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Molecular cloning and characterization of a juvenile hormone esterase gene from brown planthopper, *Nilaparvata lugens*

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ABSTRACT

Juvenile hormone (JH) plays key roles in the regulation of growth, development, diapause and reproduction in insects, and juvenile hormone esterase (JHE) plays an important role in regulating JH titers. We obtained a full-length cDNA encoding JHE in *Nilaparvata lugens* (NlJHE), the first JHE gene cloned from the hemipteran insects. The deduced protein sequence of *Nljhe* contains the five conserved motifs identified in JHEs of other insect species, including a consensus GQSAG motif that is required for the enzymatic activity of JHE proteins. *Nljhe* showed high amino acid similarities with *Athalia rosae* JHE (40%) and *Apis mellifera* JHE (39%). Recombinant NlJHE protein expressed in the baculovirus expression system hydrolyzed [^{3}H] JH III at high activity and yielded the specificity constants ($k_{cat}/K_M = 4.28 \times 10^6\ M^{-1}\ s^{-1}$) close to those of the validated JHEs from other insect species, indicating that *Nljhe* cDNA encodes a functional JH esterase. The *Nljhe* transcript was expressed mainly in the fat body and the expression level reached a peak at 48 h after ecdysis of the 5th instar nymphs. In the 5th instar, macropterous insects showed significantly higher *Nljhe* mRNA levels and JHE activities, but much lower JH III levels, than those detected in the brachypterous insects soon after ecdysis and at 48 h after ecdysis. These data suggest that NlJHE might play important roles in regulation of JH levels and wing form differentiation.

1. Introduction

Juvenile hormone (JH) plays major roles in the control of growth, development, metamorphosis, diapause and reproduction in insects (Riddiford et al., 2003). The regulation of JH titers is thus critical in the entire life of the insect. One key event is the clearing of JH that generally precedes the molt from the last larval stage to the pupal stage of holometabolous insects (Campbell et al., 2001). The very low JH titer at this time is generally achieved by the combined effect of reduced JH synthesis and scavenging by JH degrading enzymes (Roe and Venkatesh, 1990). Neurosecretory cells in the brain release allatotropic and allatostatic factors that regulate the synthesis and secretion of JH (Stay, 2000). Additionally, some important enzymes also play key roles in the synthesis and regulation of JH, such as JH methyl transferase and JH epoxidase (for a review, see Bellés et al., 2005). Degradation of JH in the tissues and in the hemolymph is another major way in which hormone titers are regulated, and JH esterase (JHE) has been thought to play key roles in the metabolism of JH (de Kort and Granger, 1996).

Some progress had been achieved in insect JHEs, such as JHE protein purification, JHE cDNAs cloning, recombinant JHE protein studies and JHE protein structure. JHE proteins have been purified from *Heliothis virescens* (Hanzlik et al., 1989), *Manduca sexta* (Venkatesh et al., 1990), *Trichoplusia ni* (Hanzlik and Hammock, 1987), *Drosophila melanogaster* (Campbell et al., 1998), *Tenebrio moliter* (Thomas et al., 2000) and *Bombyx mori* (Shiotsuki et al., 2000). Cloning of JHE cDNAs has been performed for two dipteran species *D. melanogaster* (Campbell et al., 2001) and *Aedes aegypti* (Bai et al., 2007), for two coleopteran species *T. moliter* (Hinton and Hammock, 2003b) and *Psacothea hilaris* (Munyiri and Ishikawa, 2007), and for several lepidopteran species *H. virescens* (Harshman et al., 1994), *T. ni* (Jones et al., 1994), *Choristoneura fumiferana* (Feng et al., 1999), *M. sexta* (Hinton and Hammock, 2001) and *B. mori* (Hirai et al., 2002). Recombinant JHEs have been expressed and biochemically characterized in *H. virescens* (Harshman et al., 1994), *C. fumiferana* (Feng et al., 1999), *B. mori* (Hirai et al., 2002), *M. sexta* (Hinton and Hammock, 2003a), *T. molitor* (Hinton and Hammock, 2003b) and *A. aegypti* (Bai et al., 2007). In order to perform detailed studies on the interaction between JHE and its substrates or inhibitors, the crystal structure of the JHE protein from *M. sexta* has

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been solved, which showed that JHE contains a long hydrophobic binding pocket with solvent-inaccessible catalytic triad (Wogulis et al., 2006). However, no information about JHE gene or protein from hemipteran insects has been obtained.

The brown planthopper (*Nilaparvata lugens*), a hemipteran insect, is a major rice pest in many parts of Asia. Wing polymorphism in this species is known to be a common and ecologically important trait. Although the genetic basis of wing polymorphism in insects generally is not well understood, it is presumed to be under polygenic control in this group (Denno and Roderick, 1990). It is assumed that these genes determine the level of juvenile hormone so that it is present above or below a certain threshold, leading to the production of short or long wing forms, respectively (Roff, 1986). In 4th and 5th instar nymphs of *N. lugens*, the brachypterous individuals (short wing) showed significantly higher JH titers and lower JHE activity than the macropterous (long wing) (Dai et al., 2001). Application of exogenous chemicals, such as JH, its agonists and antagonists, could regulate JH titers and JHE activity in *N. lugens*, which resulted in the production of short or long wing forms in different percentages (Ayoade et al., 1999). Here, as part of the study on the basic mechanism of wing polymorphism in *N. lugens*, we report the cloning of full-length cDNA encoding a functional JHE, tissue and developmental expression of the mRNA and its response to the exogenous JH application. Expression levels for both macropterous and brachypterous nymphs have been determined, together with JHE activity and JH titer.

2. Materials and methods

2.1. Experimental insects

Insects, *N. lugens*, were kept in laboratory cages at 25 ± 1 °C, humidity 70–80% and 16 h light/8 h dark photoperiod. The developmental stages were synchronized at each larval molt. Fat body, midgut, and other tissues were dissected from the 5th instar larvae in phosphate buffered saline (PBS) treated with 0.1% diethylpyrocarbonate (DEPC) and stored at −70 °C prior to use.

2.2. Amplification of a JHE cDNA fragment

Total RNA was isolated from 10 individuals of the 5th instar female using a Trizol kit (Invitrogen). Synthesis of first-strand cDNAs was carried out according to the reverse transcriptase XL (AMV) (TaKaRa) protocol with oligo dT_{18}. The first strand cDNA (1 μL) was used as a template for PCR. Degenerate primers, BP1 (ATHCCNTAYGCNAARCCNCC) and BP2 (GCNSCNCCNGCNYWY TGNCC), were designed from the conserved regions of insect JHEs, which were IPYAKPP (BP1) and GQSAGG(A)A (BP2) respectively. The components of PCR were PCR reaction buffer containing 0.1 mM dNTP, 5 μM each primer, and 1.0 unit of Ex-Taq DNA polymerase (Promega) in a total volume of 20 μL. Thermal cycling conditions were 95 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 50 °C for 1 min and 72 °C for 1 min. The last cycle was followed by final extension at 72 °C for 10 min. The amplified product was separated onto agarose gel and purified using the Wizard PCR Preps DNA Purification System (Promega). Purified DNA was ligated into the pGEM-T easy vector (Promega) and several independent subclones were sequenced from both directions. The full-length cDNA was obtained by the rapid amplification of cDNA ends (RACE) according to the Smart Race kit (Clontech) protocol with gene-specific primers (GSPs) for 5′-RACE (5′-GSP1: CCAAATACCTGTAGATGCTAAGC; 5′-GSP2: CTGTCACATTCCTAGGTTCACTCC) and 3′-RACE (3′-GSP1: GTTGTTGTCAACTATAGACTCG; 3′-GSP2: GGAGACTCGAATATGGTGTCATTGG).

2.3. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

mRNA levels were measured by qRT-PCR using the One Step SYBR PrimeScript RT-PCR Kit (Takara). Total RNAs were treated by DNase I (Sigma). qRT-PCR was performed in a 25 μL total reaction volume containing 5 ng of total RNA, 0.5 μL primer mix containing 10 μM each of forward and reverse gene specific primers, 0.5 μL of Ex TaqTM HS (5 U/μL), 0.5 μL of PrimeScript RT Enzyme Mix, 12.5 μL of 2× One Step SYBR RT-PCR Buffer and 8.5 μL of H_2O. Two kinds of negative controls were set up: non-template reactions (replacing total RNA by H_2O) and minus reverse transcriptase controls (replacing PrimeScript RT Enzyme Mix by H_2O). qRT-PCR was done with the following cycling regime: initial incubation of 42 °C for 5 min and 95 °C for 10 s; 40 cycles of 95 °C for 5 s, 60 °C for 20 s and 72 °C for 15 s. Standard curves were obtained using a ten-fold serial dilution of pooled total RNAs from 20 individuals. β-Actin (EU179846) was used as an internal control, which had been recognized as a suitable normalization gene by Northern blotting test (data not shown). mRNA levels of *Nljhe* were quantified in relation to the expression of β-actin. The primer pair of each gene was designed to amplify about 200 bp PCR products, which were verified by nucleotide sequencing. In order to avoid genomic DNA contamination, the specific primers for JHE gene were designed to span an intron region (1154 bp). Only data that showed good efficiency (≥85%) and correlation coefficient (≥95%) were included in the analysis. Means and standard errors for each time point were obtained from the average of three independent sample sets. Gene specific primers for JHE and β-actin were listed as: JHE-F: AAGTAACTGGCAGATTCAACC; JHE-R: CTCGAATAGATGTGCTGCAGG; β-F: TGGACTTCGA GCAGGAAATGG; β-R: ACGTCGCACTTCATGATCGAG.

2.4. JH titer determination

Tang et al. (2001) used GC–MS to show that JH III was the main JH in *N. lugens*, so JH III levels were determined as the JH titers here according to the GC–MS method. The GC–MS system consisted of a Hewlett Packard HP6890 series II gas chromatograph and a mass selective detector (model 5973MS). 10 mg (about 6 individuals of the 5th instar female) *N. lugens* whole bodies were dried in Modulyod-230 Freeze Dryer (Thermo Electron) and were homogenized in 0.5 mL Hexane. The contents were centrifuged at 13,000 × *g* and 4 °C for 10 min. The supernatant was dried using a stream of N_2 gas and diluted to 25 μL in hexane. GC operating conditions: column HP-5, 25 m × 0.2 mm i.d., film thickness 0.2 μm; column temperature programmed from 120 °C (isothermal for 2 min) to 230 °C (15 °C/min); carrier gas helium, flow rate 40 mL/min; injector temperature 250 °C; volume injected 1 μL. The standard JH III was purchased from Sigma–Aldrich.

2.5. JHE activity determination

[10-^{3}H (N)]-JH III (17.5 Ci/mmol) was purchased from PerkinElmer Life Sciences. Juvenile hormone esterase assay was performed by the partition method of Hammock and Sparks (1977) as modified by Lefevere (1989). The substrate [^{3}H] JH III was dissolved in ethanol and stored at −20 °C prior to assay. 30 mg (about 18 individuals of the 5th instar female) *N. lugens* whole bodies were homogenized in 1 mL 0.1 mol/L phosphate buffer (PH 7.0) at 0 °C. The contents were centrifuged at 6000 × *g* and 4 °C for 10 min and the supernatant was collected for JHE solution. 100 μL aliquot of whole body supernatant were incubated with [^{3}H] JH III (130,000 DPM) and unlabeled JH III (final JH III concentration was 5×10^{-4} M) for 30 min at 30 °C. The reaction was terminated by