Juvenile hormone (JH) esterase activity but not JH epoxide hydrolase activity is downregulated in larval *Adoxophyes honmai* following nucleopolyhedroviruses infection

Yasumasa Saito a,b, Shizuo G. Kamita b, Bruce D. Hammock b, Yasuhisa Kunimi a, Maki N. Inoue a, Madoka Nakai a,*

a Department of Biological Production Science, United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

b Department of Entomology and Nematology, and Comprehensive Cancer Center, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

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**ABSTRACT**

Juvenile hormones (JHs) and ecdysteroids are critical insect developmental hormones. JH esterase (JHE) and JH epoxide hydrolase (JHEH) are JH-selective enzymes that metabolize JH and thus regulate the titer of JH. Baculoviruses are known to alter host endocrine regulation. The nucleopolyhedroviruses, AdhoNPV and AdorNPV, are known to have slow and fast killing activity against Adoxophyes honmai (Lepidoptera: Tortricidae), respectively. Here we found that when penultimate (4th) instar *A. honmai* are inoculated with AdhoNPV or AdorNPV, the mean survival time is 9.7 and 8.2 days, respectively. The larvae molted once but did not pupate. The AdhoNPV- or AdorNPV-infected larvae did not show a dramatic increase in JH activity as was found in mock-infected larvae, instead they showed a marked decrease in JH activity. In contrast, both viral infections had no effect on JHE activity. In order to further characterize the JHE activity, the JHE-coding sequence of *A. honmai* (*ahjhe*) was cloned and confirmed to encode a biologically active JHE. Quantitative real-time PCR analysis of *ahjhe* expression in 4th and 5th instar *A. honmai* revealed that AdhoNPV and AdorNPV are able to reduce *ahjhe* expression levels.

**1. Introduction**

Insect metamorphosis is predominantly regulated by juvenile hormones (JHs) and ecdysteroids such as 20-hydroxyecdysone. JHs are considered to maintain larval feeding behavior. During the final instar, a dramatic decrease in JH titer and spikes in ecdysone titer are required for the induction of pupation. The dramatic decrease in JH titer results from both a decrease in JH biosynthesis and an increase in its metabolism (Hammock, 1985). JH is metabolized to JH acid or JH diol by two hydrolytic enzymes, JH esterase (JHE) and JH epoxide hydrolase (JHEH), respectively (Kamita and Hammock, 2010; Morisseau and Hammock, 2005).

Normal endocrinological regulation of insect development can be altered following infection by baculoviruses. Baculoviruses are large (genome size from 80 to 180 kbp), arthropod-specific, double-stranded DNA viruses (van Oers, 2011). The family *Baculoviridae* is traditionally composed of two groups, nucleopolyhedrovirus (NPV) and granulovirus (GV). Many baculoviruses carry a gene that encodes an ecdysteroid UDP-glucosyltransferase (EGT), an enzyme that conjugates sugar molecules to ecdysteroids and making them inactive (O'Reilly and Miller, 1989). The result of ecdysteroid inactivation is that the baculovirus-infected host is unable to complete the normal molting process. Baculoviruses (and other insect viruses) have also been shown to alter host development by downregulating JHE activity (Hajós et al., 1999; Nakai et al., 2002, 2004). In the case of the smaller tea tortrix *Adoxophyes honmai* (Lepidoptera: Tortricidae), downregulation of JHE activity has also been observed following infection with an entomopoxvirus (Nakai et al., 2004). Downregulation of JHE activity may keep a relatively high level of JH at the sensitive period to prevent pupal metamorphosis and leads to a status quo condition. It is hypothesized that both inactivation of ecdysteroids and maintaining JH titer at status quo levels are beneficial to the virus pathogen because the infected insect will likely continue to feed to facilitate the production of viral progeny.

*A. honmai* is a major pest of tea plants in Japan. The baculovirus *Adoxophyes orana* GV (AdorGV) has been used as a biological
insecticide to control *A. hommari* in Japanese tea fields. AdorGV was originally found from *A. orana* (Lepidoptera: Tortricidae) and has subsequently been developed into the commercial product Hamaki-Tentikai® (Arysta LifeScience Corp., Tokyo, Japan) and registered as a biopesticide in Japan in 2003 (Kunimi, 2007). Other entomopathogenic viruses that infect *A. hommari* (e.g., *A. hommari* GV (AdhoGV), *A. hommari* NPV (AdhoNPV), and *A. orana* NPV (AdorNPV) from *Baculoviridae*; and *A. hommari* entomopoxvirus (AHEV) from *Entomopoxvirinae*) have been isolated and studied as potential biological insecticides against *A. hommari*. These studies have shown that, except for AdorNPV, these viruses are slow-killing by inhibiting the development to pupal stage and kill the host only during the final instar (Hilton and Winstanley, 2008a; Ishii et al., 2002; Nakai et al., 2004). In contrast, AdorNPV is relatively fast-killing (Takahashi et al., 2008). In this study, we investigate how the slow-killing AdhoNPV and fast-killing AdorNPV differently regulate JH metabolism by inhibiting JHE or JHEH. Furthermore, we identified the JHE-coding sequence of *A. hommari* (**ahjhe**) and investigate the expression levels of **ahjhe** in *A. hommari*.

## 2. Materials and methods

### 2.1. Insects and cells

Eggs of *A. hommari* (Lepidoptera: Tortricidae) were obtained from Agro-Kanesho Co. Ltd. (Saitama, Japan) and used to establish a continuous colony that was maintained at 25 °C under a 16 h light: 8 h dark photoperiod. Experiments using *A. hommari* were also performed under these rearing conditions. Larvae were reared on an artificial diet (Silkmate 2S; Nihon Nosan-Kogyo Co. Ltd., Kanagawa, Japan).

SF-9 (Invitrogen, Carlsbad, CA) and High Five™ (Invitrogen) cells were cultured at 27 °C in ExCell 420 (SAFC Biosciences, Lenexa, KS) medium supplemented with 2.5% fetal bovine serum and serum free ESF921 medium (Expression Systems, Davis, CA), respectively.

### 2.2. Viruses and viral inoculation

Isolates of NPVs of *A. hommari* (AdhoNPV) (Ishii et al., 2003; Nakai et al., 2003) and *A. orana* (AdorNPV) (Hilton and Winstanley, 2008b) were used in this study. Larval *A. hommari* are permissive to both AdhoNPV and AdorNPV (Takahashi et al., 2008). Larval *A. hommari* were inoculated with AdhoNPV or AdorNPV using a modified droplet feeding method (Kunimi and Fuxa, 1996). Newly molted fourth instar larvae were allowed to feed on a liquid inoculum containing 1.0 × 10^5 occlusion bodies (OBs)/ml (a > LC_{95} dose), 10% (w/v) sucrose, and 5% (w/v) red food coloring. The same solution without virus was used as a mock inoculation in control experiments.

### 2.3. Measurement of JHE activity in hemolymph

Hemolymph samples were collected from virus- or mock-infected 4th instar larvae at 24 h intervals and diluted in 100 mM sodium phosphate buffer (pH 8.0). Hemocytes were removed from the diluted hemolymph by centrifugation (10,000×g, 1 min, 4 °C) and the supernatant from this centrifugation was diluted again in 100 mM sodium phosphate buffer containing 0.1 mg/ml BSA (pH 8.0). The clarified and diluted hemolymph (100 μl) was transferred to a 10 × 75 mm glass test tube and the JHE activity in the diluted hemolymph was determined by a partition assay as described previously (Hammock and Sparks, 1977). To initiate the enzyme reaction 1 μl of JH III substrate, a mixture of racemic, unlabelled JH III (Sigma-Aldrich, St. Louis, MO) and [3H]-labelled JH III (PerkinElmer, Boston, MA), was added to the diluted hemolymph.

The final concentration of JH III was 5.0 μM. Following the addition of substrate, the reaction mixture was incubated at 30 °C for 60–300 min so that no more than 20% of the JH III was hydrolyzed during the incubation. The reaction was stopped by the addition of 100 μl of stop solution (methanol:water:ammonium hydroxide, 10:9:1). Then, iso-octane (250 μl) was added to the stopped reaction in order to partition JH III in the organic phase and JH III acid in the aqueous phase. An aliquot (50 μl) of the aqueous phase was transferred to 1 ml of ScintiVerse BD liquid scintillation counting cocktail (Fisher Chemical, Fair Lawn, NJ). A Tri-Carb 2810 TR (PerkinElmer) scintillation counter was used to determine counts per minute (CPM) during a 2 min-long counting cycle. All of the assays were performed using hemolymph that was obtained from three to seven individual larvae.

### 2.4. Measurement of JHEH and JHE activity in fat body

Fat body tissues were collected in parallel from the same larvae that were used to collect the hemolymph samples (Section 2.3). The mass of the fat body tissues was determined and the tissues were homogenized in 300 μl of 100 mM sodium phosphate buffer containing 0.1 mg/ml BSA (pH 8.0). The homogenized tissues were then diluted in 100 mM sodium phosphate buffer containing 0.1 mg/ml BSA (pH 8.0). The diluted fat body homogenate (100 μl) was transferred to a 10 × 75 mm glass test tube and 1 μl of 1 mM OTFP (3-octylthio-1,1,1-trifluoropropan-2-one), a JHE-selective inhibitor (Abdel-Aal and Hammock, 1985) or ethanol (EtOH) was added to each sample. The homogenized fat body sample was pre-incubated with the OTFP (or EtOH) at 30 °C for 10 min prior to the addition of JH III substrate as described above. The amount of hydrolysis of the ester and epoxide moieties of JH III was determined as described above. JH III-hydrolytic activity in the presence of OTFP was assumed to result from the activity of JHEH, whereas JHE activity was calculated by subtracting JHEH activity from the total JH III-hydrolytic activity in the absence of OTFP. All of the assays were performed using fat body homogenates prepared from four to nine individual larvae.

### 2.5. RNA isolation and cDNA synthesis

Total RNA was extracted from the fat body tissues of virus- or mock-infected *A. hommari* using ISOGEN (Nippon Gene, Tokyo, Japan). The total RNA samples were treated with RNase-free, recombinant DNase I (TaKaRa, Shiga, Japan) in order to remove contaminating chromosomal DNAs. The DNase I treated total RNAs were used as template for cDNA synthesis using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa).

### 2.6. Cloning and sequencing of **ahjhe**

A full-length cDNA encoding the JHE of *A. hommari*, **ahjhe**, was identified by 3’-RACE and 5’-RACE using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa) and 5’-Full RACE Core Set (TaKaRa). In order to identify primers for 3’- and 5’-RACE, an internal region of **ahjhe** was first amplified using two degenerate primers, lepJHES-F and lepJHES-R (Table 1). These primers were designed on the basis of highly conserved regions that are found in five reported lepidopteran JHES (Feng et al., 1999; Hanzlik et al., 1989; Hinton and Hammock, 2001; Shiotsuki et al., 2000; Teese et al., 2010). The internal sequence of **ahjhe** was obtained by RT-PCR using lepJHES-F and lepJHES-R and template cDNAs generated from fifth instar day 3 (L5D3) *A. hommari*. PCR conditions with TaKaRa ExTaq Hot Start (TaKaRa) were as follows: 94 °C 3 min; 30 cycles of 94 °C 30 s; 60 °C 30 s; 72 °C 30 s; and one cycle of 72 °C 5 min. This PCR generated a 0.3 kb-long amplicon that was gel-purified using a QiaQuick Gel Extraction Kit (Qiagen,
Hilden, Germany) and inserted into the pGEM-T vector (Promega, Madison, WI), and transformed into competent cells of *Escherichia coli* JM109 (TaKaRa). The sequence of the insert was determined by a 3130xl Genetic Analyzer (Applied Biosystem, Foster City, CA) using T7 and SP6 primers. This sequence was used to design a series of four forward primers, ahjhe-3RACE-FX (Table 1). These forward primers and Oligo dT-adaptor primer – R were used for 3'-RACE (Table 1). The amplicons generated by 3'-RACE were cloned into pGEM-T vector (Promega) and their sequences were determined as described above. Five primers, ahjhe-5RACE-XX (Table 1), for 5'-RACE were designed on the basis of the 3'-end sequence, and these primers were used for 5'-RACE using the 5'-Full RACE Core Set kit (TaKaRa).

### 2.7. Generation of AcAhJHE and expression of recombinant AhJHE

AcAhJHE, a recombinant baculovirus expression vector carrying *ahjhe* under the very late p10 promoter was generated following standard protocols (Merrington et al., 1999). Firstly, pAcUW21-ahjhe was generated by inserting *ahjhe* downstream of the p10 promoter of the baculovirus transfer vector pAcUW21 (Weyer et al., 1990) at the BgIII and EcoRI restriction endonuclease sites. Subsequently, Sf-9 cells (5 × 10^5 cells in a 35 mm-diameter culture dish) were transfected with pAcUW21-ahjhe (2 µg) and Bsu36I-digested BacPAK6 baculovirus DNA (2.3 µg) (Clontech, Mountain View, CA) using Cellfectin (Invitrogen) transfection reagent. Plaque assays on Sf-9 cell monolayers were performed to isolate AcAhJHE from the supernatant of the transfected Sf-9 cells.

In order to express recombinant JHE of *A. honmai* (AhJHE), High Five™ cells grown in suspension (1 × 10^6 cells/ml) were inoculated with AcAhJHE at a multiplicity of infection of 0.5. At 67 h post inoculation (hpi), the cell suspension was centrifuged (2000 × g, 20 min, 5 °C), and the supernatant was collected and filtered through a 0.2 micron filter. The filtrate was stored at 5 °C prior to ion exchange chromatography using anion exchange spin columns (Pierce Biotechnology, Rockford, IL). The filtrate was diluted with 3 volumes of 20 mM Tris–HCl (pH 8.0) buffer prior to loading onto the spin column. After loading, the spin column was washed with 20 mM Tris–HCl (pH 8.0) and bound proteins were eluted with 20 mM Tris–HCl (pH 8.0) containing 100, 200, 300 or 400 mM NaCl. Subsequently, Centriprep Ultracel YM-30 columns (Millipore, Bedford, MA) were used to desalt the fractions and concentrate the proteins. Protein concentrations of the preparations were estimated using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. The purity of the protein preparations was estimated using ImageJ software (Rasband, 2009) analysis of proteins that were separated using NuPAGE 10% Bis–tris gels (Invitrogen) and MOPS running buffer (Invitrogen).

### 2.8. Enzyme assays for JHE activity and inhibition by DFP or OTFP

The ability of AhJHE to hydrolyze JH III was determined as described in Section 2.3. The serine protease/esterase inhibitor diisopropyl fluorophosphate (DFP) (Sigma–Aldrich) or esterase inhibitor OTFP (Abdel-Aal and Hammock, 1985) was incorporated into some partition assays to help determine the selectivity of the JH III hydrolysis. All of the assays were performed in triplicate and repeated at least three times.

### 2.9. Quantitative real-time PCR (qRT-PCR) for *ahjhe*

The developmental profile of *ahjhe* in 4th and 5th instar *A. honmai* was determined by qRT-PCR using the comparative Ct (ΔΔCt) method (Livak and Schmittgen, 2001). Total RNA from whole body was extracted every 24 h after inoculation as described in Section 2.5. β-actin of *A. honmai* was selected as a reference gene for qRT-PCR as suggested previously (Anand et al., 2008; Gatehouse et al., 2009). Primers were designed from *ahjhe* and partial sequence of β-actin of *A. honmai*. The sequences of the primer pairs that were used for qRT-PCR analysis of *ahjhe* (ahjhe-qPCR-F and ahjhe-qPCR-R) and β-actin of *A. honmai* (ahb-actin-qPCR-F and ahb-actin-qPCR-R) are given in Table 1. The amplification efficiencies of these primer pairs was determined as described in User Bulletin #2 (Applied Biosystems). The qRT-PCR reaction (10 µl) was performed using an ABI Prism® 7000 Sequence Detection System (Applied Biosystems). The reaction mixture contained 10 ng of cDNA, 30 µM of each primer, and 5 µl of FastStart Universal SYBR Green Master Mix (ROX) (Roche, Basel, Switzerland). The qRT-PCR cycle condition was as follows: one cycle of 50 °C 2 min, 95 °C 10 min; 40 cycles of 95 °C 15 s; 60 °C 1 min. At the end of the cycles, a dissociation curve analysis of the amplified product was performed as follows: 95 °C 15 s; 60 °C 20 s; 95 °C 15 s. Relative gene expression of *ahjhe* was determined following the protocol described in User Bulletin #2 of the ABI Prism® 7000 Sequence Detection System (Applied...
Biosystems). The following calculation was used: ΔCt = (ahjhe Ct − β-actin Ct), ΔΔCt = (ΔCt sample − ΔCt control), amount of target = 2−ΔΔCt (Livak and Schmittgen, 2001). ΔCt of ahjhe at fifth instar day 1 (L5D1) of mock-infected A. honmai was set as one (a control). For each treatment, three to five individuals were examined in duplicate.

2.10. Statistical analysis

For the experiments in Sections 2.3 and 2.4, nonparametric comparisons for all pairs were performed using Steel–Dwass analysis between insects of the same developmental stage using JMP® 9.0.0 software (SAS Institute, Cary, NC).

3. Results

3.1. Larval development after viral inoculation

The effect of virus infection on the time of larval development was investigated by placing newly molted 4th instar A. honmai on diet containing a lethal concentration (>LC95) of AdhoNPV or AdorNPV. Under these conditions, AdhoNPV- or AdorNPV-infected larvae molted once (4th to 5th instar) but did not pupate. The mean survival time was 9.7 and 8.2 days for AdhoNPV- and AdorNPV-infected larvae, respectively. In contrast, mock-infected 4th instars of A. honmai molted to the 5th instar in 3 days, and became pupae by 7.1 days on average.

3.2. JHE activity in hemolymph

In order to determine whether there were any differences in the activity of JHE in AdhoNPV- or AdorNPV-infected larvae, JHE activity in the hemolymph was measured at 24 h intervals (L4D1 to L5D7) (Fig. 1). JHE activity was not detected in virus- or mock-infected 4th instars at day 1 (L4D1) or day 2 (L4D2). On day 3 (final day) of the 4th instar (L4D3), JHE activity was detectable but not statistically different in virus- and mock-infected larvae (nonparametric comparisons for all pairs using Steel–Dwass Methods: all P values > 0.05). On the first day of the 5th instar (L5D1), JHE activity increased by 3- to 5-fold but these activities were not significantly different between virus- and mock-infected larvae (all P values >0.05). On the 2nd day of the 5th instar (L5D2), JHE activity in mock-infected larvae increased by 10-fold. This activity in mock-infected larvae increased by 2-fold on the 3rd day of the 5th instar (L5D3). Mock-infected larvae became prepupae on the 4th day of the 5th instar (L5D4), and JHE activity dropped to a level similar to that found on L5D2. In contrast, JHE activity in AdhoNPV- or AdorNPV-infected L5D2 larvae did not increase, showing JHE activities that ranged from 123–679 and 370–786 pmol/min/ml, respectively. There were significant differences of JHE activity between mock- and virus-infected L5D2 to L5D4 larvae (all P values < 0.05).

3.3. JHE and JHEH activity in fat body

The activity of JHEH and JHE in the fat body tissues of mock-, AdhoNPV- or AdorNPV-infected 5th instar larvae were measured in the presence or absence of OTFP, a selective and highly potent inhibitor of JHE (Fig. 2). All JHEH activities remained constant during the 5th instar (mock-infected: 875–1028 pmol/min/mg, AdhoNPV-infected: 798–993 pmol/min/mg, and AdorNPV-infected: 737–1049 pmol/min/mg) (Fig. 2A). There was no significant

![Fig. 1. JHE activity in the hemolymph of AdhoNPV-, AdorNPV-, or mock-infected A. honmai. Newly molted 4th instar larvae were inoculated with a >LC95 dose of virus, then reared until pupation or death. Hemolymph was collected by piercing a larval proleg at the times indicated (L4D1: 4th instar day 1, L4D2: 4th instar day 2, L4D3: 4th instar day 3, L5D1: 5th instar day 1, L5D2: 5th instar day 2, L5D3: 5th instar day 3, L5D4: 5th instar day 4, L5D5: 5th instar day 5, L5D6: 5th instar day 6 and L5D7: 5th instar day 7). The hemolymph was diluted as appropriate in 100 mM sodium phosphate buffer containing 0.1 mg/ml BSA (pH 8.0) and JHE activity was determined by a partition assay using JH III as a substrate. Bars indicate standard errors of the mean of hemolymph collected from at least three individual larvae. Asterisks indicate a significant difference (P < 0.05) by nonparametric comparisons for all pairs using Steel–Dwass Methods. ND: not detected.](image-url)
difference in JHEH activity between mock- and virus-infected insects (nonparametric comparisons for all pairs using Steel–Dwass Methods: all $P$ values >0.05).

JHE activity in the fat body showed no significant difference between virus- and mock-infected larvae (all $P$ values >0.05) on the first day of the 5th instar (L5D1) (Fig. 2B). Unlike JHE activity in the hemolymph, the developmental profiles of mock-infected JHE activity in the fat body did not drop on the last day of 5th instar (L5D4). In contrast, JHE activity in the fat body from L5D2 larvae did not increase following either AdhoNPV- or AdorNPV-inoculation. As was found in the hemolymph, there were significant differences of JHE activity in fat body tissue homogenates between mock- and virus-infected L5D2 to L5D4 larvae (L5D2 $P$ values = 0.023, 0.010, and 0.603; L5D3 $P$ values = 0.016, 0.003, and 0.535; L5D4 $P$ values = 0.007, 0.007, and 0.997 for mock- and AdhoNPV-, mock- and AdorNPV-, and AdhoNPV- and AdorNPV-infected larvae, respectively).

### 3.4. Cloning and analysis of ahjhe

The activity profiles of JHE and JHEH in 5th instar A. honmai suggested that JHE but not JHEH plays a role in preventing pupation. In
order to better characterize the potential role of JHE (or its gene) activity in this phenomenon, the JHE-coding sequence of *A. honmai* (*ahjhe*) was cloned from fat body tissue. Previous studies have identified multiple *jhe*-like coding sequences in the genome of insects, however, it appears that only one of these genes encodes a biologically relevant JH-metabolizing esterase (Crone et al., 2007a). In order to clone *ahjhe*, a partial sequence was first obtained using degenerate primers, lepiJHEs-F and lepiJHEs-R. These primers were designed on the basis of protein motifs that are highly conserved in known JHEs (Kamita and Hammock, 2010). Amplification with lepiJHEs-F and lepiJHEs-R identified a 265 bp region that putatively encoded a homolog of the *jhe* gene of *Choristoneura fumiferana* (Lepidoptera: Tortricidae) (*E*-value = 2e-44). This partial sequence was used to generate nested primers (Table 1) for 3′- and 5′-RACE. The RACE approach identified a full-length cDNA (Genbank accession number: LC012033) of 2,862 nts that contained an open reading frame of 1,686 nts. The 5′- and 3′-UTR sequences of *ahjhe* were 28 and 1,148 nts, respectively. The open reading frame of *ahjhe* encoded a deduced protein (AhJHE) of 561 amino acid (aa) residues. A 22 aa residues-long signal peptide for secretion was predicted at the N-terminal of AhJHE by GENETYX ver.10 software (GENETYX Co., Tokyo, Japan). Seven aa sequence motifs (RF, DM/DQ, GQSAG, E, GxxHxxE, R/Kx(6)R/KxxxR and T) (Kamita and Hammock, 2010) are found in known JHE sequences, and all of these motifs are conserved in AhJHE (Fig. 3). Phylogenetic tree analysis of AhJHE and 16 other JHE or putative JHE sequences (Fig. 4) placed AhJHE in a clade with known lepidopteran JHEs, a clade that was distinct from the non-lepidopteran JHE clades. Within the lepidopteran JHE clade, AhJHE clustered with CfJHE, a JHE from another caterpillar within the family Tortricidae.

3.5. Expression and purification of recombinant AhJHE

In order to test if *ahjhe* encoded a biologically active JHE, a recombinant baculovirus, AcAhJHE, carrying *ahjhe* was generated. The specific activity of this preparation for JH III was 2200 ± 100 nmoles of JH III acid/min/mg of AhJHE. This represented a 20-fold increase in specific activity in comparison to that found in the supernatant (107 nmoles of JH III acid/min/mg) of High Five™ cells infected with AcAhJHE. The mature protein (AhJHE) lacking a signal peptide is predicted to be 539 aa with a calculated molecular weight of 59.08 kDa. A protein of approximately 60 kDa was clearly isolated from the 100 mM NaCl fraction following ion exchange chromatography (data not shown). The 100 mM NaCl fraction was used as the enzyme source for all of the enzyme assays.

3.6. Inhibition of AhJHE by DFP or OTFP

Historically, JHE has been characterized as esterase activity that is highly resistant to inhibition by DFP (Kamita and Hammock, 2007).
In order to better characterize recombinant AhJHE, the ability of DFP or OTFP to inhibit AhJHE was tested by adding DFP (1–10 μM) or OTFP (1–10 nM) to the standard JH assay. DFP was able to inhibit 66 ± 2% of JHE when present in the JH assay at 10 μM. Although DFP showed moderate potency against AhJHE, DFP was roughly 1000-fold less potent than OTFP (62 ± 3% inhibition at 10 nM).

3.7. Gene expression of ahjhe

qRT-PCR was used to measure the relative gene expression (RGE) levels of ahjhe message in AdhoNPV- or AdorNPV-infected larvae (Fig. 5). The expression level of ahjhe in mock-infected 5th instar day 1 (L5D1) larvae was taken as “100%” or a value of 1. The activation of ahjhe expression was consistent with the elevated JHE activity that was found in mock-infected larvae (Figs. 1 and 2B). In comparison, the RGE of ahjhe in virus- and mock-infected 4th instar A. honmai was less than 0.21 during all of the times tested. Similarly, the RGE of ahjhe in 5th instars was less than 1 in all of the virus-infected 5th instars that were tested.

4. Discussion

4.1. JHE activity in hemolymph and fat body

JHE is a protein that is produced in fat body cells and secreted into the hemolymph. Thus, hemolymph JHE activity shows a strong and positive correlation with fat body JHE activity (Anand et al., 2008). As expected a sharp increase of JHE activity was found immediately prior to the prepupal stage (L5D2–L5D4) in mock-infected larva A. honmai. The developmental profile of JHE activity in the hemolymph appeared to peak on L5D3 (Fig. 1), whereas JHE activity in the fat body remained high (Fig. 2B). In contrast, a sharp increase in JHE activity prior to the prepupal stage was not found in AdhoNPV- or AdorNPV-infected larvae. JHE activity remained constant and at relatively low levels in NPV-infected insects. AdhoNPV (Ishii et al., 2002) and AdorNPV (data not shown) infect the fat body of A. honmai, thus it appears that this reduction in JHE activity in fat body and subsequent JHE release from fat body to hemolymph are the direct result of virus infection. On the other hand, it is known that parasitoid wasps can also downregulate JHE activity.
because of the production of growth-blocking peptide (GBP) in hemolymph (Hayakawa, 1995). It would be interesting to investigate whether baculovirus infection also induces production of GBP. The kinetic parameters of known JHEs (i.e., low $K_M$ and slow substrate turnover) suggest that JHE biologically functions as a JH scavenging enzyme, thus it appears that the virus is able to sufficiently reduce JHE levels so that JH titers remain above the level that is required for normal development and metamorphosis.

4.2. JHEH activity in fat body

The metabolism of JH by JHE gives JH acid, a compound that may function as a substrate of JH acid methyl transferase (JHAMT) (Shinoda and Itoyama, 2003). The action of JHAMT can thus potentially function as an antagonist of JHE activity. In contrast, the action of JHEH results in JH diol, an irreversibly hydrolyzed metabolite (Seino et al., 2010). In the JH metabolism process, JHE is dominant in many insects (Kamita et al., 2003) and high levels of JH acid can cause metamorphosis (Ismail et al., 2000), suggesting JHE may play a greater role than JHEH in regulating JH titers (Keiser et al., 2002).

JHEs have a signal peptide and are secreted enzymes and the activity is largely found in the hemolymph, whereas JHE activity is membrane bound and found in the tissues, such as fat body and midgut (Anand et al., 2008; Kamita and Hammock, 2010). JHE activities were not detectable in the hemolymph of *Heliothis virescens* (Lepidoptera: Noctuidae) or *Bombyx mori* (Lepidoptera: Bombycidae) (Li et al., 2003; Zhang et al., 2005). Similarly, we were unable to detect JHEH activity in the hemolymph of *A. honmai* (data not shown).

During the final instar, JH metabolism by JHEH activity was lower than that of JHE and the JHEH activity remained constant regardless of mock- or virus-inoculation (Fig. 2A). It has been demonstrated that JHEH activity in fat body has no correlation with both JHE activities in hemolymph and fat body (Anand et al., 2008). From these data, JHEH is not likely to have a role in the induction of pupation in *A. honmai*. As previously described, fat body tissues of *A. honmai* can be infected with both AdhoNPV and AdorNPV, however, AdhoNPV and AdorNPV only targeted JHE but not JHEH resulting in an extension in the larval stage. This is the first report to analyze JHEH activity following baculovirus infection.

4.3. Gene expression of *ahjhe*

In order to investigate whether the inhibition of JHE occurs at the protein or gene expression levels, qRT-PCR for *ahjhe* was carried out (Fig. 5). Gene expression of *ahjhe* in mock-infected larvae was elevated from L5D1, which was one day before JHE enzyme activation (L5D2) (Figs. 1 and 2A), therefore, *ahjhe* expression appears to result in increased AhJHE activity. In contrast, both AdhoNPV- and AdorNPV-infected JHE activities in hemolymph were suppressed by more than 25-fold compared to the peak level (L5D3), whereas suppression of *ahjhe* expression was relatively lower, less than a 10-fold difference (Fig. 5). These findings are consistent with another study using *Epiphyas postvittana* (Lepidoptera: Tortricidae) infected with *E. postvittana* NPV (EppoNPV) that also showed roughly 4-fold downregulation of the expression of a putative *jhe* gene (Gatehouse et al., 2009). Interestingly, this previous study also showed downregulation of four putative JHEH-encoding genes. In contrast in this study, there was no impact on JHEH enzyme activity by virus infection. Thus, a detailed analysis of *jheh* gene expression was not performed in this study.

The mechanism of downregulation of *jhe* gene expression in baculovirus-infected caterpillars is still unknown. However, pathogen-encoded microRNAs (miRNAs) are known to play roles in pathogen–host interactions, and it is known that viral-encoded miRNAs can affect host gene expression profiles (Hussain and Asgari, 2014). As mentioned above (Section 4.1), AdhoNPV and AdorNPV infect the fat body, and *ahjhe* gene expression is reduced by viral infection (Fig. 5). We propose that small interfering RNAs may play a role in the downregulation of *jhe* gene expression in...
baculovirus-infected caterpillars, however, this hypothesis must still be tested. Also, in a previous study, JHE activity was shown to be reduced in *H. virescens* that were infected by a recombinant *Autographa californica* multiple NPV (AcMNPV) that carried a jhe gene in an antisense direction, suggesting that antisense RNA interference (RNAi) effects may be responsible for the reduction in JHE activity (Hajós et al., 1999).

The sequential effects of NPV infection on JH metabolism in *A. honmai* are summarized below. Larval *A. honmai* ingest AdhoNPV or AdorNPV. Following the initial infection of midgut cells or hemocytes, the progeny viruses initiate a systemic infection of fat body cells and other tissues. In cells that are infected with the virus, AhJHE is not produced because of the lack of ahjhe message. Because of the dramatic reduction in JHE activity, JH titer may remain above a level that maintains the status quo (i.e., the larval stage is maintain and pupation is repressed). In this study, there was no difference of JHE regulation following infection of NPVs showing different killing speeds. Furthermore, a correlation between JHE and JHE activities was not found, and JHE activity was unaffected by NPV infection.

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References


