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In vitro expression and biochemical characterization of juvenile hormone esterase from *Manduca sexta*

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Abstract

Juvenile hormone esterase (JHE) is a selective enzyme that hydrolyzes the methyl ester of juvenile hormone. This enzyme plays an important role in the regulation of metamorphosis in caterpillars, and is implicated in additional roles in development and reproduction in this and other orders of insect. The full length coding region of the JHE cDNA from *Manduca sexta* was subcloned into the baculovirus AcMNPV genome under the control of the p10 promoter. The recombinant virus demonstrated the expression of high levels of JHE activity when infected into Hi5 cells from *Trichoplusia ni*. The recombinant protein was partially purified by anion exchange chromatography and its biochemical characterization showed similar features to the wild type protein. The recombinant JHE has an estimated MW of 66,500 Da. Some heterogeneity with the enzyme was observed when analyzed by isoelectric focusing, although the peak of JHE activity was observed at pI = 6.0. It is highly sensitive to trifluoroketone inhibitors and certain phosphoramidothiolates, while relatively insensitive to other common esterase inhibitors. Incubating the enzyme with various organic solvents and detergents showed that the enzyme is activated at lower concentrations of solvents/detergents and remains significantly active even at high concentrations. The high tolerance of organic solvents may make this JHE enzyme useful in future applications as a synthetic catalyst.

Keywords: *Manduca sexta*; Juvenile hormone; Esterase; Protein expression; Baculovirus; Inhibitor

1. Introduction

Juvenile hormone (JH) is a sesquiterpenoid hormone that regulates development and reproduction in insects. In lepidopteran hemolymph, its primary route of metabolism is the hydrolysis of the methyl ester moiety (Hammock, 1985). Juvenile hormone esterase (JHE) is a member of the α/β-hydrolase fold family of enzymes, and it is thought to be the key enzyme that is responsible for the formation of the JH acid metabolite. This reaction has been demonstrated to be essential for proper onset of metamorphosis in caterpillars (Riddiford, 1980; Sparks and Hammock, 1980; Roe and Venkatesh, 1990). Reports by Ismail et al. (1998, 2000) have proposed that JH acid specifically is required as a hormone in order to induce metamorphic competence in some larval tissues. Thus, JHE can also be considered a biosynthetic enzyme as well as a degradative enzyme. This laboratory has sought to manipulate the importance of this regulatory enzyme via expression from recombinant baculoviruses. The rationale for this was to execute an anti-JH effect in early larval instars in order to disrupt normal development. Recombinant *Autographa californica* multiply occluded nucleopolyhedrosis virus (AcMNPV) that carried the JHE gene from *Heliothis virescens* demonstrated a slight increase in insecticidal efficiency (Hammock et al., 1990; Bonning et al., 1995). Since then it was established that JHE in vivo is actively degraded, and the short in vivo half life was implicated as a potential limiting factor in the insecticidal efficiency of the recombinant virus (Ichinose et al., 1992; Booth et al., 1992). Through purification and cloning of JHEs from other species of insects, and subsequent comparative studies, we have since sought to gain new information on the physical properties and regulation of this important regu-

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latory enzyme. Ultimately such knowledge could be applied towards the design of new and improved insecticidal agents.

The activity of JHE throughout the life cycle of *Manduca sexta* has been well characterized, and several reports have described biochemical characterization of the principal enzyme that is found at peak levels in final instar hemolymph (Coudron et al., 1981; Sparks et al., 1983; Abdel-Aal and Hammock, 1985; Venkatesh et al., 1990; Jesudason et al., 1992). Recently, the cDNA of the JHE from 5th instar fat bodies has been cloned and sequenced. The deduced amino acid sequence had significant homology to other JHEs that had been reported in Lepidoptera (Hinton and Hammock, 2001). In this study, we report the subcloning of the coding region of the cDNA into the baculovirus genome for in vitro expression, and then demonstrate that the recombinant enzyme has many similar characteristics to the wild type protein which had been previously isolated from hemolymph.

2. Materials and methods

2.1. Recombinant virus construction

The full length cDNA sequence of JHE was amplified by RT-PCR using previously isolated mRNA from the fat body of *M. sexta* (Hinton and Hammock, 2001). In this case the PCR primers had the restriction sites *Bgl*II or *Eco*RI inserted into their 5′ ends to facilitate cloning into the baculovirus transfer vector, pAcUW21 (Pharmingen, San Diego, CA). The primers were synthesized by Gibco Life Technologies Inc. (Gaithersburg, MD) with the following sequences: Ms5′Bgl2:5′-CACGTCTAGATCTGCCACCATGGACGCGCGGCG ACAAGTG-3′ and Ms3′*Eco*R1:5′-TATAGCGGCCG CGAATTCTGCCAACGCGCTCCACACATCCAC-3′. The cloning site on the pAcUW21 vector is just downstream of the p10 promoter. The first step of the RT-PCR procedure was a primer annealing step which included 10 pmol of the Ms3′*Eco*R1 primer with 100 ng of mRNA in 5 μl total. This mixture was heated to 80 °C and then slowly cooled to 4 °C. The volume was then raised to 40 μl in Reverse Transcriptase buffer (50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 2 mM DTT) (Gibco Life Technologies) and the following components were added: 20 U/μl Superscript II reverse transcriptase (Gibco Life Technologies), 2 U/μl RNase inhibitor (Promega, Madison, WI), and 0.25 mM dNTP (TaKaRa, Otsu, Japan). The cDNA synthesis reaction was carried out at 42 °C for 50 min, 50 °C for 20 min, then stopped at 94 °C 5 min. Two microliters of the first reaction were used as template for the following 100 μl PCR reaction, which contained 100 pmol each of primer Ms5′Bgl2 and Ms3′*Eco*R1, 2.5 U of LA Taq DNA Polymerase (TaKaRa), 0.2 mM of each dNTP (TaKaRa), and 1X LA Taq polymerase buffer (proprietary components by TaKaRa). Thermocycling parameters were three cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 69 °C for 30 s, and 72 °C for 3 min. The thermocycling was performed in a PTC-100 thermocycler from MJ Research, Inc. The resultant PCR product was desalted in a Qiagen PCR cleanup column (Valencia, CA) and then digested with *Bgl*II (Promega) and *Eco*RI (New England Biolabs, Beverly, MA) at 37 °C in a single reaction mixture using NEB buffer 3 (New England Biolabs). The pAcUW21 DNA was digested in the same manner in a separate test tube, except that calf intestinal alkaline phosphatase (New England Biolabs) was also added. Both digestion mixtures were desalted in a Qiagen PCR purification column, and then approximately 250 ng of the PCR product was mixed with 50 ng pAcUW21 DNA with T4 DNA ligase in Fast-Link ligation buffer (Epicentre Technologies, Madison, WI). Two microliters of the ligation mix were used to transform *Escherichia coli* DH5α from Gibco Life Technologies. Several colonies were selected and the plasmid DNA was isolated using the Ultraclean miniprep kit (MO BIO Laboratories, Inc., Solana Beach, CA). DNA sequencing using an ABI DNA sequencer verified sequence of the cDNA insert. The recombinant virus was generated by Lipofectin reagent (Gibco Life Technologies) mediated transfection of 500 ng of recombinant transfer plasmid (pAcMs7JHE) with 1 μg of BacPak6 DNA (Gibco Life Technologies) that was linearized by digestion with *Bsu*36I (New England Biolabs). The two DNAs were mixed with 8 μl Lipofectin (Gibco Life Technologies) in 200 μl of serum free ExCell 401 medium (JRH Biosciences, Lenexa, KS), incubated at room temperature for 15 min, and then added to a monolayer culture of Sf21 cells in a 35 mm diameter tissue culture plate. Recombinant virus, AcMs7JHE was purified by two rounds of plaque purification and then amplified in Sf21 cells as previously described (O'Reilly et al., 1992).

2.2. Enzyme preparation

Two separate purification schemes are described in the study. The first scheme was sufficient for concentration of the enzyme, and the resultant enzyme preparation was used for all enzyme characterizations described in this report. The second scheme demonstrates increased purity, and is presented only in the purification scheme (Table 1) and in the SDS-PAGE and IEF analyses. The second scheme differs from the first by the addition of protease inhibitors to the harvested cell culture medium, a raised pH, and the addition of a polyethylene glycol (PEG) precipitation step. JHE preparation #1: AcMs7JHE virus was used at multiplicity of infection (MOI) of five to infect Hi5 cells in 50–250 ml of a sus-

Table 1
Purification of JHE from viral infected cell culture medium by PEG precipitation and Q-Sepharose ion exchange chromatography (enzyme preparation #2)

	Volume (ml)	Total protein (mg)	Total activity (nmol JH III min^{-1})	Specific activity (nmol JH III min^{-1} mg^{-1})	Yield activity (%)	Purification factor
10,000×*g* supernatant	390	82	29,250	357	100	1
PEG 15% supernatant	557	40	33,977	859	116	2
Q-Sepharose 200 mM NaCl	3	8	4232	540	14	2
Q-Sepharose 350 mM NaCl	3	12	18,677	1556	64	4
Q-Sepharose 500 mM NaCl	12	4	588	136	2	0
Combined NaCl fractions	18	24	23,497		80	

pension culture in serum free medium (ESF 921, Expression Systems LLC, Woodland, CA). At 48 hpi (hours post-infection), the viral infected cell culture medium was harvested and centrifuged at 10,000 × *g* for 10 min in order to remove cells and cell debris. The supernatant was diluted 1:4 in cold 10 mM Tris buffer, pH 8.0 and loaded onto a 5 ml Q-Sepharose anion exchange column (Amersham Biosciences, Piscataway, NJ). Close to 100% of detectable JHE activity bound to the column in one passage. The column was washed with 20 ml of 10 mM Tris buffer and then the loaded protein was eluted stepwise in increasing NaCl (50, 100, 200, 300, 500 mM, 1 M). The majority of JHE activity eluted in the 300 mM NaCl fraction with a smaller amount in the 200 and 500 mM fractions. The 300 mM fraction was concentrated further and simultaneously desalted by replacement with 50 mM phosphate buffer, pH 7.4 in a Centricon-30 device (Millipore, Bedford, MA) with a 30,000 MW cutoff. JHE preparation #2: Infection of Hi5 cells was the same as with the first preparation. When harvesting the cell culture medium at 57 hpi, the following proteases were added: Pepstatin A (Sigma, St Louis, MO) at 1 μM, TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone, Sigma) at 10 μM, EDTA at 1 mM, and E64 (*trans*-Epoxysuccinyl-L-leucylamido[4-guanido]butane, Sigma) at 10 μM. The medium was centrifuged at 2000 × *g* and 10,000 × *g*. To the supernatant, Tris–HCl (pH 9.0) was added to a final concentration of 5 mM, and a 50% (w/v) solution of PEG, MW 3350 (Sigma) was slowly added until the final concentration was 15%, and gently stirred at 4 °C for 45 min. This solution was centrifuged at 10,000 × *g* for 10 min. The supernatant was then diluted 1:4 in 5 mM Tris–HCl, pH 9.0 and loaded to the same Q-Sepharose column described above. Greater than 96% of detectable JHE activity bound to the column in one passage. The column was washed with 20 ml of 10 mM Tris buffer and then the loaded protein was eluted stepwise in increasing NaCl (50, 100, 200, 350, 500 mM, 1 M). The majority of JHE activity eluted in the 200 and 350 mM NaCl fractions, which were then concentrated and desalted in a Centricon-30 filtration device (Millipore).

2.3. Protein analysis

Measurement of protein concentration was done using the BCA assay (Pierce, Rockford, IL) with BSA fraction V protein (Sigma) to derive a standard curve. Polyacrylamide gel electrophoresis (PAGE) was done using Novex precast polyacrylamide gels (Invitrogen Corp., Carlsbad, CA) for both SDS-PAGE analysis and isoelectric focusing. SDS-PAGE gels were stained with GelCode Silver Stain SNAP Stain Kit from Pierce. Narrow range (3–7) isoelectric focusing gels were divided into lanes which were stained for general esterase activity, or cut into slices for JHE activity. The former was done by soaking the gel at 35 °C in 50 mM phosphate buffer, pH 6.8, containing 0.05% Fast Blue RR salt (Sigma) with 0.02% α-naphthyl acetate (Sigma) and 0.02% β-naphthyl acetate (Sigma). For JHE analysis, the lane was cut into 5 mm slices and placed into 50 mM phosphate buffer, pH 7.4, and the enzyme was eluted at 4 °C for 14 h. An aliquot was then assayed for JH III hydrolysis as described in Section 2.4. An additional lane was sliced and placed into 5 mM KCl solution, which was later used for pH measurement. The pH was measured at 4 °C using a Corning model 430 pH meter.

2.4. Enzyme analyses

JH III hydrolysis was assayed by the method described by Hammock et al. (1977). All enzyme assays were done with a proper dilution of the enzyme so that the rate of product formation observed was linear within the time frame of the assay. Briefly, this assay uses tritiated JH III (New England Nuclear Research Products, Boston, MA: 17 Ci/mmol), the metabolite of which is partitioned into an aqueous phase after organic extraction and then detected in a scintillation counter. The labeled JH III is diluted cold JH III (Sigma) and brought to 0.5 mM in ethanol (Croston et al., 1987). One microliter of this JH stock solution is added to a 100 μl solution (final concentration is 5 μM) of diluted enzyme in phosphate buffer, pH 7.4, and then incubated at 30 °C for 15 min. Enzyme assays were performed in triplicate.

For assays utilizing *p*-nitrophenyl acetate (Sigma), *p*-nitrophenyl valerate (Sigma) and *p*-nitrophenyl palmitate (Sigma), 20 μl of enzyme solution was added to 278 μl of 50 mM phosphate buffer, pH 7.4. The reaction was initiated at 30 °C with the addition of 2 μl of substrate dissolved in either ethanol or 1-propanol (the latter only with the palmitate derivative) and the formation of *p*-nitrophenol was monitored for 2 min at 405 nm in a Molecular Devices microtitre-plate reader (kinetic mode). The final substrate concentration was 1×10^{-3} M. For conversion of mOD/min to μmol/min a standard curve was created using *p*-nitrophenol (Sigma) by adding 2 μl of substrate with final concentration 13.3–333 μM into the same buffer solution as was used in the assay. For assays utilizing α-naphthyl acetate and α-naphthyl caprylate, 20 μl of enzyme solution was added to 278 μl of 50 mM phosphate buffer, pH 7.4 with 0.062% w/v Fast Blue RR dye (Sigma). The reaction was initiated at 30 °C with the addition of 2 μl of substrate and the formation of α-naphthol was monitored for 2 min at 450 nm in a Molecular Devices microtitre-plate reader (kinetic mode). Substrates were dissolved in ethanol except for the palmitate derivative, which was dissolved in 1-propanol. The final substrate concentration was 1×10^{-3} M. For conversion of mOD/min to μmol/min a standard curve was created using α-naphthol (Aldrich). Two microliters of α-naphthol was added to final concentration 13.3–267 μM and measured at 450 nm in the same buffer solution as was used in the assay with substrate. The JH analog, HEPTAT, was synthesized and assayed as described by McCutchen et al. (1993), except that we performed the assay with final substrate concentration of 500 μM in 50 mM phosphate buffer, pH 7.4, at 30 °C.

2.5. K_m determinations

The Michaelis constant (K_m) for JH III was done using the tritiated JH assay as described above, except that the same enzyme dilution was tested with different substrate concentrations, and at lower concentrations the time of incubation was shortened in order to observe initial velocity (less than 15% of substrate consumed). After the range of the K_m was determined, five substrate concentrations which bracketed the estimated K_m value were used (from 10–250 nM). The K_m values for α-naphthyl acetate and *p*-nitrophenyl acetate were also done as described above, using the same enzyme dilution at substrate concentrations ranging from 25–1000 μM. The K_m value for HEPTAT was done as described except that the same enzyme dilution was tested at substrate concentrations ranging from 10–500 μM. Each assay was performed with samples in triplicate and then the assay was repeated 2–3 times. The K_m values were then determined using Lineweaver–Burk plots (1/[substrate] vs. 1/velocity). The mean value and standard deviation of at least three K_m determinations are reported for each substrate.

2.6. pH analysis

The ^{3}H-JH III partition assay was performed as described above with the following modifications. The enzyme was diluted in water until the final dilution step in the following buffers to give a final buffer concentration of 50 mM with 25 μg/ml BSA. One buffer was prepared for each pH at a concentration of 100 mM in a range from 4 to 11.0 in 0.5 pH unit increments. Ammonium acetate was used for pH 4–5.5. Bis-Tris was used for pH 6–6.5. Phosphate was used for pH 7–7.5. Tris was used for pH 8.0–8.5. Borate was used for pH 9.0–10.0. CAPS buffer was used for pH 10.5–11. The pH was rechecked after the addition of enzyme solution to ensure that the buffering capacity was sufficient for the added protein. Buffer dependent hydrolysis was checked at each pH. As anticipated from the structure of JH, nonenzymatic hydrolysis was not significant.

2.7. Inhibitor studies

All inhibition experiments were carried out using ^{3}H-JH III as the substrate. These assays were performed as described above except that the individual 100 μl assay tube contained 1 μl of a stock solution of inhibitor dissolved in 100% ethanol. Inhibition assays were compared with ethanol controls. At each inhibitor concentration, the assays were run in triplicate. The median inhibitor concentrations (IC_{50}) were determined by regression of at least four points in the linear region of the curve on either side of the IC_{50}. The mean value of three separate curves is given in the table. The trifluoroketone inhibitors, OTFP (1,1,1-trifluoro-3-octylsulfanyl-propan-2-one) and DETFP (1,1,1-trifluoro-3-decylsulfanyl-propan-2-one) were synthesized in this laboratory as previously described (Hammock, 1985). The dihydroxysulfone inhibitors, $OTFPdOHSO_2$ (1,1,1-trifluoro-3-(octane-1-sulfonyl)-propan-2,2-diol) and $DETFPdOHSO_2$ (1,1,1-trifluoro-3-(decane-1-sulfonyl)-propan-2,2-diol), were also synthesized in this laboratory as previously described (Wheelock et al., 2001). EPPAT (*O*-ethyl-*S*-phenyl phosphoramidothiolate) was also synthesized in this laboratory as previously described (Sanborn and Fukuto, 1972). Methamidophos (*O*,*S*-dimethyl phophoramidothiolate) and carbaryl (α-naphthyl *N*-methylcarbamate) were purchased from Chem Service (West Chester, PA). Phenylmethysulfonylfluoride (PMSF) was purchased from Sigma.

2.8. Protease inhibitor assays

Each inhibitor was tested at the higher end of commonly recommended working concentrations for inhi-

bition of JH III hydrolysis. All assays were in 50 mM phosphate buffer, pH 7.4. Control reactions were conducted under the same concentration of ethanol as the test samples. PMSF (Sigma) was tested at 1 mM. TPCK (Sigma) protease inhibitor was tested at 100 μM and 1% ethanol. Pepstatin A (Sigma) was tested at 2 μM and 1% ethanol. Leupeptin (Sigma) was tested at 100 μM. EDTA (Fisher, Pittsburgh, PA) was tested at 5 mM. E64 (Sigma) was tested at 100 μM.

2.9. Solvent and detergent activation studies

These experiments were done using the ^{3}H-JH III partition assay as described above except the enzyme buffer, which was 50 mM Tris–HCl, pH 7.8 (where indicated), did not contain BSA, and for each experiment contained the indicated amount of organic solvent or detergent. For each concentration of solvent a control tube was added to ensure that JH III did not partition into the aqueous phase independently of enzyme under these conditions, although this concern had already been addressed previously with regard to organic solvents (Croston et al., 1987).

3. Results

The coding region of JHE from *M. sexta* was PCR amplified and subcloned into the pAcUW21 transfer vector. The insert was verified by sequencing of the plasmid DNA. The transfer vector was then cotransfected with digested AcMNPV viral DNA into Hi5 cells, and following two rounds of plaque purification, a plaque was amplified and tested positive for JHE activity. Fig. 1 shows the time course of JHE expression in a 100 ml culture of Hi5 cells. Cell culture medium from cells infected with wild type virus had no detectable esterase activity (data not shown). Although activity continued to increase with time until 72 hpi, for subsequent protein expression experiments the medium was harvested at 48–60 hpi in order to avoid protease activity. Concentration of the enzyme in the cell culture medium varied according to the size of the culture. Using various sizes of Erlenmeyer flasks as cell culture vessels which were agitated upon a shaker, the optimum production observed was from 25 ml of cell culture, and would then gradually decrease as the volume of cells increased. Activity in the viral infected cell culture medium ranged from 65–110 nmol/min/ml. Greater than 96% of JHE activity from the original cell culture medium was detected in the supernatant after the debris was pelleted at 10,000 × *g*.

In this report we used two different enzyme preparations. The first one consisted of a partial purification by anion exchange chromatography. This method resulted in enzyme that was concentrated sufficiently

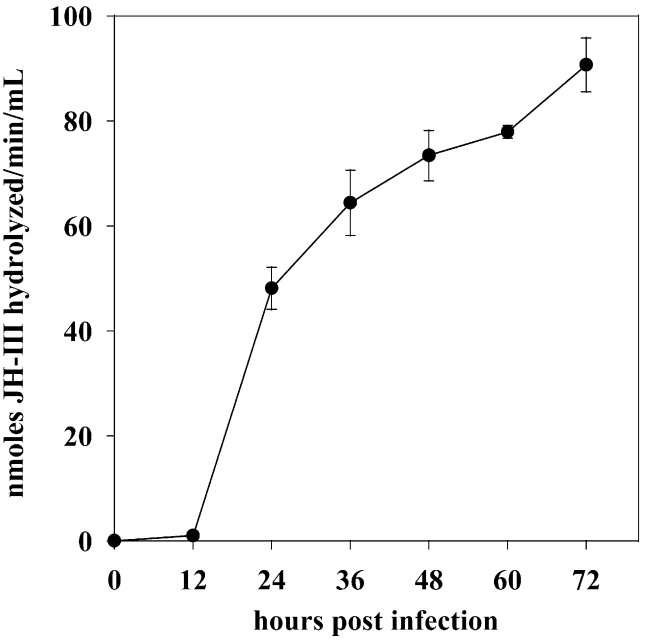

Fig. 1. Time course of JHE expression in Hi5 cells. About 100 ml of cells (1×10^6 cells/ml) was infected with AcMs7JHE at MOI = 5 in serum free medium in a 250 ml Erlenmeyer flask. One milliliter of viral infected cell culture medium was taken at each time point, and the 10,000×*g* supernatants were frozen. Samples from all time points were simultaneously thawed, diluted and assayed in triplicate for hydrolysis of JH III as described. Vertical bars indicate standard deviation of the mean. In separate experiments, JHE in cell culture medium was shown to be stable to repeated freeze thaw. The same trend of expression was observed with volumes ranging from 50–200 ml.

enough to do the enzymatic analyses that follow in the report. However, electrophoresis revealed some heterogeneity in the JHE protein, which caused concern over protease degradation during the purification. Thus, we revised the enzyme preparation by raising the pH, including protease inhibitors, and by adding an early PEG precipitation step. The inclusion of the PEG precipitation in the second enzyme preparation resulted in an improved purification scheme, which is illustrated in Table 1 and in Fig. 2. Greater than half of the protein in the cell culture medium was removed by PEG precipitation (compare lanes E and F in Fig. 2) with no loss of activity. The ion exchange step that followed was necessary to concentrate the enzyme, and further increased the specific activity by nearly a factor of 2. The addition of PEG apparently did not affect the efficiency of binding of JHE to the Q-Sepharose column. A significant amount of JHE eluted from the Q-Sepharose column in the 200 mM NaCl fraction, which by SDS-PAGE analysis appears almost as pure as the 350 mM NaCl fraction (Fig. 2, lanes B and C). However, the specific activity of the 350 mM fraction is almost three times higher than the 200 mM NaCl fraction (Table 1), indicating greater purity of active enzyme. It is possible that some of the activity that was lost during the ion exchange step had eluted into the 200 mM NaCl fraction as inactive JHE

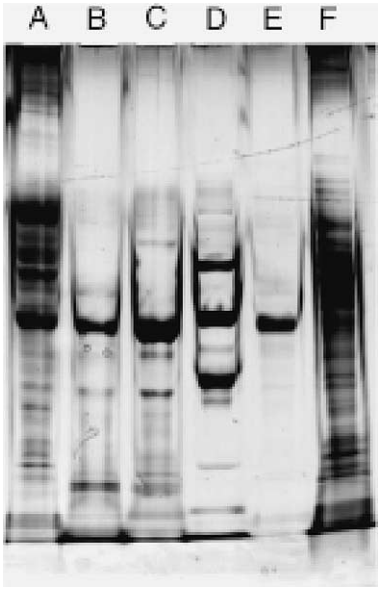

Fig. 2. SDS-PAGE analysis of recombinant JHE from *M. sexta*. Samples were run on a Novex precast 10% Tris–Glycine gel, and stained with Pierce GelCode Silver Stain. Lane A: 40 μg of AcMs7JHE infected cell culture medium, 57 hpi, 10,000 × *g* supernatant. Lane B: 20 μg of eluate from Q-Sepharose column (200 mM NaCl fraction). Lane C: 30 μg of eluate from Q-Sepharose column (350 mM NaCl fraction). Lane D: MW markers phosphorylase B (97,000), albumin (67,000), and ovalbumin (45,000). Lane E: 20 μg of supernatant from 15% PEG precipitation. Lane F: 40 μg of pellet from 15% PEG precipitation.

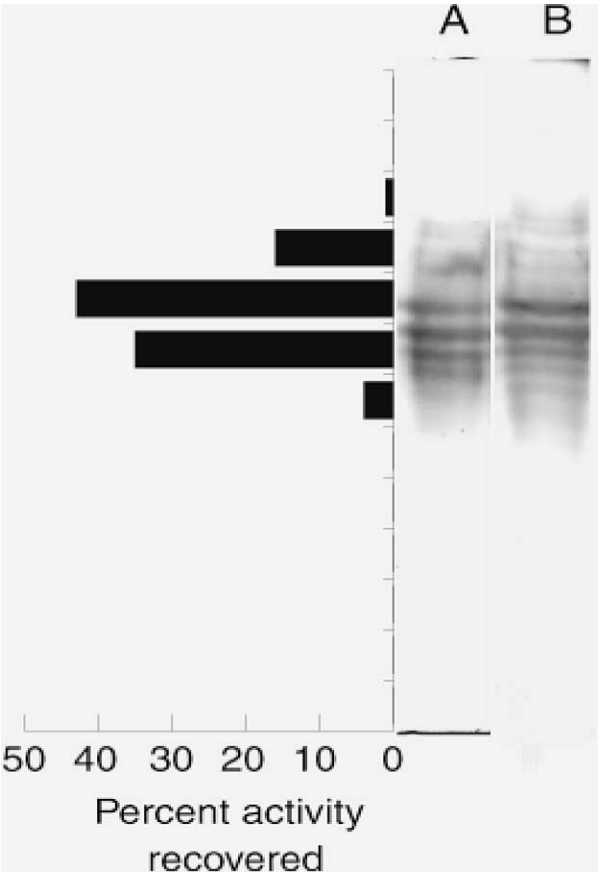

Fig. 3. IEF analysis of recombinant JHE from *M. sexta*. Lanes A and B were stained with α-naphthyl acetate, α-naphthyl acetate and Fast Blue as described in Section 2. Samples were run on a Novex precast isoelectric focusing gel, narrow range (pH 3–7). Lane A contains 1.5 μg of 300 mM salt fraction from first enzyme preparation. Lane B contains 1.5 μg of 350 mM salt fraction from second enzyme preparation. For lane A, two neighboring lanes were loaded with equal amount of enzyme. The lanes were cut into 5-mm slices and analyzed for either JHE activity or pH. The graph indicates JHE activity recovered from each 5-mm slice, which is aligned on the *Y*-axis with the stained region of the gel. The peak slice on the graph next to lane A corresponds to pH 6.0. Sixty-eight percent of the applied JHE activity was recovered from the gel. Samples from cell culture infected with wild type virus did not show any staining with naphthyl acetate (not shown).

protein (total yield of 80% activity with all fractions combined). The 350 and 200 mM NaCl fractions yielded 64 and 14%, respectively, of the activity that was loaded onto the Q-Sepharose column. Following, ion exchange chromatography, the peak fractions were further concentrated and desalted using a Centricon-30 device. The final specific activity was 1556 nmol/min/mg protein in the concentrated 350 mM salt fraction. Based upon estimation of band density of JHE in SDS-PAGE analysis of the purified protein, we calculate that in vitro JHE production produced 41 mg JHE per liter of cell culture. Based on the catalytic activity observed in solution and the specific activity of JHE from this and previous studies we estimate that approximately 47 mg of JHE is produced per liter of cell culture.

When analyzing the protein sample from preparation #2 by SDS-PAGE, the molecular weight of the JHE protein was estimated by comparison with molecular weight markers. The prominent band (Fig. 3, lane C) runs just below the 67,000 Da albumin marker, and therefore we estimate the mass of JHE to be about 66,500 Da. The isoelectric point (pI) of the partially purified JHE was determined by running it on narrow range (3–7) IEF gels. Since there are numerous proteins other than JHE present in the cell culture medium, we identified the JHE band both by staining with naphthyl acetate and by determining JHE activity in gel slices. The position of the naphthyl acetate stain corresponded to the peak of JHE activity, which was identified by cutting the gel into 5 mm slices and then assaying the gel slices for JHE activity. As can be seen in Fig. 3, the isoelectric focusing resulted in a smear rather than a distinct peak. The peak of JHE activity was found in the gel slice corresponding to pH of 6.0. Sixty-eight percent of the total activity that was loaded on the gel was recovered. Replicates of narrow range IEF gels yielded the same peak pI value for JHE, although measurements on wide range IEF gels (3–10) were more variable, giving pI values ranging from 6.1–6.5.

Staining of cell culture medium from wild type AcMNPV did not show any staining by this method

(data not shown), which indicates that all proteins stained in the IEF gels correspond to the products of the JHE cDNA. To address the concern that the heterogeneity of JHE observed by IEF might arise from partial degradation (either proteolysis or deglycosylation), we did a second enzyme preparation, which included measures to prevent protease activity, and then compared the two preparations (Fig. 3). The JHE preparation was altered by adding several protease inhibitors, raising the pH of the purification buffer, and adding an early PEG precipitation step in order to remove proteins more quickly. It had been previously demonstrated that several protease activities are associated with baculovirus infected cells in culture, which are more active at low pH (Naggie and Bentley, 1998). Hu and Bentley also showed that several protease inhibitors could be used to help stabilize recombinant proteins during in vitro expression. With the exception of PMSF, none of the protease inhibitors they used (Pepstatin A, TPCK, EDTA, and E64) affected JHE activity in vitro (data not shown), and JHE also retains near optimum activity at high pH. Thus, we employed these precautionary steps in order to prevent any degradation of the JHE protein. However, the second purification scheme seems to have made no difference in the heterogeneity of JHE forms that had been previously observed. Comparison of the two JHE purification strategies by isoelectric focusing (Fig. 3) showed that heterogeneous forms of JHE exist in both enzyme preparations. Following PNGase F digestion (to remove any *N*-linked carbohydrates), we failed to show any mobility shifts on either SDS-PAGE or IEF gels (data not shown). Thus, we were unable to demonstrate that the cause of heterogeneity arises from proteolysis or differential glycosylation, although the analyses were not extensive enough to rule them out completely.

The ability of JHE of *M. sexta* to hydrolyze a variety of substrates was tested and the specific activities are listed in Table 2. The highest specific activity by the recombinant JHE was observed with HEPTAT, a synthetic JH analog (McCutchen et al., 1993). The enzyme also showed activities against common esterase substrates such as *p*-nitrophenyl acetate and α-naphthyl acetate which were greater than the activity observed for the endogenous substrate, JH III. However the observed K_m value for JH III is 2–3 orders of magnitude lower than the K_m values observed for the other substrates tested. Since we did not have a homogenous preparation of the enzyme, we did not estimate k_{cat} but rather used the observed V_{max} values in relation to K_m for assessment of substrate specificity. When considering the ratio of V_{max} to K_m as an indicator of substrate specificity, the ratio for JH III is also higher than those of the other substrates tested (JH III⩾HEPTAT > α - naphthyl acetate > *p* - nitrophenyl acetate).

The measured K_m values for *p*-nitrophenyl acetate and α-naphthyl acetate are so high that they are close to the limit of solubility for these substrates in an aqueous solution. Thus, the specific activities listed for these two model substrates in Table 2 are close to the maximal velocity, but do not actually achieve the theoretical upper limit of reaction velocity for these substrates. Because of these solubility problems with naphthyl esters and *p*-nitrophenyl esters, we did not attempt to measure K_m values for the esters with longer aliphatic side chains, but we did want to illustrate that they could be hydrolyzed by JHE. In the case of *p*-nitrophenyl palmitate, we had to alter the conditions of the assay to allow for this. The *p*-nitrophenyl palmitate analog was initially tested but yielded no detectable activity under similar conditions to that of the other substrates (substrate delivered in ethanol such that final concentration of ethanol is 1% within 50 mM phosphate buffer, pH 7.4). However, when the concentration of isopropanol was increased in the assay to 10%, activity was observed with this hydrophobic substrate.

JHE was incubated with ^{3}H-JH III in the presence of a range of pH buffers to create a pH curve (Fig. 4). JHE retains activity over a wide range of pH, with as much as 80% of optimal activity from pH 6.5–11.0.

The partially purified JHE was assayed in the presence of several common esterase inhibitors such as PMSF,

Table 2
Kinetic parameters of recombinant JHE in hydrolyzing some model substrates

Substrate	K_m (μM)	V_{max} (μmol/min/mg protein)	V_{max}/K_m (ratio)
JH III	0.052 ± 0.007	1.4 ± 0.07	26.9
HEPTAT	61 ± 9	31.1 ± 2.1	0.51
α-Naphthyl acetate	410 ± 150	21.2 ± 3.3	0.04
p-Nitrophenyl acetate	250 ± 30	8.3 ± 1.2	0.03
α-Naphthyl caprylate	ND	1.5 ± 0.18	ND
p-Nitrophenyl valerate	ND	1.3 ± 0.10	ND
p-Nitrophenyl palmitate	ND	0.2 ± 0.02[a]	ND

Enzyme was partially purified as described (preparation #1). The same enzyme preparation was used for each assay. Assay conditions for each substrate are described in Section 2. Results are presented as the mean ± SD (n = 3–4). ND, not determined.

[a] For the indicated substrate, increased solvent was used to facilitate the reaction.

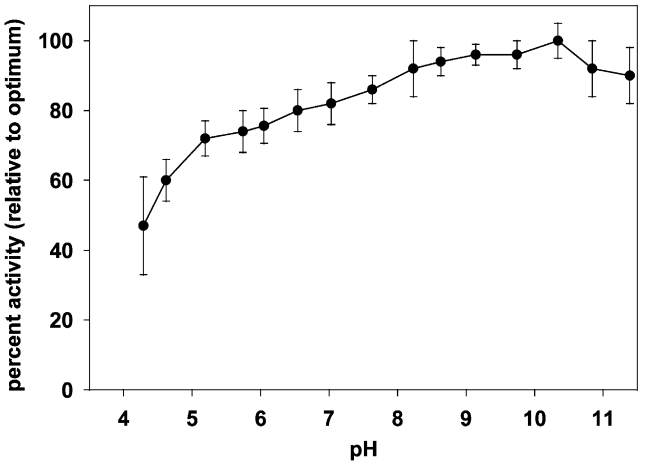

Fig. 4. Effect of the incubation pH on the enzyme activity of recombinant JHE. Mean values are represented in relation to JHE activity at pH optimum (pH 10.3). Vertical bars indicate standard deviation of the mean. JH III hydrolysis assays were performed in triplicate according to the conditions described in Section 2. Corrections for chemical hydrolysis at each pH were made for each calculation of enzymatic hydrolysis.

carbaryl, methamidophos, EPPAT, OTFP, DETFP (Table 3). The calculated IC_{50} (concentration at which the enzyme is 50% inhibited) values for this enzyme preparation are all within an order of magnitude of the values that have been reported in various reports in the literature. The inhibitory effects of common protease inhibitors were also tested at standard working concentrations. With the exception of PMSF, none of the protease inhibitors tested showed any inhibition of JHE at the concentrations used.

JHE activity was also tested in the presence of a few organic solvents such as methanol, ethanol, isopropanol, acetone, and acetonitrile (Fig. 5). All solvents tested showed an activation of JHE activity at low concentrations. The highest amount of activation (915% of control) shown was by acetonitrile at 10% (v/v) solvent in Tris buffer. With the exception of methanol, JHE retained more than 100% normal activity at solvent concentrations as high as 20% (v/v). JHE was also tested in the presence of some detergents and emulsifying agents: sodium dodecyl sulfate (SDS), Triton X-100 (TX-100), polyoxyethylene sorbitan monolaurate (Tween-20) and it was discovered that at low concentrations these also have an activation effect on the JHE enzyme (Fig. 6). SDS differs from the other two in that it has an activating effect at very low concentration (0.1%), but raising the concentration any higher results in a dramatic loss of activity. On the other hand, with TX-100 and Tween-20 the enzyme retains near 100% activity at concentrations as high as 5%.

Table 3
Effects of inhibitors upon recombinant JHE of *M. sexta*

Inhibitors	IC_{50} (M)
OTFP	$9.1 \pm 0.26 \times 10^{-9}$
DETFP	$2.8 \pm 0.22 \times 10^{-9}$
$OTFPdOHSO_2$	$3.7 \pm 0.17 \times 10^{-6}$
$DETFPdOHSO_2$	$7.0 \pm 0.42 \times 10^{-7}$
EPPAT	$1.4 \pm 0.16 \times 10^{-9}$
Methamidophos	$3.3 \pm 0.21 \times 10^{-6}$
PMSF	$3.2 \pm 0.54 \times 10^{-5}$
Carbaryl	$>10^{-4}$

The median inhibitor concentrations (IC_{50} = concentration at which hydrolysis of JH III is 50% inhibited) were determined by regression of at least four points in the linear region of the curve on either side of IC_{50}. The mean value, ±SD, of three separate curves is given in the table. Enzyme preparation, abbreviations, and JHE assay are described in Section 2.

4. Discussion

We have demonstrated that the full length cDNA sequence of JHE from *M. sexta*, which was reported by Hinton and Hammock (2001), represents a protein which is active towards the hydrolysis of JH III when expressed

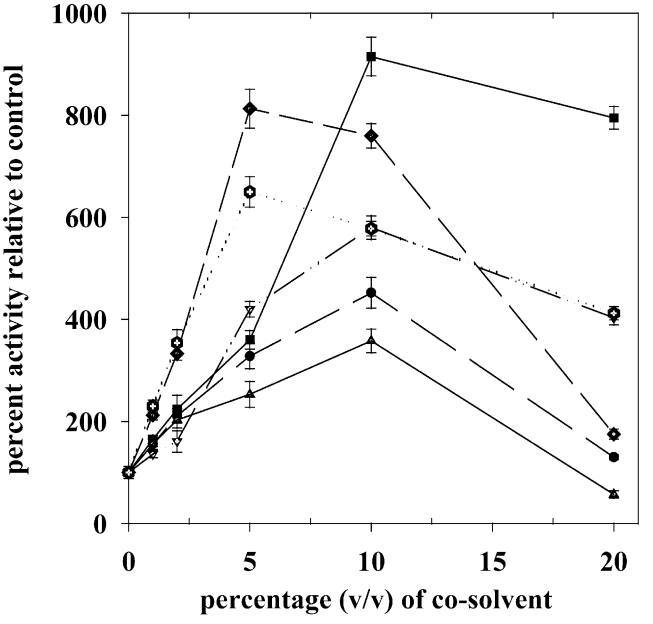

Fig. 5. Effect of organic co-solvents on recombinant JHE catalyzed hydrolysis of JH III. JH III hydrolysis assays were performed in triplicate according to the conditions described in Section 2. Enzyme was diluted in Tris–HCl, pH 7.8, prior to the addition of solvent. Activity is expressed as levels in comparison to control JHE in Tris buffer with no solvent added. Percent co-solvent is expressed on a volume/volume basis. Vertical bars indicate standard deviation of the mean. Solvents are represented by symbols as follows: acetone (circle), acetonitrile (square), methanol (triangle), ethanol (upside down triangle), isopropanol (diamond), *n*-propanol (hexagon).

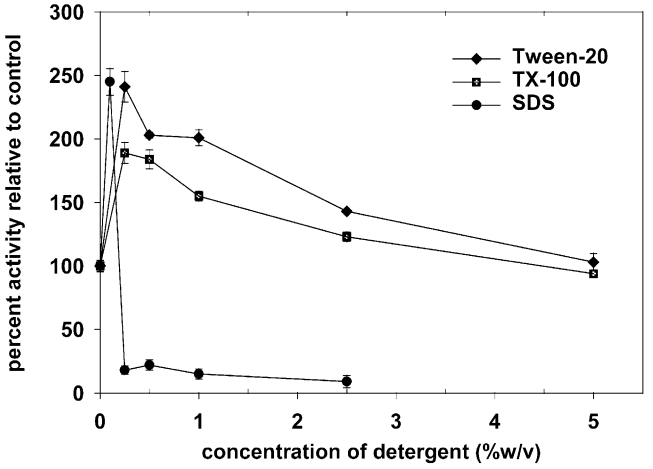

Fig. 6. Effect of detergents of recombinant JHE catalyzed hydrolysis of JH III in Tris–HCl, pH 7.8. JH III hydrolysis assays were performed in triplicate according to the conditions described in Section 2. Amount of each detergent; SDS, TX-100, and polyoxyethylene sorbitan monolaurate (Tween-20); is expressed on a volume/volume percentage basis. Activity is expressed as levels in comparison to control JHE in Tris buffer with no detergent added. Vertical bars indicate standard deviation of the mean.

in the baculovirus in vitro expression system. The recombinant enzyme displays various biochemical properties that indicate that it is the same protein that this laboratory isolated by biochemical purification from the hemolymph of 5th instar *M. sexta* (Hinton and Hammock, 2001). The majority of JHE activity is secreted into the extracellular medium in the viral infected cell culture. This is in accordance with the fact that the cDNA was isolated from the fat body, while the purified protein was obtained from hemolymph. Furthermore, the deduced protein sequence contains a putative signal peptide for secretion, and agrees with the partial amino acid sequence obtained from the purified protein which was isolated from the hemolymph (Hinton and Hammock, 2001).

The final specific activity of the second enzyme preparation (1556 nmol/min/mg protein) compares favorably to earlier estimates of specific activity ranging from 442–1020 nmol/min/mg protein for JHE preparations by affinity chromatography from hemolymph of *M. sexta* (Abdel-Aal and Hammock, 1985; Venkatesh et al., 1990; Hinton and Hammock, 2001). Although PAGE analysis in each of these reports demonstrates proteins of apparent homogeneity, the lower specific activity could be explained by incomplete recovery of enzyme activity following its elution by a tight binding inhibitor.

The molecular weight we observed with recombinant JHE is consistent with previously observed literature values which range from 63,000–68,000 Da (Hinton and Hammock, 2001; Coudron et al., 1981). The pI of the recombinant JHE protein, although not found in a distinct band, is also found to approximate a range of measured values that have been observed in the literature. Various reports in the literature give pI values for JHE isolated from the hemolymph of larvae of *M. sexta* (Abdel-Aal and Hammock, 1986; Venkatesh et al., 1990; Jesudason et al., 1992; Hinton and Hammock, 2001) which range from 5.5–6.1, and often give more than one peak of activity from the same sample in an IEF gel. The fact that we also observed heterogeneity in our samples as well, which were derived from a single gene expressed in cell culture, indicates the possibility that differential processing and/or post-translational modification is an inherent property of the JHE gene within an individual cell type.

The wide pH optimum range we observed with recombinant JHE (Fig. 5) is in agreement with Coudron et al. (1981), who previously reported that hemolymph JHE of *M. sexta* had high stability from pH 5–9. Using a different method for assaying JHE activity and longer incubation times, their report stated that above pH 9 the background hydrolysis was too high for accurate measurement, but using the radiometric assay we were able to demonstrate that JHE is stable at even higher pH than was previously observed. This may be a common feature among JHE proteins, because studies of JHE from *Trichoplusia ni* (Hammock et al., 1977) and *Tenebrio molitor* (Hinton et al., unpublished) also showed wide pH curves from other JHE enzymes.

The observation that the JHE enzyme had a higher specific activity (8.6:1 relative specific activity) towards HEPTAT than it did for JH III is in accordance with previous observations that the spectrophotometric JH analog was well designed and that other purified JHE proteins also showed up to 30-fold higher activity towards HEPTAT than to JH itself (McCutchen et al., 1993). The recombinant JHE of *M. sexta* also showed the ability to hydrolyze a variety of ester compounds, with significant activity towards *p*-nitrophenyl acetate, naphthyl acetate and other analogs of these surrogate substrates (Table 2). It had been previously reported that JHE from *M. sexta* is not active towards α-naphthyl acetate (Coudron et al., 1981), but that analysis was done with JHE of lower specific activity (84 nmol JH/min/mg as opposed to 1368 nmol JH/min/mg) than was used in the current study. Although it apparently requires the use of very concentrated JHE such as is available in this study in order to detect the hydrolysis of naphthyl esters and *p*-nitrophenyl esters, it is worth noting that the relative activity as compared to JH III is actually very high, but the reason JH III was detected more easily was due to the differences in sensitivities of the assays involved with each substrate. JHE enzymes from other lepidopterans, such as *T. ni* (Hanzlik and Hammock, 1987), have been reported with low activity against both naphthyl acetate and *p*-nitrophenyl acetate. JHE's ability to hydrolyze other common esterase substrates may have also been overlooked in other systems due to its relatively low abundance.

The observation that JHE in vitro has a specific activity towards a variety of substrates that is comparable towards JH simply shows that it is a potentially versatile enzyme, and should not confuse the issue of its role as a JH selective enzyme in vivo. The 2,3-unsaturated carbonyl of JH makes it very stable to both nonenzymatic as well as enzymatic degradation. In general with esterases and other α/β-hydrolase fold enzymes, turnover or k_{cat} decreases with the hydrophobicity of the acyl enzyme. One would anticipate that the acyl enzyme of JH would be turned over very slowly. The acyl enzyme of natural JH also has a 2,3 unsaturation leading to high transition state energy in the deacylation step. JH could almost be considered an inhibitor for JHE in the sense that carbamates are considered inhibitors of acetylcholinesterase. Thus, it is not surprising that the maximal velocity of even JHE for JH is relatively low. The initial rates of the substrates used in Table 3 reflect in part the fact that α-naphthol and *p*-nitrophenol are excellent leaving groups. The surrogate substrate HEPTAT uses a thioether to mimic the 2,3 olefin of JH and thus reduces the activation energy of hydrolysis of the ester. As is common among esterases, less lipophilic acid moieties lead to higher turnover of the substrate and this observation was used in the design of HEPTAT as a surrogate substrate for JHE. Moreover, in assessing the specificity of an enzyme, one must consider the k_{cat}/K_m ratio rather than the turnover rate alone. The JHE enzyme in larvae of *M. sexta* only appears at key timepoints in which JH titers must decline, and the high k_{cat}/K_m ratio of JHE is likely to be a physiologically essential factor. Since JH is already decreasing at the time JHE activity arises (Nijhout, 1975; Baker et al., 1987), the concentration of the enzyme surpasses the low levels of JH soon after it first appears. Although the JHE of *M. sexta* has a low K_m value for JH, the titer of JH will fall well below the K_m also. In such a situation, one could argue that the k_{cat} value has less biological relevance since the JHE molecule will unlikely have a JH already bound, or to exist in the acylated form, when it encounters each new molecule of JH.

Despite the fact that JHE is capable of hydrolyzing substrates other than JH, relatively few enzymes besides JHE can hydrolyze JH at all. Other general esterases that have been found in larval hemolymph do not have significant catalytic activity towards JH in vitro (Sparks and Hammock, 1979, 1980). Also, the activity of JH binding protein in the hemolymph is thought to further increase the selectivity of JH–JHE interaction by decreasing the ability of any general esterases to access the lipophilic substrate, while at the same time serving to enhance the JH–JHE interaction by preventing adsorption of JH into lipophilic tissues where JHE could not have complete access to the substrate (Goodman, 1990; Touhara et al., 1995). Cloning and expression of the JHE of *M. sexta* will facilitate experiments defining the interaction among the JH binding protein (Touhara et al., 1993), JH epoxide hydrolase (Debernard et al., 1998) and JHE. The JHBP has long been suspected of protecting JH from degradation by nonspecific enzymes. Clearly it can protect JH from sequestration in pools where the substrate is refractory to degradation. Thus the JHE-JHBP combination at certain developmental stages can be seen as a system to clear JH rapidly from the insect.

A reasonable working definition of a JHE is an esterase with high apparent specificity for JH whose catalytic activity correlates with expected variations in titer. The hypothesis of a physiological role of a putative JHE can be further tested in vivo by artificially decreasing or increasing the titer of JHE and examining effects on the physiology of the insect. The blood of some insects shows very high esterase activity on some commonly used esterase model substrates such as *p*-nitrophenyl acetate and α-naphthyl acetate. Thus, using these surrogate substrates to monitor a low abundance JHE against a background of general esterase activity can be dangerous. If one is interested in JHE activity, the unequivocal substrates to use are JH homologs. However, surrogate substrates can be very useful for rapid and less expensive monitoring of the enzyme. The naphthyl acetate stain is very useful for monitoring transgenic expression of JHE because general esterase activity is very low in the parent cell lines. Therefore, a variety of substrates can be used to monitor transgenic JHE but one must be very cautious when using substrates other than JH in blood or tissue homogenates.

The various inhibitors that were tested yielded data that are variably consistent with previous observations in the literature using the purified, wild type enzyme. Sparks and Hammock (1980) measured an IC_{50} value of 2×10^{-9} M for EPPAT, the most potent inhibitor found for JHE prior to the development of trifluoroketone inhibitors. The value with the recombinant JHE, 1.4×10^{-9} M, is in very good agreement. We also measured IC_{50} values for carbaryl ($> 1\times10^{-4}$ M) and PMSF (3.2×10^{-5} M) that are very close to the respective values ($> 1\times10^{-4}$ M and 6.6×10^{-5} M) of Sparks and Hammock (1980). The JHE of *H. virescens* was also found to be very susceptible to PMSF (Hammock et al., 1990), while that of *T. ni* was resistant (Sparks and Hammock, 1980). In addition, some trifluoroketone inhibitors were tested which agree with the trend indicated by Wheelock et al. (2001), in which JHE from *T. ni* is more sensitive to thioether forms than to sulfone derivatives. The small sample of representatives from this series shows that the octyl ($OTFPdOHSO_2$) and decyl ($DETFPdOHSO_2$) analogs of the sulfone forms of trifluoroketone have IC_{50} values a few orders of magnitude higher than observed for the same chain lengths of the thioether forms (OTFP and DETFP). Previous reports of inhibition by OTFP of JHE from *M. sexta* have shown more variability in their IC_{50} measurements and also

revealed more than one catalytic site on the purified enzyme from hemolymph (Abdel-Aal and Hammock, 1985; Venkatesh et al., 1990). The IC_{50} values range from 1.2×10^{-9} to 7.8×10^{-9} M for the more sensitive form of the enzyme (Venkatesh et al., 1990; Abdel-Aal and Hammock, 1985), and values from 3.8×10^{-6} to 6.0×10^{-6} M for the less sensitive form (Abdel-Aal and Hammock, 1985; Venkatesh et al., 1990). These previous results come from affinity purified JHE from hemolymph, which otherwise would seem homogenous except for subtle differences in isoelectric points in one report (Venkatesh et al., 1990). Thus, the results indicated either more than one catalytic site per protein, or that closely related forms of the JHE gene exist. The report by Abdel-Aal and Hammock (1986) had compared the inhibition curves for purified JHE from a few lepidopterans. With OTFP, JHE from *M. sexta* had a wider inhibition curve that set it apart from the other JHEs (including *H. virescens*). Upon closer inspection two curves could be distinguished, giving one for each putative catalytic site. During this study, since we were using recombinant JHE and also had *H. virescens* JHE in recombinant form, we compared the curves of both enzymes using OTFP and revealed that with the recombinant enzymes, the inhibition curves appear similar (data not shown). Also, our measured IC_{50} value for recombinant JHE of *M. sexta* is 9.21×10^{-9} M, which would likely correspond to a single, highly sensitive catalytic site rather than a median value between two different sites. Since the recombinant baculovirus contains only one copy of the JHE gene, it seems likely that the previous reports utilized samples containing isoforms of the same enzyme arising due to genetic polymorphisms, and such a phenomenon is not possible in the in vitro system described here.

We tested the inhibitor methamidophos, since it is a very widely used pesticide, and much work has been done to develop assays to detect the pesticide at low levels. In recent years, acetylcholinesterase has been used to develop inhibition based tools for the low level detection of methamidophos and other inhibition based insecticides (Nunes et al., 1998; Nunes et al., 2001). We observed here that the inhibition of recombinant JHE of *M. sexta* ($IC_{50} = 3.3 \times 10^{-6}$ M) is comparable to inhibition of many of the cholinesterase enzymes tested. Sparks et al. (1983) had previously measured an IC_{50} of 5.1×10^{-6} M for JHE from hemolymph of *M. sexta*. In light of this information, in combination of the stability of the JHE enzyme in the presence of various solvents, we propose that the enzyme might be a suitable candidate for future development of inhibitor based insecticide assays. Other studies have shown JHEs from lepidopteran species to be surprisingly sensitive to phosphoramidothiolates such as methamidophos, and surprisingly resistant to many other organophosphate esterase inhibitors.

One note to make in reference to the JHE partition assay is that this enzyme remains active at 50% methanol and also in fairly basic solution. Therefore, in order to truly terminate the enzyme reaction using the partition assay (Hammock et al., 1977), it is necessary to follow the 'stop' solution (methanol:H_2O:NH_4OH) immediately with the extraction step in order to completely denature the enzyme and remove substrate from the reaction. We undertook studies with solvents in order to verify that the recombinant enzyme shared properties that had been observed previously using the wild purified JHE from hemolymph (Croston et al., 1987). The results shown in Fig. 5 show a similar trend on JHE activation by organic solvents, which was observed in the previous report (Croston et al., 1987). The previous report was limited to organic solvents, but the additional observations here include its higher activity in detergents and emulsifying agents (Fig. 6). These results confirm that it is possible to maintain high JHE activity using the recombinant enzyme. This is independent of any necessary cofactors or stabilizing agents, which might have been present in vivo. JH, the natural substrate of JHE is also fairly lipophilic, but in vivo there exist other water soluble proteins such as JHBP which are thought to facilitate the transport of JH in order to make it available to target receptors or metabolizing enzymes alike (Touhara et al., 1995). In vitro, such useful agents are not as common. In the case of JH it is soluble enough to do experiments at low concentrations with an enzyme that has a very low K_m value for its substrate. However, with regard to other substrates that are either more hydrophobic or have higher K_m values, it is fortunate to have enzymes which can remain active in the presence of co-solvents that commonly cause most enzymes to denature even at low concentrations. Also, as we have demonstrated, the enzyme can be used to hydrolyze esters that are only soluble in aqueous conditions in the presence of co-solvents. Note that under normal buffer conditions no hydrolysis was observed with *p*-nitrophenyl palmitate, but after raising the concentration of isopropanol to increase the solubility of substrate, activity was detected (Fig. 5). Also, there are reports of JHE from several species to have a substrate preference for certain ester chain lengths (Hammock et al., 1977; McCutchen et al., 1993; Grieneisen et al., 1997). It is possible that the enzyme could be used to selectively hydrolyze a single site on a bifunctional ester, or simply to perform ester hydrolysis or transesterification of lipophilic compounds that are not compatible with standard aqueous buffers.

In conclusion, we have been successful in expressing an active JHE enzyme from the cDNA clone that was previously reported, and through its characterization have verified the identity of the cDNA as a functional JHE gene. Also, we have introduced an enzyme that can be easily produced in abundance in vitro, and new ideas for the potential utility of this enzyme as both a

biosensor mechanism and as a synthetic enzyme. It should also prove useful as a tool in the future study of the regulation of JHE biology, its stability in vivo as well as in vitro, and by adding to the information and molecular tools which can be utilized for design of JHE mutants in biopesticide designs.

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References

Abdel-Aal, Y.A.I., Hammock, B.D., 1985. Apparent multiple catalytic sites involved in the ester hydrolysis of juvenile hormones by the hemolymph and by an affinity-purified esterase from *Manduca sexta* Johannson (Lepidoptera: Sphingidae). Archives of Biochemistry and Biophysics 243, 206–219.

Abdel-Aal, Y.A.I., Hammock, B.D., 1986. Transition state analogs as ligands for affinity purification of juvenile hormone esterase. Science 233, 1073–1076.

Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., 1987. In vivo fluctuation of JH, JH acid, and ecdysteroid titer and JH esterase activity, during development of fifth stadium *Manduca sexta*. Insect Biochemistry 17, 989–996.

Bonning, B.C., Hoover, K., Booth, T.F., Duffey, S., Hammock, B.D., 1995. Development of a recombinant baculovirus expressing a modified juvenile hormone esterase with potential for insect control. Archives of Insect Biochemistry and Physiology 30, 177–194.

Booth, T.F., Bonning, B.C., Hammock, B.D., 1992. Localization of juvenile hormone esterase during development in normal and recombinant baculovirus-infected larvae of the moth *Trichoplusia ni*. Tissue and Cell 24, 267–282.

Croston, G., Abdel-Aal, Y.A.I., Gee, S.J., Hammock, B.D., 1987. Activation of crude and homogenous juvenile hormone esterases by organic solvents. Insect Biochemistry 17, 1017–1021.

Coudron, T.A., Dunn, P.E., Seballos, H.L., Wharen, R.E., Sanburg, L.L., Law, J.H., 1981. Preparation of homogenous juvenile hormone specific esterase from the haemolymph of the tobacco hornworm, *Manduca sexta*. Insect Biochemistry 11, 453–461.

Debernard, S., Morisseau, C., Severson, T.F., Feng, L., Wojtasek, H., Prestwich, G.D., Hammock, B.D., 1998. Expression and characterization of the recombinant juvenile hormone epoxide hydrolase (JHEH) from *Manduca sexta*. Insect Biochemistry and Molecular Biology 28, 409–419.

Goodman, W.G., 1990. Biosynthesis, titer regulation and transport of juvenile hormones. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods. Rutgers University Press, New Brunswick, pp. 83–124.

Grieneisen, M., Mok, A., Kieckbusch, T., Schooley, D., 1997. The specificity of juvenile hormone esterase revisited. Insect Biochemistry and Molecular Biology 27, 365–376.

Hammock, B.D., Sparks, T.C., Mumby, S.M., 1977. Selective inhibition of JH esterase from cockroach hemolymph. Pesticide Biochemistry and Physiology 7, 517–530.

Hammock, B.D., 1985. Regulation of juvenile hormone titer: degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7. Pergamon Press, Oxford, pp. 431–437.

Hammock, B.D., Bonning, B.C., Possee, R.D., Hanzlik, T.N., Maeda, S., 1990. Expression and effects of the juvenile hormone esterase in a baculovirus vector. Nature 344, 458–461.

Hinton, A.C., Hammock, B.D., 2001. Purification of juvenile hormone esterase and molecular cloning of the cDNA from *Manduca sexta*. Insect Biochemistry and Molecular Biology 32, 57–66.

Ichinose, R., Nakamura, A., Yamoto, T., Booth, T.F., Maeda, S., Hammock, B.D., 1992. Uptake of juvenile hormone esterase by pericardial cells of *Manduca sexta*. Insect Biochemistry and Molecular Biology 22, 893–904.

Ismail, S.M., Satyanarayana, K., Bradfield, J.Y., Dahm, K.H., Bhaskaranand, G., 1998. Juvenile hormone acid: evidence for a hormonal function in induction of vitellogenin in larvae of *Manduca sexta*. Archives of Insect Biochemistry and Physiology 37, 305–314.

Ismail, S.M., Goin, C., Muthumani, K., Kim, M., Dahm, K.H., Bhaskaran, G., 2000. Juvenile hormone acid and ecdysteroid together induce competence for metamorphosis of the Verson's gland in *Manduca sexta*. Journal of Insect Physiology 46, 59–68.

Jesudason, P., Anspaugh, D.D., Roe, R.M., 1992. Juvenile hormone metabolism in the plasma, integument, midgut, fat body, and brain during the last instar of the tobacco hornworm, *Manduca sexta* (L.). Archives of Insect Biochemistry and Physiology 20, 87–105.

McCutchen, B.F., Uematsu, T., Szekacs, A., Huang, T.L., Shiotsuki, T., Lucas, A., Hammock, B.D., 1993. Development of surrogate substrates for juvenile hormone esterase. Archives of Biochemistry and Biophysics 307, 231–241.

Naggie, S., Bentley, W.E., 1998. Appearance of protease activities coincides with p10 and polyhedrin-driven protein production in the baculovirus expression system: effects on yield. Biotechnology Progress 14, 227–232.

Nijhout, H.F., 1975. Dynamics of juvenile hormone action in larvae of the tobacco hornworm, *Manduca sexta* (L.). Biological Bulletin 149, 568–575.

Nunes, G.S., Skladal, P., Yamanaka, H., Barcelo, D., 1998. Determination of carbamate residues in crop samples by cholinesterase-based biosensors and chromatographic techniques. Analytica Chimica Acta 362, 59–68.

Nunes, G.S., Montesinos, T., Marques, P.B.O., Fournier, D., Marty, J.L., 2001. Acetylcholine enzyme sensor for determining methamidophos insecticide: evaluation of some genetically modified acetylcholinesterases from *Drosophila melanogaster*. Analytica Chimica Acta 434, 1–8.

Riddiford, L.M., 1980. Interactions of ecdysteroids and juvenile hormone in the regulation of larval growth and metamorphosis of the tobacco hornworm. In: Hoffman, J.A. (Ed.), Progress in Ecdysone Research. Elsevier, Amsterdam, pp. 409–430.

Roe, R.M., Venkatesh, K., 1990. Metabolism of juvenile hormone: degradation and titer regulation. In: Gupta, A.P. (Ed.), Recent Advances in Comparative Arthropod Morphology, Physiology, and Development, vol. 1. Rutgers University Press, New Brunswick, pp. 127–179.

Sanborn, J.R., Fukuto, T.R., 1972. Insecticidal, anticholinesterase, and hydrolytic properties of S-aryl phosphoramidothioates. Journal of Agricultural and Food Chemistry 20, 926–930.

Sparks, T.C., Hammock, B.D., 1979. A comparison of the induced and naturally occurring juvenile hormone esterases from last instar larvae of *Trichoplusia ni*. Insect Biochemistry 9, 411–421.

Sparks, T.C., Hammock, B.D., 1980. Comparative inhibition of the juvenile hormone esterases from *Trichoplusia ni*, *Tenebrio molitor* and *Musca domestica*. Pesticide Biochemistry and Physiology 14, 290–302.

Sparks, T.C., Hammock, B.D., Riddiford, L.M., 1983. The haemolymph juvenile hormone esterase of *Manduca sexta* (L.)—inhibition and regulation. Insect Biochemistry 13, 529–541.

Touhara, K., Lerro, K.A., Bonning, B.C., Hammock, B.D., Prestwich, G.D., 1993. Ligand binding by a recombinant insect juvenile hormone binding protein complex. Biochemistry 32, 2068–2075.

Touhara, K., Bonning, B.C., Hammock, B.D., Prestwich, G.D., 1995. Action of juvenile hormone (JH) esterase on the JH–JH binding protein complex. An in vitro model of JH metabolism in a caterpillar. Insect Biochemistry and Molecular Biology 25, 727–734.

Venkatesh, K., Abdel-Aal, Y.A.I., Armstrong, F.B., Roe, R.M., 1990. Characterization of affinity-purified juvenile hormone esterase from the plasma of the tobacco hornworm, *Manduca sexta*. Journal of Biological Chemistry 265, 21727–21732.

Wheelock, C.E., Severson, T.F., Hammock, B.D., 2001. Synthesis of new carboxylesterase inhibitors and evaluation of potency and water solubility. Chemical Research in Toxicology 14, 1563–1572.