatic esters at physiological concentrations. While not as low as the <100 nM K_M for JH III, the low K_M for α-naphthyl nonanoate raises the intriguing possibility that JHE from *Gryllus assimilis* might play physiological roles other than the regulation of the juvenile hor-

mone titer, as has been speculated previously for JHE from *M. sexta* (Abdel-Aal and Hammock, 1985). Recent studies suggest that some well-studied esterases, such as acetylcholine esterase, may have unsuspected novel functions such as proteolytic regulation of cell growth and development (Small, 1990). The types of compounds tested as potential JHE substrates by homogeneously purified enzyme have typically been limited to general esterase substrates (e.g., α naphthyl or p-nitrophenyl esters). We tested a wider range of substrates but found that purified JHE from *Gryllus assimilis* exhibited no detectable acetylcholinesterase, aminopeptidase, acid phosphatase, and only weak alkaline phosphatase activities (Table 4).

In summary, we have undertaken the first purification to homogeneity and characterization of JHE from a hemimetabolous insect. This enzyme exhibits many similarities with the well-characterized lepidopteran and dipteran JHEs (e.g., subunit M_r, turnover number, Michaelis constant, and k_{cat}/K_M), as well as some notable differences (dimeric quaternary structure and degree of amino acid sequence difference among isoforms). The present study represents a critically important step in our evolutionary-genetic studies of JHE activity variation in natural populations. Indeed, we have recently isolated and sequenced a *Gryllus assimilis* cDNA using an oligonucleotide probe derived from the N-terminal amino-acid sequence of *Gryllus assimilis* JHE presented in Table 2 (A. J. Zera *et al.*, unpublished data). The availability of this cDNA will allow us to begin studies on the evolution of the structure and regulation of the JHE gene in *Gryllus*.

ACKNOWLEDGMENTS

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