Characterization and Expression Profiles of Juvenile Hormone Epoxide Hydrolase From *Lymantria dispar* (Lepidoptera: Lymantridae) and RNA Interference by Ingestion

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Abstract

Juvenile hormone epoxide hydrolase (JHEH) is an important enzyme in the degradation pathways of juvenile hormone (JH) in insects. It converts JH to JH diol and hydrolyses JH acid to JH acid diol. JHEH titers regulate the entire process of insect development. In this study, full length *ldjheh* cDNA (2101 bp) was cloned from the Asian gypsy moth *Lymantria dispar* (L.; Lepidoptera: Lymantridae), and provisionally designated *ldjheh1*. LdJHEH1 was characterized by predicted molecular weight of 52.64 kDa, theoretical isoelectric points of 6.87 and contains a transmembrane domain at the N-terminus. The transcriptional profiles of *ldjheh1* were detected by qRT-PCR. The *ldjheh1* was found to be expressed throughout all developmental stages with maximum expression levels occurring in fourth instar larvae. The *ldjheh1* mRNA was detected in the heads, thoraces, and abdomens of gypsy moth larvae on day 2 of the third instar. The *ldjheh1* was also detected in bodies of third instar larvae stage, with the highest peaks occurring at 24 h after ecdysis. The *ldjheh1* gene was successfully knocked down by oral delivery dsRNA in the third instar larvae of *L. dispar*. The dsRNA targeting *ldjheh1* was produced in vitro. Ingesting dsRNA for *ldjheh1* only slightly delayed larval development.

Key words: Asian gypsy moth, JHEH, transcriptional profiles, qRT-PCR, RNAi

Juvenile hormones (JHs) are composed of sesquiterpenoids, which are synthesized primarily by the insect corpora allata and then released into the hemolymph. Here, they play multiple and important roles in the regulation of larval metamorphosis (Riddiford 1994) and adult insect reproduction, e.g., by regulating pheromone synthesis and vitellogenesis (Wyatt and Davey 1996). Accurate regulation of JH concentration is, therefore, crucial for orderly development in insects. Many studies have shown that JH titers are controlled mainly by changing the rates of its synthesis and degradation (Gilbert et al. 2000, Li et al. 2003). JH synthesis is regulated by neuropeptides, such as allatotropins and allatostatins, secreted from the brain (Stay et al. 1994). JH degradation occurs over a very short period and is mediated by at least three enzymes: juvenile hormone esterase (JHE) (Hammock and Sparks 1977), juvenile hormone epoxide hydrolase (JHEH) (Share and Roe 1988), and juvenile hormone diol kinase (JHDK) (Maxwell et al. 2002a,b). In insects, JH is principally degraded by JHEs and JHEHs, which have high substrate specificity. JHE is a member of the carboxylesterase family. It hydrolyzes the methyl ester moiety of JH to juvenile hormone acid (JHa), which can be reverted back to JH (Hammock 1985, Hirai et al. 2002, Tan et al. 2005), and catalyzes juvenile hormone diol (JHd) to produce juvenile hormone acid diol (JHad) (Share and Roe 1988). As one of the epoxide hydrolases, JHEH transforms epoxides to compounds with decreased chemical reactivity, increased water solubility, and changed biological activity (Arand et al. 2005, Morisseau and Hammock 2005). In certain organs and tissues, JHEH degrades JHa to JHad (Share and Roe 1988), and hydrolyses JH to JHd, which is an irreversible hydrolysis reaction (Roe and Venkatesh 1990, Wojtasek and Prestwich 1995, Anspaugh and Roe 2005, Newman et al. 2005).

Initially, research about JH degradation concentrated on the mechanism of action of JHE (Kamita et al. 2003). However, since a specific partition assay was developed to simultaneously determine the activity of JHE and JHEH (Share and Roe 1988), rapid progress has been made in determining how JHEH participates...
in JH degradation (Touhara and Prestwich 1993, Wojtasek and Prestwich 1996, Debernard et al. 1998, Severson et al. 2002). Many assays had revealed that JHEH plays an important role, as critical as that of JHE, in degrading JH in certain insects (Campbell et al. 1992, Halarnkar et al. 1993, Lassiter et al. 1995, Khlebodarova et al. 1996, Debernard et al. 1998). The extent of the roles that JHE and JHEH play in JH degradation depends on the species and the developmental stages of the insect (Keiser et al. 2002, Kamita et al. 2003). Thus, JHEH inhibitors are considered to be potential insecticides for certain insects in which JHEH plays major function in JH degradation (Severson et al. 2002). This is further supported by findings that higher concentrations of norharman could inhibit the activity of JHEH in Reticulitermes speratus (Kolbe; Isoptera; Rhinotermitidae) (Itakura et al. 2008). In addition, several jheh or jheh-like genes have been cloned from lepidopteran insects, such as Manduca sexta (L.; Lepidoptera; Sphingidae) (Wojtasek and Prestwich 1996), Bombyx mori (L.; Lepidoptera; Bombycidae) (Hirai et al. 2002, Zhang et al. 2005, Seino et al. 2010, Cheng et al. 2014) and Danaus plexippus (L.; Lepidoptera; Nymphalidae) (Mackert et al. 2010, Cheng et al. 2014). A jhe gene has been documented in Lymantria dispar (L.; Lepidoptera; Lymantridae) (Nussbaumer et al. 2000), however, little characterization of a jhe gene in L. dispar has been done. Thus, in this study, we focused on the characterization of a jheh gene.

Materials and Methods

Insect Rearing

L. dispar eggs were collected from the Liangshui Natural Reserve of Xiaojiang’an Mountain (128°53′20″E, 47°10′50″N) and maintained at 4°C. When the egg masses were ready to hatch, they were disinfected with 5% methanol. Hatched gypsy moth larvae were reared in sterilized Petri dishes (9 cm in diameter and 1.5 cm in height) on leaves of Populus alba × Populus glandulosa under a 14:10 (L:D) h photoperiod, and at constant temperature (25 ± 1°C) and humidity (60%). Thirty first instar, 20 second instar, and 10 third instar larvae were reared per sterilized Petri dish. The leaves were replaced every day and botanical sponges soaked in water were used to keep the leaves fresh. Gypsy moth larvae that were healthy and similar in size were used for the experiments.

Cloning and Identification of the ldjheh1 Gene

Total RNA was isolated from the third instar larvae using Invitrogen TRizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Promega) to remove any contaminating genomic DNA. RNA concentrations were measured using a spectrophotometer and RNA integrity was checked by analysis on a 1% w/v agarose gel. One microgram of total RNA was reverse transcribed using reverse transcriptase (Takara Bio., Dalian, China).

RNA was submitted to GenBank (accession number MF996338).

Gene Characterization and Polygenetic Analysis

ExPaSy Proteomics Server (http://cn.expasy.org/tools/pi_tool.html) was used to compute the molecular weights and isoelectric points of the deduced protein sequences. TMHMM 2.0 (www.cbs.dtu.dk/services/TMHMM) was used to predict transmembrane domains. JHEH amino acid sequences from other insects were retrieved from NCBI and aligned with the predicted LdJHEH1 using ClustalX (1.83) (Thompson et al. 1997). Phylogenetic trees were generated using the neighbor-joining method in MEGA 5.1 software (Tamura et al. 2007). The reliability of the NJ tree topology was evaluated by bootstrapping a sample of 1,000 replicates.

RNA Isolation and Synthesis of First-Strand cDNA

Total RNA was isolated using a TRizol reagent (Invitrogen) according to the manufacturer’s protocol and subsequently treated with RNase-free DNase I (Promega) to remove any contaminating genomic DNA. RNA concentrations were measured using a spectrophotometer and RNA integrity was checked by analysis on a 1% w/v agarose gel. One microgram of total RNA was reverse transcribed using reverse transcriptase (Takara Bio., Dalian, China).

Quantitative Real-Time PCR Analysis

In the developmental expression analysis, cDNA templates were prepared from eggs, larvae, prepupae, day 5 pupae (P5D) and adults to compare transcription levels of ldjheh1 in different developmental stages. cDNA templates were also derived from the heads, thoraces, and abdomens of gypsy moth larvae on day 2 of the third instar for an initial survey of the tissue expression. For the temporal analysis, cDNA templates were derived from the whole bodies of the third instar larvae at different times (hours) post-ecdysis (I3H0, I3H12, I3H24, I3H36, I3H48, I3H60, I3H72, I3H84, I3H96, I3H108, I3H120) to test the transcription levels of the ldjheh1 gene. For each sample, RNA was extracted from five individuals and repeated in biological triplicate. cDNAs used as templates for qRT-PCR were synthesized using 1 μg of total RNA and 1 μM oligodeoxynucleotidyde primer, diluted ten times with RNase-free water. Primer sequences are listed in Table 1, the efficiency of the PCR (Eff%) and

| Table 1. Primers used in ORF verification, qRT-PCR, and dsRNA synthesis |
|-----------------------------|-----------------------------|
| Experiments                | Primer names and sequences (5′ to 3′) |
| ORF verification           | LdJHEH1F: ACTTAAGATGGGTACGAGAATG |
|                            | LdJHEH1R: ACCAGGCTGGGAAATAGAA |
| qRT-PCR                    | qLdJHEH1F: AGAGATGATCTCCGAGTCTT |
|                            | qLdJHEH1R: TGGATTACGAGAGGATCT|
| dsRNA synthesis            | qActin F: ATGTGATATGATCGAGGCTAG |
|                            | qActin R: GCATGATCTGAGGAGGCA |
|                            | dsRNAJHEH1F: TATAACGCTACTATAGGGGGGCAAGTTCATCCAAAGA |
|                            | dsRNAJHEH1R: TATAACGCTACTATAGGGGGCCATCATATAGGCTGCAATCC |
|                            | dsRNAJHEPPE: TATAACGCTACTATAGGGGGCCAGGCTGAGGAGGAGG |
|                            | dsRNAJHEPP: TATAACGCTACTATAGGGGGCCAGGCTGAGGAGGAGG |

Boldfaced sequences indicate the T7 promoter.
RNAi Analysis

Synthesis of dsRNA (513 bp) based on the full-length ldjheh1 gene was performed in vitro using the MEGAscript T7 High Yield Transcription kit (Ambion). Specific primers were designed with the T7 promoter to amplify the ldjheh1 gene (listed in Table 1). The dsRNA was purified with phenol/chloroform followed by ethanol precipitation. The dsRNA of a green fluorescent protein (GFP) gene was generated using a plRES2-EGFP plasmid as template. The dsRNA was confirmed by electrophoresis on 2% agarose gel. The dsRNA was diluted with RNase-free water to 4 μg/μl and the 3μl dsRNA solution was dripped onto the P. alba × P. glandulosa circular leaves (6 mm in diameter). The treated leaves were dried for 0.5 h on filter paper under airflow, and then placed in plastic cases (3 cm in diameter and 4.5 cm in height). Leaves treated with RNase-free water and the GFP dsRNA were used as control. Each third instar larva was confined to one plastic cases with one of the treated circular leaves. Each replicate was fed on a treated leaf, after feeding completely then kept on normal leaves. Each treatment was repeated 60 times; 30 replicates were used to extract the total RNA and 30 were used to observe the duration of larval stages.

Results

cDNA Cloning and Characterization of the ldjheh1 Gene

One full length cDNA of a putative ldjheh gene was cloned in L. dispar and provisionally designated ldjheh1. The ldjheh1 cDNA consisted of 2101 bp. The ORF length was 1380 bp, which encodes a 459 amino-acid protein, and the predicted molecular weight was 52.64 kDa with the theoretical isoelectric point of 6.87. We predicted the presence of a transmembrane domain using the software TMHMM 2.0, and, as expected, LdJHEH1 contains a transmembrane domain at the N-terminus (Fig. 1).

We analyzed and compared the amino acid sequences of seven JHEH and JHEH-like proteins from other insect species. The N-terminal segments of LdJHEH1 and other four Lepidoptera insects contain two conserved motifs (‘WWG’ and ‘HGWP’), catalytic triad (Asp229, Glu402 and His429) and two tyrosine residues (Tyr299 and Tyr373). The Phe57 and Pro121 conserved in the JHEH of other species were also present in LdJHEH1 (Fig. 2).

Phylogenetic Analysis

A phylogenetic tree was constructed to evaluate the evolutionary relationship of JHEH proteins derived from the cDNAs of 15 insect species (Fig. 3). The highly conserved JHEH proteins formed separate order-based clades for Siphonaptera, Hymenoptera, Lepidoptera, and Diptera. As expected, the JHEH of L. dispar belonged to the Lepidoptera clade (Fig. 3).

Expression of the ldjheh1 Gene

The transcription levels of ldjheh1 in different developmental stages, including eggs, larvae, prepupae, pupae and adults, showed that the ldjheh1 gene is expressed throughout all developmental stages. Expression levels of ldjheh1 in the eggs, prepupae and adults were lower than those in the larvae. The highest peak of ldjheh1 expression occurred at 60 h into the fourth larval instar stage (Fig. 4). The ldjheh1 mRNA was also detected by qRT-PCR in the heads, thoraces and abdomens of gypsy moth larvae on day 2 of the third instar. Expression levels of ldjheh1 mRNA were higher in the thoraces and abdomens than in the heads (Fig. 5). The qRT-PCR analyses were performed every other 12 h to test the temporal expression profile of ldjheh1 cDNA in the third instar larvae. Higher peaks of ldjheh1 expression occurred 12–24 h after ecdysis, mRNAs decreased from 36 to 120 h, and the highest peaks occurred 24 h after ecdysis (Fig. 6).

Effect of dsRNAJHEH on the expression of the ldjheh1 gene

After ingesting dsRNAJHEH, third instar larvae examined after 12, 36, and 48 h showed a decreased ldjheh1 mRNA abundance by a factor of 0.28, 0.68, and 0.88, respectively, however, third instar larvae examined after 24 and 72 h showed an increased ldjheh1 mRNA abundance by a factor of 0.25 and 3.28, respectively, when compared with the correspondent blank control (by the time after ingesting Rnase free water) (Fig. 7). Ingesting dsRNAJHEH slightly delayed the duration of the third instar larval by 0.38 d, compared with the control group of 5.43 d.

Discussion

The physiological function of JH in insect development is strictly regulated through synthesis and degradation pathways (Hammock 1985). In this paper, we cloned a jheh gene in L. dispar. Multiple

![Fig. 1. Transmembrane domains of JHEH in L. dispar. The domains were predicted using TMHMM 2.0.](image-url)
Fig. 2. Alignment of seven insect JHEH or JHEH-like proteins. The N-terminal membrane anchor motif ‘WWG’ is labeled as black triangles. The HGWP motif, catalytic triad (Asp229, Glu402 and His429) and two tyrosine residues (Tyr299 and Tyr373) are labeled as stars. The tyrosine residues (Phe57 and Pro121) are labeled as white triangles (the amino acid position is in LdJHEH). All sequences were downloaded from the NCBI database (www.ncbi.nlm.nih.gov). The sequences used, with the NCBI accession number codes in parentheses, are: Bombyx mori JHEH-like protein (B.mor, NP_0011598617), Plutella xylostella JHEH-like protein (P.xyl, XP_011556635), Amyelois transitella JHEH-like protein (A.tra, XP_013192198), Papilio xuthus JHEH protein (P.xut, KP04294), Manduca sexta JHEH protein (M.sex, AAC47018), Drosophila melanogaster JHEH protein (D.mel, ACV04637).

Fig. 3. Phylogenetic analysis of JHEH or JHEH-like homologs from different insect species based on amino acid sequences. JHEH or JHEH-like proteins originated from one Siphonapteran, Ctenocephalides felis (C.fel); three Hymenopterans, Camponotus floridanus (C.flo), Harpegnathos saltator (H.sal), and Athalia rosae (A.ros); seven Lepidopterans, Lymantria dispar (L.dis), Bombyx mori (B.mor), Plutella xylostella (P.xyl), Amyelois transitella (A.tra), Papilio xuthus (P.xut), Manduca sexta (M.sex), and Danaus plexippus (D.ple); and four Dipterans, Aedes aegypti (A.aeg), Culex quinquefasciatus (C.qui), Drosophila melanogaster (D.mel), and Bactrocera dorsalis (B.dor).
Fig. 4. Developmental expression patterns of the ldjheh1 gene. cDNA templates were derived from the whole bodies of the first (I1H60), second (I2H60), third (I3H60), fourth (I4H60), fifth (I5H60), and sixth (I6H60) instar larvae, prepupae, pupae and adults. The bars represent the 2^ΔΔCt method (±SE) normalized to the geometrical mean of housekeeping gene expression. Different letters above each bar denote significant differences between treatments; P < 0.05, LSD using ANOVA.

Fig. 5. The expression patterns of the ldjheh1 gene in heads, thoraces, and abdomens. cDNA templates were derived from the heads, thoraces and abdomens of gypsy moth larvae on day 2 of the third instar. The bars represent the 2^ΔΔCt method (±SE) normalized to the geometrical mean of housekeeping gene expression. Different letters above each bar denote significant differences between treatments; P < 0.05, LSD using ANOVA.

sequence alignments of JHEH with homologs from other insects suggested that LdjHEH1 contains a conserved ‘WWG’ motif, which has been found to function as an anchor for membrane association (Gilbert et al. 2000). BmJHEH expressed in Sf9 cells was found to be membrane-bound (Zhang et al. 2005). TMHMM 2.0 predicted LdjHEH1 contains a transmembrane domain at the N-terminus (Fig. 2). These data suggest that LdjHEH1 is a membrane-bound protein in L. dispar. BmJHEH, as a typical α/β hydrolase, contains a conserved catalytic triad (Asp227, Glu403, and His430) and two tyrosine residues (Tyr298 and Tyr373), which stabilize and donate protons to the oxygen atom of the epoxide ring (Yamada et al. 2000). The ‘WWG’ motif, catalytic triad and two tyrosine residues were also present in JHEH and five JHEH-r of B. Mori, while only BmJHEH and BmJHEH-r1, -r2, and -r5 show hydrolytic activity with JH (Seino et al. 2010). However, the Phe55, Pro119, and Trp228 were not found in BmJHEH-r3 and BmJHEH-r4, which did not show JHEH activity. Phe55 and Pro119, which conserved in the JHEH of other species reported to have hydrolytic activity on JH, might contribute to proper assembly of the substrate binding pocket. The Trp228 might be an important site for substrate selectivity. The catalytic triad around Asp227 in JHEH of L.dispar was QAGDWG, as was BmJHEH-r1. All of this importance catalytic triads and residues known for JHEH activity were also present in LdjHEH1 (Fig. 2). The enzymatic activities of JHEH in L. dispar were not examined in this study, but the importance catalytic triads and residues in the amino acid sequence of LdjHEH1 suggest that LdjHEH1 may be involved in JH degradation.

The results also showed that ldjheh1 transcription is detectable in all developmental stages. The expression levels of ldjheh1 in the eggs, prepupae and adults were lower than in the larvae (Fig. 4). High transcription levels of jheh have been detected in the Malpighian tubules, fat bodies, midgut and epidermis of L. decemlineata (Lü et al. 2015) and B. mori (Yang et al. 2011). This indicates that JHEH might degrade JH in various tissues (Lü et al. 2015). Midgut, Malpighian tubules and most fat bodies are located in insect abdomens. In day 2 third instar larvae, ldjheh1 expression was highest in the abdomen (Fig. 5).

Temporal patterns of ldjheh1 gene expression in the third instar showed that the ldjheh1 mRNA content significantly increased, by 2.86-fold in 12 h and 4.03-fold in 24 h compared with 0 h, then decreased to about the initial level in 36 and 48 h. Thereafter, the ldjheh1 mRNA content dropped rapidly and remained at relatively low levels until the end of the instar (Fig. 6). The temporal pattern of ldjheh1 expression has high similarity to that of B.mori jheh mRNA in the fat body in the early stage of the fifth (last) instar (Zhang et al. 2005). The same expression profile of jheh was also observed in Trichoplusia ni (L.; Lepidoptera: Noctuidae) (VanHook Harris et al. 1999). In holometabolous insects, high JH titers elicit larval-stage-to-larval-stage molting, whereas a pulse of 20E and a drop in JH during the final larval instar triggers larval-to-pupal metamorphosis (Dubovsky 2005). The ldjheh1 mRNA content rapidly decreased at 36 and 48 h and remained at low levels at 60–120 h in the third instar. These results are compatible with the common idea that decreases in jheh mRNA levels cause high levels of JH, which elicit larval-stage-to-larval-stage molts.

The last step in this study was to detect expression of the ldjheh1 gene after ingesting dsRNA and further, to evaluate the possible effects on larval development at the third larval instar stage in L. dispar. Our results showed that RNAi-mediated knockdown of the ldjheh1 gene significantly decreased the expression levels of ldjheh1. After ingesting dsRNA, the expression did not decrease 72 h post-feeding (Fig. 7) because dsRNA might be degraded, and the duration of
the third instar larval development was slightly delayed by 0.38 d, compared with the Rnase free water treated group of 5.43 d. RNAi of the ldjheh1 gene, using this oral delivery method and this dsRNA concentration, did not affect the survivorship and phenotype of the third instar larval. Feeding of dsRNA as a non-invasive approach is more attractive than hemocoel injection, and furthermore opens the possibility of new methods to control pest through the production of species-specific hairpin RNAs against pests in transgenic plants (Price and Gatehouse 2008). In 24 h starved larvae, dsRNA-degrading activity in the midgut was greatly decreased and could be an important factor for the increased sensitivity to dsRNA (Rodrı́guez-Cabrera et al. 2010). In addition, gene silencing by feeding dsRNA requires high concentrations for success (Terenius et al. 2011). Therefore, low levels of silencing were obtained in L. dispar after ingested dsRNAJHEH, may be caused by the dsRNA-degrading activity in the midgut, and the concentration of dsRNA need added for high silencing.

Acknowledgments

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