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Isolation of juvenile hormone esterase and its partial cDNA clone from the beetle, *Tenebrio molitor*

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Abstract

Juvenile hormone esterase (JHE) plays an essential role in insect development. It is partially responsible for the clearance of juvenile hormone (JH) which regulates various aspects of insect development and reproduction. Because of its role in regulating JH titer, this enzyme has been targeted for development of biologically-based insecticides. JHE was partially purified from the beetle, *Tenebrio molitor*, using a transition state analog as the affinity ligand. Two forms of JHE were characterized by activity analysis, isoelectric focusing, two-dimensional SDS–PAGE and N-terminal sequence analysis. The esterase is associated with two proteins of sizes 71 and 150 kDa, both of which are active on JH III. A partial cDNA clone for the enzyme was isolated based on the sequence of N-terminal and internal peptides. Its sequence indicates that JHE from *T. molitor* and *Heliothis virescens* may have a common origin.

Keywords: Esterase; Affinity chromatography; Juvenile hormones; Insects; Hormones; *Tenebrio molitor*

1. Introduction

Juvenile hormones (JHs), such as JH III (methyl(2E, 6E)-[10R]-10, 11-epoxy-3, 7, 11-trimethyl-2, 6-dodecanoate) (Fig. 1), are insect hormones that are involved in the regulation of the development of insects. These hormones are also required in the adult form of many insects for the regulation of reproductive processes such as oogenesis (Sehnal, 1985). In order for metamorphosis to occur in holometabolous insects, the JH titer must decrease during the last larval stage, resulting in pupation. JH titers must remain low during metamorphosis of the pupa in butterflies and moths (Lepidoptera). This decrease in JH is modulated, in part, by juvenile hormone esterase (JHE), which hydrolyzes the methyl ester of JH to the corresponding carboxylic acid (Fig. 1). There is now indication that the JH acid metabolite also may have intrinsic biological activity (Ismail et al., 1998).

JHE (EC 3.1.1.1), a member of the the carboxylesterase family, has been purified and characterized from several species of insects, mainly those in the lepidopteran order including *Trichoplusia ni* (Abdel-Aal and Hammock, 1988), *Heliothis virescens* (Hanzlik and Hammock, 1987) and *Manduca sexta* (Jesudason et al., 1990). However, there are only a few examples of characterization of this enzyme from other insect orders, such as beetles (order Coleoptera) (Connat, 1983; Stauffer et al., 1997 in *Tenebrio molitor*; Vermunt et al., 1998a,b in *Leptinotarsa decemlineata*). The JHEs of Lepidoptera have the same kcat/km ratio on the three major forms of JH homologues (Abdel-Aal and Hammock, 1985; Abdel-Aal et al., 1988). Recent studies on JHE enzymes have utilized JH III as a substrate due to the fact that it is now the only commercially available JH in radiolabeled form. However, JH III is thought to be the sole JH of Coleopterans (Grieneisen et al., 1997; Baker, 1990). A Dipteran JHE from *Drosophila melanogaster* was recently characterized by Campbell et al. (1998) and is likely to be the same JHE reported by Rauschenbach et al. (1995) and Khlebodarova et al. (1996). Recently, Vermunt et al. (1998b) reported a dimeric form of JHE from *L. decemlineata* by native

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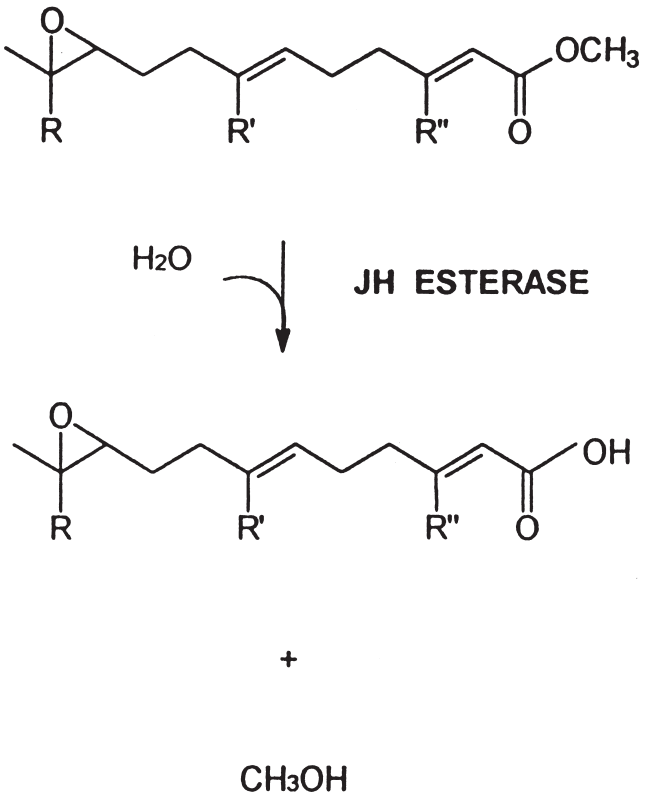

Fig. 1. Enzymatic reaction catalyzed by JHE. Note that the JH ester is in a chemically stable α,β-conjugated system. JH 0: R=R′=R″=ethyl; JH I: R=R′=ethyl; R″=methyl; JH II: R=ethyl, R′=R″=methyl; JH III: R=R′=R″=methyl.

gradient PAGE and the cloning of two JHE-related genes from this insect (Vermunt et al., 1998a). The sequence of this protein is quite different from the JHEs reported from *H. virescens*; it does not contain the characteristic GQ**S**AG sequence which contains the active site serine (underlined and bold). This raises the question of whether JHE evolved from a monophyletic or polyphyletic origin in beetles and moths.

JHE from *H. virescens* has been particularly well characterized due to interest in exploitation of this enzyme for use as a biopesticide (Bonning and Hammock, 1996). Three cDNA clones have been isolated, and the recombinant form of the enzyme expressed in a nuclear polyhedrosis virus has been shown to reduce caterpillar feeding and increase the speed of kill over wild type virus (Bonning et al., 1995). Both the natural and recombinant JHE cause anti-JH effects upon injection into the caterpillar, *Manduca sexta* (Hammock et al., 1988; Philpott and Hammock, 1990). Modified forms of JHE recombinantly produced in baculovirus have been shown to be more highly insecticidal than the wild-type recombinant JHE in *T. ni* and *H. virescens* (Bonning et al., 1997).

Our goal is to characterize JHEs from insects that are evolutionarily distant from *H. virescens* and other Lepidoptera. It is our hope that one could design biopesticides based on JHE by exploiting unusual or otherwise different properties of JHEs found in diverse organisms. Herein, we describe partial purification by affinity chromatography, subsequent characterization of a putative dimeric form of JHE, and partial cloning of the gene.

2. Materials and methods

Chemicals: C-10[^{3}H]-JH III was purchased from New England Nuclear Research (Boston, MA) at a specific radioactivity of approximately 16.4 Ci/mmol and was mixed with unlabeled JH III (5×10^{-4} M), which was purchased from Calbiochem (San Diego). 3-Octylthio-1,1,1-trifluoropropan-2-one (OTFP, $C_8H_{17}SCH_2C(O)CF_3$) and 3-pentylthio-1,1,1-trifluoro-2-propanone (PTFP, $C_4H_9SCH_2C(O)CF_3$) were used as inhibitors to elute JHE from the affinity column and were synthesized as described in Hammock et al. (1984). 3-(4-Mercaptobutylthio)1,1,1-trifluoropropan-2-one (MBTFP, $HSC_4H_8SCH_2C(O)CF_3$) was used as a ligand for the affinity gel (Abdel-Aal and Hammock, 1986).

2.1. Insects

Larvae of *T. molitor* were purchased from Carolina Biological Supply (Burlington, NC) and were reared at 28°C on a 18L:6D photoperiod and fed a diet of Cheerios™ and potatoes. Purification was performed on pupae that were 0–48 h post-pupation and frozen at −20°C until further use. Enzyme activity was stable under these storage conditions. Repeated freeze–thaw was found to result in no detectable loss of JHE activity.

2.2. JHE purification

All biochemical procedures were performed at 4°C unless otherwise specified. The 0–48 h pupae were placed in 0.2 M $NaPO_4$(pH 7.4), 5%(w/v) sucrose, 0.02%(w/v) NaN_3, 2 mM EDTA, 10^{-7} M pepstatin, 0.02% 1-phenyl-2-thiourea, 1 mM β-mercaptoethanol (purification buffer) at a volume of 1.3 pupae/ml buffer. Pupae were homogenized using a Polytron homogenizer (Brinkmann Instruments, New York) for 5×1 min on ice, with a 1 min pause between each homogenization to avoid heating the samples. The homogenates were centrifuged at 10,000***g*** for 20 min, the pellet was resuspended and centrifuged again. The combined supernatants were filtered through cheesecloth and were centrifuged at 100,000***g*** for 60 min. It was routinely found that approximately 80% of the total enzymatic activity was recovered in the 100,000***g*** supernatant. The supernatant was diluted three-fold with purification buffer (previous trials had shown that three-fold dilution of samples resulted in optimal binding to affinity gel) and was immediately loaded in batch onto MBTFP–

Sepharose. This was done by combining the crude extract with the gel in a polypropylene tube and then rotating on a wheel at 4°C. At every 30 min the tube was set upright and the gel was allowed to settle. An aliquot of the supernatant was assayed for JHE activity. This method was repeated until more than 80% of the JHE activity had bound to the gel. This typically took about 6 h. The affinity gel varied in epoxide activation from approx. 6–9 μmol/g gel. A typical purification procedure started with an extract from approximately 200 pupae. The resulting soluble fraction was diluted threefold and was loaded onto ~3 ml of affinity gel. Affinity purification of JHE was accomplished similarly to the method of Abdel-Aal et al., 1988 (Abdel-Aal and Hammock, 1986). 1-Octyl-β-D-glucoside (OG) (0.1% (w/v)) was used to encourage elution of the protein by minimizing hydrophobic interaction with the gel. This concentration provided the greatest yield of JHE elution from the column in the presence of the trifluoroketone inhibitor, OTFP, at a final concentration of 1 mM (Abdel-Aal and Hammock, 1986).

The enzyme was eluted batchwise in several steps in the presence of several milliliters of fresh buffer and inhibitor; the elutions were carried out for 4–8 h with shaking at 4°C. Eluates were concentrated by using Centricon concentrators (Amicon, Beverly, MA). Eluates were reactivated by dialysis in purification buffer at 4°C for 96 h in the presence of exogenously added ovalbumin as a carrier at a final concentration of 200 μg/ml. Further dialysis resulted in some additional reactivation (approx. 22%) and the activity was stable to further dialysis for up to 7 days.

2.3. Electrophoresis

SDS–PAGE and native Tris–glycine gels were run using either 10% polyacrylamide gels or prepared 8–16% gradient gels (Novex, San Diego). The following standards were run on the native Tris–glycine gels: ovalbumin (43 kDa), bovine serum albumin (67 and 134 kDa) and catalase (232 kDa). Wide-range protein standards (Novex) were run on SDS–PAGE. Isoelectric focusing (IEF) was performed using precast slab gels (Pharmacia) in a range of pH 3–7 and 3–10. IEF Wide Range Standards (Pharmacia) were used to calibrate the gels. JHE activity from both crude fractions and in 2 mm gel slices from IEF was verified by both partition assay (Hammock and Sparks, 1977) and TLC (Casas et al., 1991; Stauffer et al., 1997). Gels were stained with Coomassie Brilliant Blue according to the method of Sambrook et al. (1989).

2.4. Renaturation of JHE from SDS–PAGE

p71 and p150 forms of JHE were renatured following SDS–PAGE on 8–16% gradient gels (Novex, San Diego) as described above. The gel was stained with Nile Red (Sigma) according to the procedure of Daban et al. (1996). The gel was agitated for 5 min in a solution of 40 μg/ml Nile Red in distilled water. The stained bands were promptly excised from the gel under UV illumination and immediately placed in 500 μl 12% (v/v) Triton X-100, phosphate buffered saline (PBS) for 2 h at room temperature with shaking for renaturation. Enzymatic activity on JH III was measured by a partition assay with radiolabeled JH III (Hammock and Sparks, 1977).

2.5. Sequencing and amino acid analysis

For N-terminal sequencing, the samples were electrophoresed on precast 8–16% SDS–PAGE (Novex). The gel was transferred to Problot (Applied Biosystems), stained with Coomassie Blue R-250 according to Biorad instructions and submitted to the Protein Structure Lab (University of California, Davis) for amino acid analysis or sequence analysis on a Beckman 6300 Analyzer or a Applied Biosystems 470A Sequencer, respectively. For in-gel Lys C digestion the samples were electrophoresed on SDS–PAGE, stained with Coomassie R-250; in-gel proteolysis was performed according to the methodology of Y.M. Lee (personal communication) using Lys C endoprotease from *Achromobacter lyticus* (Wako). The peptides were separated on a 1×150 mm, 5 μm, 300 Å Michrom reverse-phase C18 Reliasil column running on a Michrom Ultrafast Protein Analyzer. A gradient of 5–70% B (A=2% acetonitrile, 0.075% (v/v) TFA, B=100% acetonitrile, 0.1% (v/v) TFA) in 70 min at a flow rate of 50 μl/min with detection at 210 nm was used. Peak fractions were collected and submitted for analysis to the Protein Structure Lab (University of California, Davis).

2.6. Cloning of JHE

PolyA RNA was isolated from fat bodies of three 0–48 h old pupae of *T. molitor*. Approximately 350 mg of wet tissue was used, and yielded 6.65 μg of poly A RNA. mRNA was isolated using the Poly(A) Pure kit from Ambion per manufacturer's instructions. This mRNA was used as template for Reverse Transcriptase—PCR. The degenerate oligonucleotides, TmGPA (5′–TAY–ACN–AAR–TAY–GGI–CCI–GC–3′), TmAIG (5′–AAR–TAY–GGI–CCI–GCI–ATH–GG–3′) were constructed as sense primers by Gibco Life Technologies. These primers were based upon the peptide sequence obtained by in-gel Lys C digestion that was nearest the N-terminus, TPAIGLTEVSR, based on the alignment with the JHE sequence from *H. virescens*. Likewise, the oligonucleotides, TmPNP (5′–AAN–GGR–TTI–GGR–TTI–GCI–GC–3′) and TmFEK (5′–NII–YTT–YTC–RAA–IGG–RTT–IGG–3′) were generated as antisense primers based upon the internal peptide

sequence, TAANPNPFEK. For the first strand of cDNA synthesis, 500 ng of mRNA was hybridized with 10 pmol of the oligonucleotide, TmFEK in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) in a total volume of 5 μl. The mixture was incubated at 70°C for 5 min, cooled slowly to 46°C and then placed on ice. The RNA-:oligonucleotide hybrid mixture was then mixed in a 20 μl reaction containing 20 U/μl Superscript Reverse Transcriptase 2 (Gibco Life Technologies), 2 U/μl of RNasin ribonuclease inhibitor (Promega), and Reverse Transcriptase buffer (50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 2 μM DTT) (Gibco Life Technologies) and 0.25 mM dNTP (TaKaRa). The reverse transcriptase reaction was carried out at 42°C for 50 min followed by 50°C for 20 min. An aliquot (0.5 μl) of this first strand reaction was used for the first round of PCR amplification in a 100 μl reaction containing 100 pmol each of the primers, TmFEK and TmGPA, 2.5 U of Taq DNA Polymerase (TaKaRa), 0.2 mM of each dNTP (TaKaRa), and Taq polymerase buffer (10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$) (TaKaRa). The thermocycling was performed in a PTC-100 thermocycler from MJ Research, Inc. The reaction was carried out with an initial 2 min denaturing step at 94°C followed by 4 cycles of 30 set at 94°C 30 set at 61°C and 2 min at 72°C; then 6 rounds of 30 s at 94°C, 30 s at 59°C 2 min at 72°C; then 8 cycles of 30 s at 94°C, 30 s at 57°C, 2 min at 72°C; and finally 19 rounds of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C. A second round of nested PCR was performed using 2 μl of the first reaction with the same conditions as above except that the primers TmAIG and TmPNP were used. The second amplification resulted in an 800 base-pair PCR product, which was then cloned into pPCR-Script Amp SK(+) plasmid according to manufacturer's instructions using the PCR-Script Amp Cloning Kit (Stratagene). The recombinant plasmid, pPNP, was amplified in *Epicurian coli* XL1 Blue MRF'™ cells (Stratagene). pPNP DNA was purified from the recombinant bacteria using published techniques (Sambrook et al., 1989). The subcloned PCR product was sequenced from pPNP DNA using an ABI sequencing machine Model #377 (Perkin Elmer).

3. Results

3.1. JH metabolites in crude homogenates

All experiments in this paper utilized 0–48 h old pupa, in part because larvae are difficult to stage. Previous reports by J.L. Connat (1983) and Sparks and Hammock (1980) have demonstrated that during development of *T. molitor*, JHE activity is high in early pupae. Whole body homogenate was used in this case because *T. molitor* consumes little water and thus, inherently has relatively low quantities of hemolymph where most JHE is typically located in other species.

Quantitative experiments indicate that approximately 98% of the total esterase activity on JH III is found in the soluble crude pupal fractions following 10,000***g*** spins and filtering. Of this soluble activity 80% is recovered in the 100,000***g*** supernatant and approx. 1.0% is recovered in the 100,000***g*** pellet fraction. Thus, 19% of activity is lost during ultracentrifugation. Similar results were obtained in a second independent experiment.

Crude 100,000***g*** supernatants and pellets of insect pupae were analyzed for JH-esterase and JH epoxide hydrolase activities using TLC to separate metabolites of radiolabeled JH. JH-acid was found to be the major metabolite present in the 100,000***g*** supernatant; in a 30 min incubation 87% of the radioactivity was present as JH-acid, the balance of the activity was present as JH-diol and JH-diol-acid. No parent JH was detected at this time point. The 100,000***g*** membrane fraction, incubated with substrate under the same conditions, yielded 74 and 26% JH-acid and JH-acid-diol, respectively. A second independent experiment yielded similar results. These results indicate the presence of a low amount of epoxide hydrolase activity, and for this reason we assayed the subsequent affinity eluate for epoxide hydrolase activity.

3.2. Purification by affinity chromatography

JHE from *T. molitor* was purified by an affinity chromatography method designed for JHEs from extracts of other insects. This method relies on the use of a trifluoroketone-based transition state analog bound to Sepharose CL-6B to selectively bind JHE with subsequent elution using a stronger inhibitor. This technique typically yields homogeneous JHE from crude preparations of many different insects in a single step (Abdel-Aal and Hammock, 1986).

In this report, we present results from our attempts to purify and characterize JHE from *T. molitor* using the classical MBTFP–Sepharose affinity system (Abdel-Aal and Hammock, 1986). MBTFP–Sepharose was used in an attempt to improve upon the purification of Stauffer et al. (1997). In all cases, including the purification reported here, it was found that approx. 60–90% of the total JHE activity (as measured either by spectrophotometric assay or partition assay with radiolabeled JH III) bound to the column after 4–6 h (McCutchen et al., 1993; Hammock and Roe, 1985). Table 1 includes the results of affinity chromatography of a crude 100,000***g*** supernatant. After dialysis for 72 h, a specific activity of approximately 286 nmol/min mg total protein was achieved. Further dialysis did not appreciably improve activity, and the activity was stable under these conditions for up to seven days of dialysis at 4°C. A 20.1-fold purification was achieved with a total recovery of 1.8 and 0.1% in activity and protein, respectively. The

Table 1
Purification table for JHE from *T. molitor*

	Total protein (mg)	Specific activity (nmol/min mg)	Fold purification	Recovery activity (%)
100,000***g*** supernatant	1243	13.9	1	100
Affinity step[a]	2.02	N.D.[a]	N.D.[a]	N.D.[a]
Reactivation[b]	1.11	286.1	20.1	1.8

[a] Sample was inhibited with the trifluoroketone, OTFP.
[b] Sample was dialyzed in the presence of 1.6 g/ml ovalbumin for 3 days.

observed recoveries are close to a previous purification in *T. molitor* (49-fold purification and 1.56% yield of activity) that was reported by Stauffer et al. (1997). Fig. 2 shows SDS–PAGE analysis of the affinity eluate using OTFP which reveals that eight major bands were purified by this procedure. SDS–PAGE analysis of purification using the inhibitor, PTFP, yielded similar results. The inhibitor, OTFP, was used for all subsequent purifications because of its low I_{50} relative to PTFP (two orders of magnitude less than that of PTFP) (Stauffer et al., 1997). The sample proved refractory to further purification by several methods that included ion

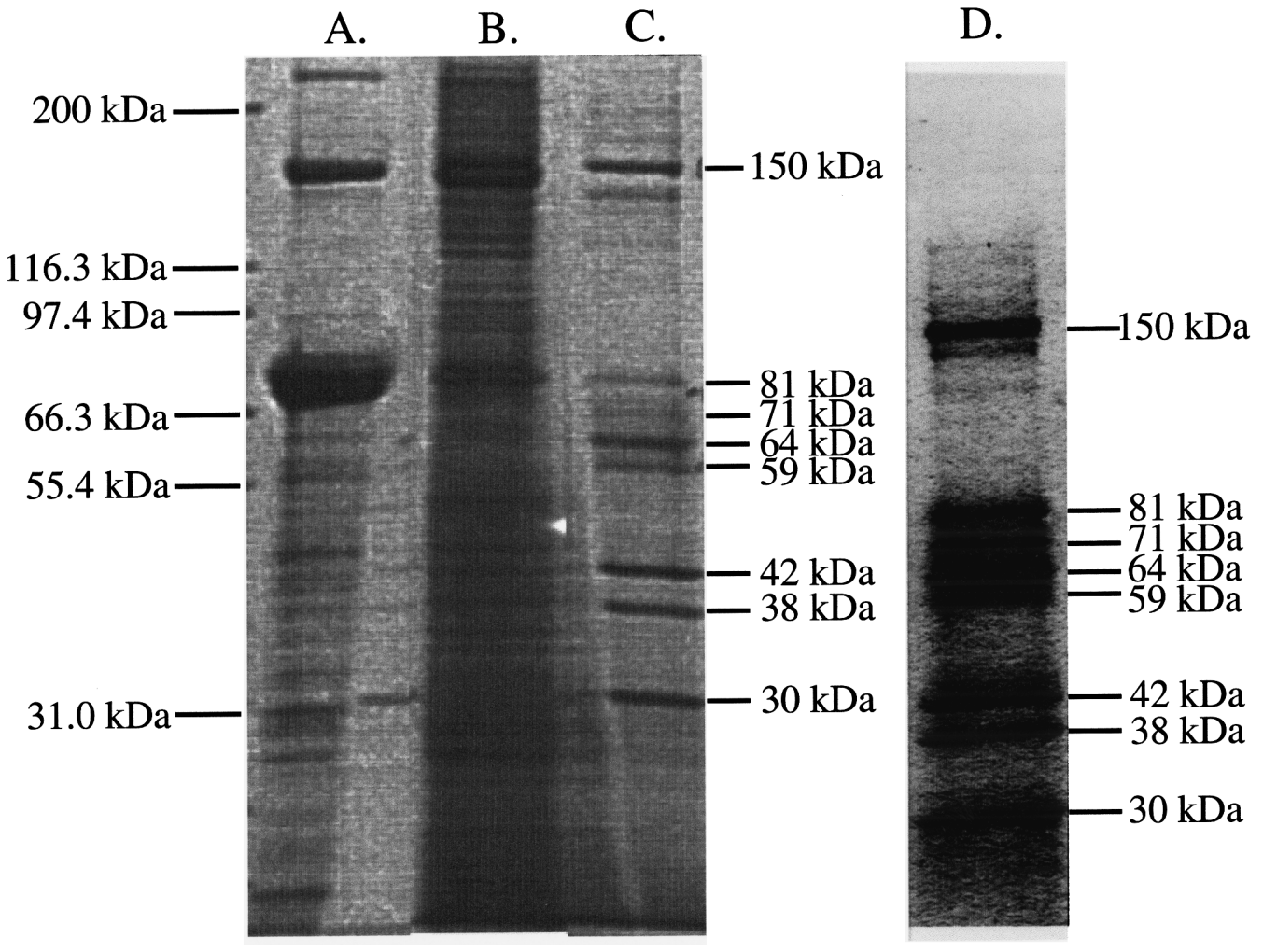

Fig. 2. SDS–PAGE analysis of 100,000***g*** supernatant (A), insoluble precipitate from affinity purification (B) and affinity eluate (C) from *T. molitor*. An 8–16% SDS–PAGE was run in which 12 and 3.1 μg total protein of the crude supernatant and affinity eluate, respectively, and 30 μl of a SDS-solubulized suspension were loaded onto the gel. The resulting gel was stained with Coomassie Blue R-250. Numbers on the left-hand side show positions of molecular weight markers and on the right-hand side show estimated molecular weights of bands discussed in the text. Lane D contains the same affinity eluate as in lane C, but in this lane the resolution is much improved such that each band can clearly be visualized.

exchange, hydrophobic interaction and lectin chromatography.

We observed during the affinity purification of JHE from *T. molitor* that a large amount of protein precipitates out of solution during incubation with the trifluoroketone inhibitor used for elution. In a single experiment 50% of the total protein following an elution was present as an insoluble material as measured by Biorad protein assay. SDS–PAGE analysis showed that a substantial amount of proteins ranging from 71 to 150 kDa appeared to be present in this fraction with a much greater proportion of p150 relative to the amount seen in nonprecipitated affinity eluate (Fig. 2).

Solubilization of the pellet was attempted with both the non-ionic and ionic detergents, Triton X-100 and sodium dodecyl sulfate (SDS), respectively. Of the reagents tried, only SDS was capable of fully solubilizing the pellet. Thus a major factor in the apparent poor yield may be due to aggregation of JHE that subsequently precipitates out of solution.

3.3. Identification of JHE on isoelectric focusing

JHE from the affinity eluate of *T. molitor* was identified and characterized by isoelectric focusing (pH 3–10), two-dimensional SDS–PAGE and activity analysis. Fig. 3 shows the IEF for the affinity eluate and corresponding activity profile as measured by the radiometric substrate, [^{3}H]-JH III, across an IEF gel for both the 100,000***g*** supernatant and the purified affinity eluate. Two major areas of activity were identified at a pI=4.9 and 6.7, which correspond to the Coomassie Blue stained areas on the IEF gel (Fig. 3). All of the enzyme activity that was loaded onto the gel was recovered in these two peaks in a ratio of 46 and 54% for the pI 6.7 and 4.9 forms, respectively, for the crude extract and 5% and 95% for the pI 6.7 and 4.9 forms, respectively, for the affinity eluate.

A TLC analysis of products formed by incubation of JH III with the two pI forms was accomplished to test whether the enzymatic activity of the two pI forms is due to ester hydrolysis. Both pI forms produce JH-acid as the major product on TLC analysis with no evidence of any JH-diol or JH-diol-acid that would be indicative of epoxide hydrolase activity (data not shown).

A two-dimensional SDS–PAGE of the affinity eluate after the components were separated based on their pIs is shown in Fig. 4. These data show that the pI 4.9 form appears to be the one that consists of the 71 and 150 kDa proteins. The pI 6.7 form appears to contain only proteins smaller than 81 kDa, and appears to be associated with at least the majority of the 30 kDa protein that copurifies on affinity chromatography. These results suggest more than one of the protein bands in Fig. 2 are JHEs.

3.4. N-terminal sequence analysis

An N-terminal sequence analysis was performed on several bands of the affinity eluate. Table 2 shows the N-terminal sequence data for the bands of interest in this study. The N-terminal sequence of the 71 kDa protein and the 150 kDa protein are identical. A BLAST database search (Karlin and Altschul, 1990) using each N-terminal sequence as a query shows similarity of the 71 kDa protein and 150 kDa protein to the JHE of *H. virescens* (Hammock et al., 1988). Thus we tested the hypothesis that the 71 kDa band is a JHE, and that the 150 kDa protein represents a dimer of the the 71 kDa protein. Identification of homologous proteins in the BLAST database for the remaining three bands which migrate close to the 71 kDa band, namely, the 81, 64 and 59 kDa bands, was not possible with the present data.

3.5. Characterization of 150 kDa protein as a putative JHE dimer

We tested the hypothesis that the 150 kDa protein is a dimer of the 71 kDa protein. Surprisingly, both the p71 and p150 proteins were detected on SDS–PAGE even under strongly denaturing conditions. We could not separate the p71/p150 couple, and the two forms appear to interconvert on the time scale of many of our experiments. Interconversion can be demonstrated by concentrating a sample of JHE that eluted from an S200 gel filtration column at a molecular weight of 71 kDa in which no 150 kDa protein was initially present as examined by SDS–PAGE. Concentration of this sample 12-fold (65–780 μg/ml) caused partial interconversion to the p150 form as measured by SDS–PAGE (data not shown). Because of the inability to separate the p71/p150 couple under native conditions, we resorted to separation on SDS–PAGE, followed by renaturation in the presence of Triton X-100 and measurement of renatured bands for enzyme activity on JH III. Not surprisingly, renaturation after incubation in the ionic detergent, SDS, was associated with a low recovery of JHE activity and in all cases only a few percent of the total activity loaded on the gels was recovered after renaturation. Fig. 6 shows a time course of activity on JH III for the p71 and p150 bands following SDS–PAGE and renaturation in the presence of Triton X-100. The enzyme activity for both p71 and p150 is essentially linear with time over a period of 6 h (Fig. 6).

The 150 kDa protein was also examined to ascertain whether it is stabilized via electrostatic interaction or if a covalent linkage such as a disulfide bond is present. We found that under conditions of heating at 90°C for 10 min or heating plus the addition of reducing agents, the 150 kDa protein does not dissociate into smaller proteins (data not shown). These results argue that the putative dimer is not likely to be linked via a disulfide bridge

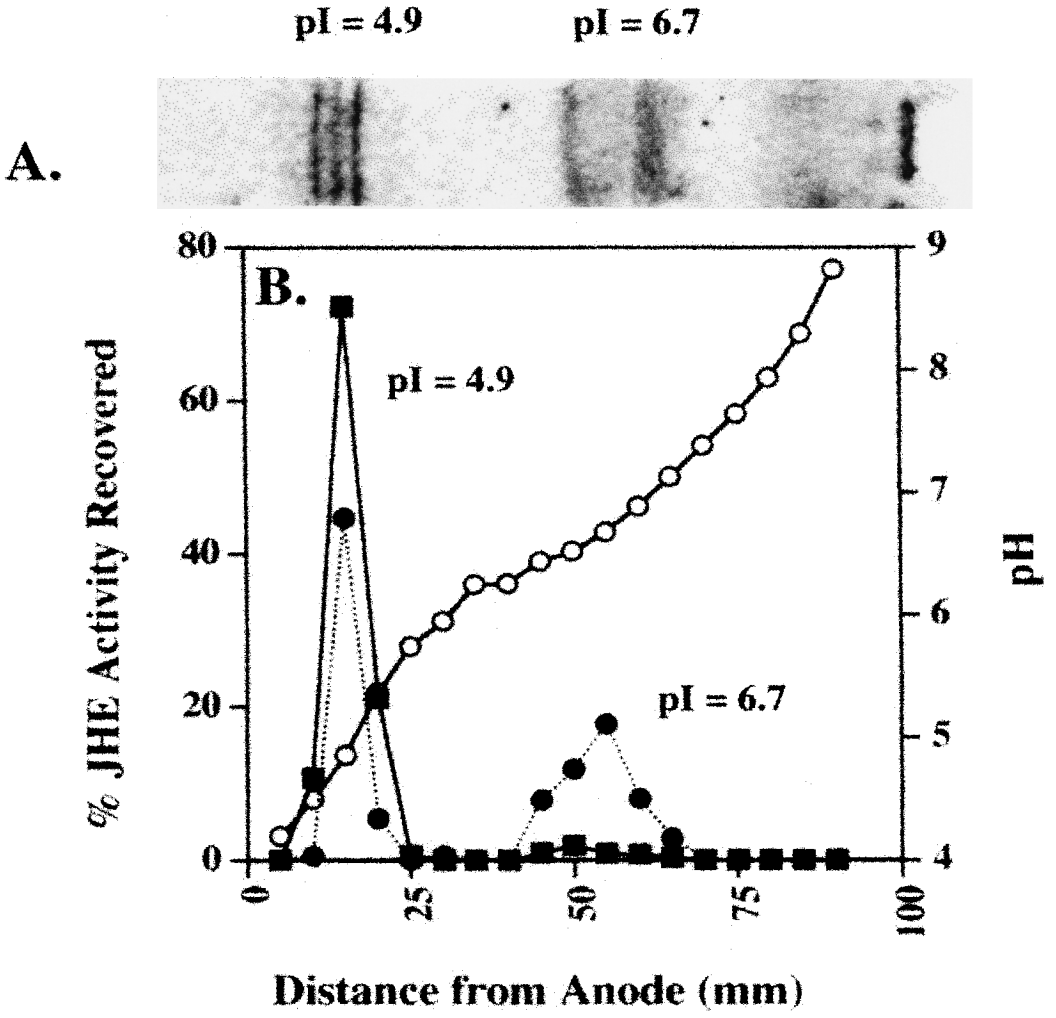

Fig. 3. Enzymatic activity analysis of JHE affinity eluate and crude 100,000*g* supernatant on isoelectric focusing (IEF), pH 3-10 (Pharmacia). (A) 1.1 μg affinity eluate and 6 μg crude material (not shown) were electrophoresed and stained with Coomassie Blue R-250. (B) Esterase activity on JH III as a function of distance in mm from the anode. (●) and (■) represent gel slices from crude homogenate and affinity eluate, respectively. Activity is displayed as a percent of total activity initially loaded onto the gel. pH gradient is shown as a function of the distance from the anode, indicated by ○ (mm).

and are similar to those obtained by Vermunt et al. on the JHE of *L. decemlineata* (Vermunt et al., 1998b). Thus the possibility of an electrostatic interaction between the two putative monomers was tested by serial dilution of the affinity eluate up to 100-fold; this did not result in interconversion. Finally, the affinity eluate was incubated in the presence of 1 mM β-mercaptoethanol at 90°C in a time course up to 4 h. We found that not only did extensive heat and reducing agent not dissociate the 150 kDa protein into smaller proteins, but the 150 kDa protein appeared to produce higher order aggregates that did not migrate into an 8–16% SDS–PAGE. Thus the results indicate the possibility that a putative dimer associates by an uncharacteristically strong electrostatic interaction.

3.6. Cloning of JHE

The putative JHE (p71) was further characterized by partial cloning of the protein coding region from cDNA generated from pupal mRNA. Primers for obtaining the clone were synthesized based on peptide sequences obtained from in-gel Lys C digestion of the 71 kDa band on SDS–PAGE. Fig. 5 shows the deduced protein sequence of the partial clone of JHE from *T. molitor* which comprises roughly the N-terminal half of the protein and its alignment with that from *H. virescens*, While this clone displays 42 and 40% identity to JHEs of *H. virescens* and *M. sexta* (Hanzlik et al., 1989; A. Hinton, unpublished), it displays only 21% identity to the JHE-related proteins from *L. decemlineata* reported by Ver-

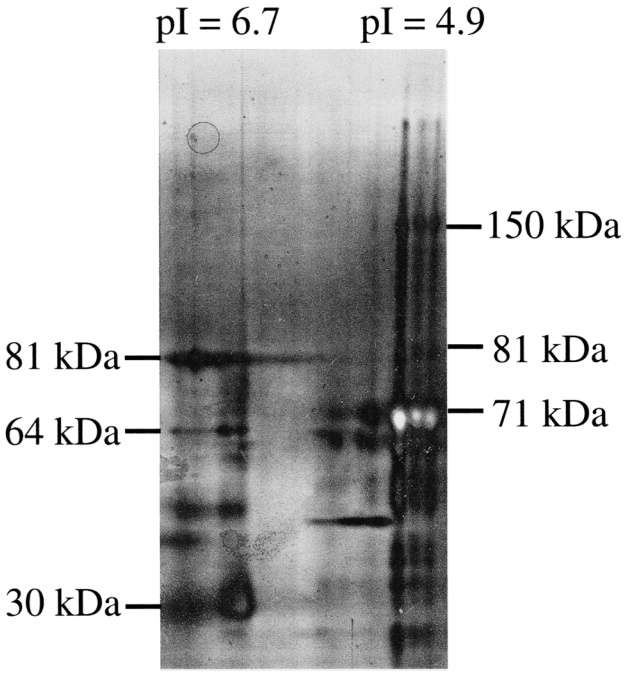

Fig. 4. Two-dimensional SDS–PAGE of JHE affinity eluate. 8–16% 2D SDS–PAGE (Novex) was run on the JHE affinity eluate following separation in the first dimension by IEF (pH 3–7) (Novex) in the first dimension. Approx. 2.9 μg total protein was run on the first dimensional gel. The IEF gel was prepared for loading onto the subsequent 2D SDS–PAGE per instructions (Novex). Numbers on the left and right of the gel show estimated molecular weights of bands of the pI isoforms discussed in the text. Thus both the 150 and 71 kDa JHE bands can be detected in the band focusing at pI=4.9.

Table 2
N-terminal sequence analysis of proteins inthe affinity eluate from *T. molitor*

Molecular weight[a]	Sequence
150	FNTLSPWDKEVIYNWKA
81	HSVHSTNYAQKDV
71	FNTLSPWDKEVIYNWKA
64	YAPKSPIVY
59	PPEVTIEQGKLR

[a] Molecular weights of proteins sequences were determined on a 8–16% SDS–PAGE.

munt et al. (1998a). Further analysis by the CLUSTAL W alignment program (Thompson et al., 1994) for the JHEs of *H. virescens*, *T. molitor* and *L. decemlineata* yielded homology scores of 34 for the alignment of *H. virescens* and *T. molitor*, 4 for the alignment of *T. molitor* and *L. decemlineata* and 5 for the alignment of *H. virescens* and *L. decemlineata*. These results suggest that while JHE from *T. molitor* has significant similarity to that of JHE from H. virescens, the JHE reported from *L. decemlineata* does not. The clone reported here shows similarity to the other JHEs studied, and contains the characteristic GQ**s**AG sequence around the active site serine (underlined and bold) (Fig. 5). The other two members of the catalytic triad were not identified due to an incomplete clone. All of the peptides obtained from Lys C digestion gave sequences which exactly matched the corresponding amino acid sequence derived from the cDNA clone sequence.

4. Discussion

Several JHEs have been successfully purified to homogeneity by the MBTFP–Sepharose-based affinity purification method, however, these successes have been largely limited to insects of the Lepidopteran order. JHEs from insects of other orders have proven more difficult to purify. This was recently indicated by Stauffer et al. (1997) and Vermunt et al. (1998b) for JHE of *L. decemlineata*. In this study, we purified JHE from the coleopteran, *T. molitor*, using a trifluoroketone-based affinity purification system, but failed to obtain a homogeneous preparation of JHE. Upon further study, however, we found some rather interesting biochemical characteristics of this JHE which are presented in the results.

In the application of the affinity chromatography technique to whole body homogenate of *T. molitor*, our consistent result was the purification of a small number of proteins. SDS–PAGE analysis (Fig. 2) reveals the presence of major protein bands at eight different molecular weights. The fact that more than one protein eluted from the affinity gel could either indicate multiple forms of JHE, other esterase enzymes, or unrelated proteins.

As the purification table (Table 1) indicates, there is a 20-fold increase in specific activity as measured by radiometric JHE assay, and a 1.8% yield in total JHE activity. Though greater than 90% of the soluble JHE activity bound to the affinity gel, elution and reactivation of the enzyme was difficult. The low yield of catalytically active enzyme from the affinity purification scheme, in this case, is likely due to two causes. The first reason is the apparent aggregation and precipitation of the enzyme following purification under the conditions presented in this paper. The precipitate was resistant to resolubilization, and the analysis of partially solubilized precipitate by SDS–PAGE revealed a significant amount of 150 kDa protein. Possibly, the smaller JHE protein(s) has a tendency to dimerize and then precipitate out of solution, or the majority of JHE activity exists as a 150 kDa protein in vivo, but is unstable under the in vitro conditions that have been reported here.

A second reason for low yield of activity is the nature of the affinity gel and the elution buffer. Elution requires incubation of the gel with a very tight binding inhibitor. The trifluoroketone inhibitor forms a covalent bond with the enzyme's catalytic site serine. Thus, catalytically active enzyme is needed for both elution from the column and removal of the inhibitor. In addition to poor

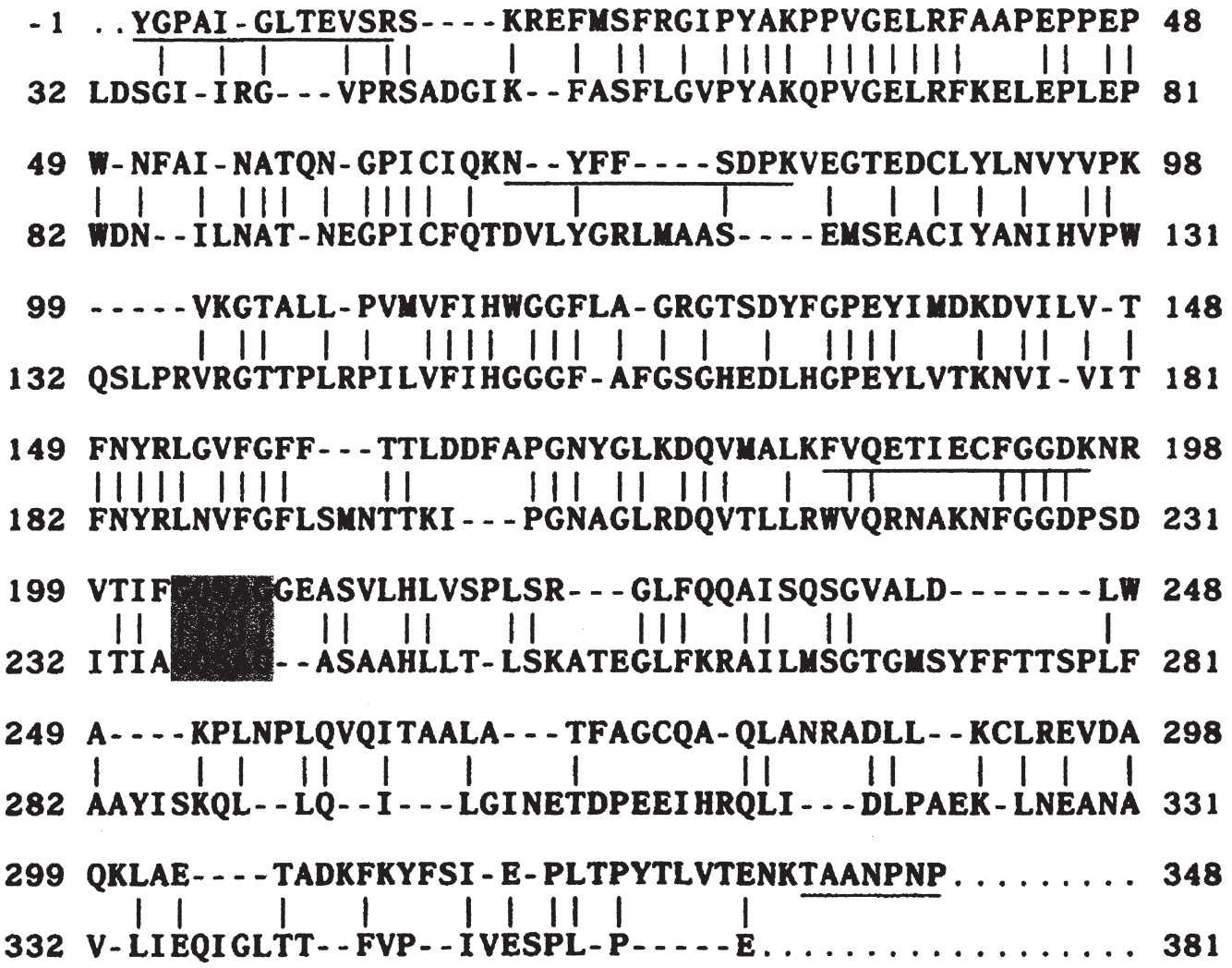

Fig. 5. Protein sequence alignment of partial clone of JHE from *T. molitor* (upper sequence) with that of *H. virescens* (Hanzlik, et al, 1989) (lower sequence). Partial clone of JHE from *T. molitor* is aligned with that of *H. virescens* using the software, DNASIS. Numbering system includes amino acids and gaps. Thus, catalytic serine 203 from JHE of *H. virescens* is 238 in this figure. Underlined residues correspond to peptides whose sequences were obtained from in-gel Lys C digests. All of the peptides obtained by this method agree exactly with this sequence. Shaded region corresponds to the catalytic serine sequence. JHE from *T. molitor* and *H. virescens* display 42% identity in the region of the clone, which comprises the majority of the N-terminal domain. The sequence from *L. decemlineata* (Vermunt et al., 1998a) did not have high enough similarity to present a meaningful alignment by either DNASIS or CLUSTAL W (Thompson et al., 1994).

elution, low observed recovery may be due to incomplete removal of the trifluoroketone inhibitor from the enzyme despite extensive dialysis. This observation has been made before with other JHEs. Trifluoroketones exhibit slow tight-binding kinetics, and OTFP has been reported to have an I_{50} of 10^{-10} M on JHE of *T. molitor* (Stauffer et al., 1997).

The affinity eluate contains two groups of JH hydrolyzing proteins which can be separated based on their pIs. The two sets at different pIs are enzymatically active and both produce JH-acid as their sole reaction product. Though the majority of JHE activity of the affinity eluate migrates at pI=4.9 with IEF analysis, part of the original JHE activity is apparently present within a group of proteins with a pI of 6.7. A higher proportion of activity in the crude homogenate migrates to pI 6.7 than in the affinity eluate. The distribution of total activity between the pI 6.7 and pI 4.9 groups goes from 46 and 54%, respectively, in the 100,000***g*** supernatant; to 5 and 95%, respectively, in the affinity eluate. This indicates that the JHE activity of pI 6.7 is even more unstable, or more difficult to reactivate after binding to affinity gel than is the activity at pI 4.9. In a previous study by Stauffer et al. (1997), only one area of JHE activity was observed with IEF. This form ran at pI=4.43, and accounted for 100% of the activity recovered from the gel. The discrepancies between these previous results and ours can be explained either by subtle differences in experimental technique, or by genetic variations between two different populations of *T. molitor*.

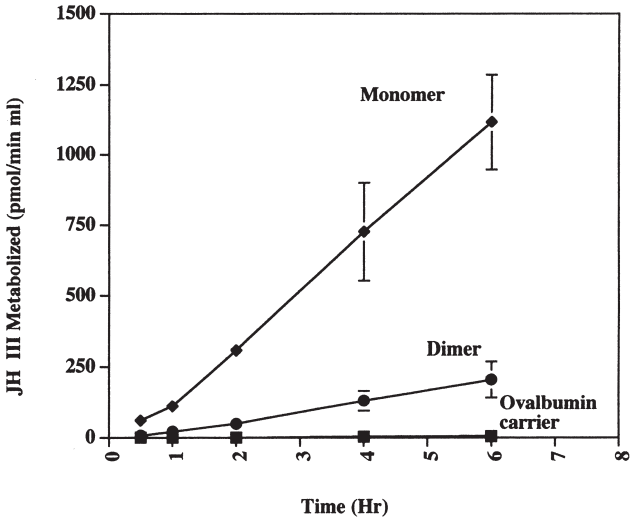

Fig. 6. Time course of activity on JH III for the putative dimer and monomer of JHE after renaturation from SDS–PAGE. Each point represents the average ±SD of two independent experiments in which the data were each collected in triplicate. A blank gel slice was averaged and subtracted from each datum point for each independent experiment.

The significance of additional forms of JHE activity remains uncertain. When both pI groups of JHE from IEF are run on a second dimension of electrophoresis using SDS–PAGE, each pI group separates into various molecular weights. There is some overlap between the 2 groups (64 and 81 kDa), but several other bands are unique to each pI group. The 71 and 150 kDa proteins seem to be limited to the pI 4.9 group of JHE activity. In the future, the thorough explanation of the roles of these different forms of JHE will likely require the production of antibodies and/or molecular cloning and expression of individual proteins within these groups. With such tools available it would be feasible to determine whether these are isoforms of the same protein, or different gene products of completely independent genes. It will also be interesting to assess the roles of each form and assess their prominence throughout development.

Under the conditions used for affinity chromatography, a variety of proteins were eluted. All major bands were eluted from SDS–PAGE, and the eluate was dialyzed and tested for esterase activity. Two of the proteins (71 and 150 kDa) are able to hydrolyze JH. One other protein (38 kDa) had catalytic activity on α-naphthyl acetate but not JH. The remainder of proteins that co-eluted from the affinity gel have no known function, as they neither exhibited any catalytic activity nor revealed any homology to known proteins by N-terminal sequence analysis. The inability to recover JHE activity from the other eluted proteins following SDS–PAGE does not rule out the possibility that these are JH hydrolyzing proteins which are less stable or which fail to reactivate under these conditions.

Despite the fact that there is more than one protein which possibly represents an independent JHE, we were able to make a logical decision to focus upon the 71 kDa protein and the 150 kDa protein for several reasons. Firstly, both of these proteins exhibited JHE activity after elution from SDS–PAGE and dialysis. Secondly, they both are represented in the group of proteins which migrates at pI=4.9 by isoelectric focusing. This area of the IEF gel represents the majority of JHE activity in the affinity eluate. Thirdly, N-terminal analysis indicates not only that the two proteins have identical N-termini, but that this protein sequence has homology to a previously cloned JHE from another species, *H. virescens*. Based upon these reasons, we decided to focus upon this pair of proteins.

Analysis of the 71 kDa band by partial cloning was accomplished. This clone, which encompasses the N-terminal domain of p71, is 42% identical to the region of *H. virescens* JHE to which it aligns. This sequence demonstrates that the isolated clone is probably that of an esterase similar to JHE based on homology to JHE of *H. virescens* and the signature sequence in the vicinity of the catalytic serine, GQSAG. Although this clone shows high homology to JHE sequences from *T. ni*, *M. sexta* (Hinton, A.C., unpublished), and *Lymantria dispar* (Nussbaumer, C., 2000), it shows low identity (21%) to the JHE-related sequences recently reported from the beetle *L. decemlineata* (Vermunt et al., 1998a). Comparison of our sequence data from *T. molitor* with JHEs from Lepidopterans strongly suggests a monophyletic origin, at least with the homologue or paralogue described here. The observation that our sequence data are not highly similar to JHE from *L. decemlineata* is unexplainable at this point (Vermunt et al., 1998a). However, Vermunt et al. (1998a) report the absence of both a catalytic serine and the characteristic GQSAG sequence in the vicinity of the active site serine in their translated sequences of the two JHE-related genes in Colorado potato beetle. Furthermore, catalytic activity on JH III has not been demonstrated for the proteins purified by Vermunt et al. (1998b). This suggests that the JHE reported herein is not the same as that isolated from *L. decemlineata* (Vermunt et al., 1998a,b), although the JHE reported by Vermunt et al. (1998a,b) may correspond to a small percentage of JHE from *T. molitor* unidentified in this study.

SDS–PAGE of the affinity eluate shows an additional band that is approximately twice the molecular weight of the 71 kDa JHE (150 kDa, Fig. 2). We tested the hypothesis that this band is a dimer of the 71 kDa protein. Fig. 4 shows, by two-dimensional SDS–PAGE following isolectric focusing, that this 140–150 kDa protein has a pI of 4.9, which is identical to that of the 71 kDa protein. Furthermore, an N-terminal sequence analysis of the putative monomer and dimer obtained from SDS–PAGE shows that both the 71 and 150 kDa proteins have

identical N-terminal sequences out to 17 amino acids (FNTLSPWDKEVIYNWKA). The N-terminal sequences of four individual bands in the molecular weight range of approx. 153–174 kDa determined following separation by SDS–PAGE appear to have identical sequences at least out to the first five amino acids, which is as far as we sequenced in these cases. These data indicate the possibility that these bands are N-glycosylated isoforms of the p150 form. We attempted to ascertain whether the putative dimer is a homo-dimer or hetero-dimer. However, peptide mapping of Lys-C digests of the dimer was unsuccessful, presumably due to challenges associated with this type of analysis on large proteins. Thus, we propose the hypothesis that the JHE dimer is a homo-dimer, based on the fact that only one sequence is obtained on N-terminal sequencing of all four bands in the 150 kDa molecular weight region. However, one cannot rule out the possibility that there is actually a heterodimer with one of the sequences N-terminally blocked.

Although the N-terminal sequence data and similar pI on isoelectric focusing would strongly suggest dimerization, the inability to dissociate this putative dimer by SDS–PAGE is disconcerting. The putative dimer was stable to strongly denaturing conditions, including incubation with SDS, urea, reducing reagents and high temperature. Most noncovalent interactions and disulfide linkages would be disrupted under these conditions. Possible interpretations of this are (1) the 150 kDa protein represents a single polypeptide (2) the 150 kDa protein represents a dimer which is covalently linked by some covalent linkage other than disulfide bonds (3) the 150 kDa protein represents two monomers which are bound by unusually strong electrostatic and/or hydrophobic interactions. The latter two possibilites are considered due to the observation that a small amount of the p71 form demonstrated conversion to p150 after separation of the two forms on S200 gel filtration column, and then concentrating the p71 sample 12-fold. The concentrated sample (65 μg/ml) revealed a small p150 band on SDS–PAGE. Thus, there is evidence that the 150 kDa protein can arise from monomers of pure 71 kDa protein.

It is now clear with the growing number of JHEs characterized outside the Lepidopteran order that JHEs can vary widely among insect species and orders. This variation comprises not only catalytic parameters but possibly also quaternary structure, as we have demonstrated here. The future cloning and comparison of JHEs from different species, as well as different forms of JHE within species, will allow discovery of mechanisms of enzyme function, degradation, and their roles in insect physiology and development.

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