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Expression pattern of enzymes related to juvenile hormone metabolism in the silkworm, *Bombyx mori* L.

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Abstract The physiological balance of juvenile hormone (JH) in insects depends on its biosynthesis and degradation pathway. Three key enzymes namely, juvenile hormone esterase (JHE), juvenile hormone epoxide hydrolase (JHEH) and juvenile hormone diol kinase (JHDK) are required for degradation in insects. Our present results showed that JHE and JHEH exhibited expression in almost all the tissues. This indicated that JHE and JHEH might degrade JH simultaneously. In addition, the highest levels of JHDK were observed in the midgut, with trace level being found in the malpighian tubule and haemocytes. Since the midgut is a digestive organ and not a JH target, it was hypothesized that both JHE and JHEH hydrolyzed JH to JH diol (JHd) which was then transported to midgut and hydrolyzed further by JHDK, to be finally excreted out of the body. Also the expression studies on JH degradation enzymes in different tissues and stages indicated that the activities of the three enzymes are specific and coincident with the JH functions in silkworm, *Bombyx mori* L.

Keywords Juvenile hormone · JHE · JHEH · JHDK · Metabolism · Silkworm *Bombyx mori* L

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Introduction

Molting hormone (MH) and juvenile hormone (JH) plays an important role in the regulation of insect growth and development. JH is synthesized primarily by the corpora allata and then released into the hemolymph to prevent metamorphosis and to regulate reproductive maturation. Its presence throughout late embryonic development ensures that ecdysteroid-induced molting yields another larval stage, thereby allowing for continued and systematic growth. For metamorphosis to initiate, the ecdysteroids must be expressed in the absence of JH. After this initial ecdysteroid action, JH may then reappear to regulate the details of metamorphosis [1, 2]. Thus, a systematic larval growth requires the JH titer in the hemolymph to be precisely regulated through its timely biosynthesis and degradation. Three metabolizing enzymes were identified in the pathway of JH degradation, named JH esterase (JHE) [3], JH epoxide hydrolase (JHEH) [4, 5] and JH diol kinase (JHDK) [6–8]. JHE (EC 3.1.1.1) is a member of the carboxylesterase family which hydrolyse the methyl ester moiety of JH to form JH acid (JHa), then JHEH (EC 3.3.2.3) hydrolyses its epoxide moiety to form JH diol (JHd), and finally the JHE degrades the JHd and the JHEH degrades the JHa to form JH acid diol (JHad). The JHDK metabolizes JH-d to the JH diol phosphate [9].

JHE is the main degradative enzyme of JH degradation pathway. It was, which isolated from insects such as *Bombyx mor*i [3], *Choristoneura fumiferana* [10], *Heliothis virescens* [11], *Manduca sexta* [12] etc. JH titer in the hemolymph of insects depends on the activity of JHE. Inhibition of JHE activity resulted in giant larvae and delayed morphogenesis in *M. sexta* [13]. Further, Baculovirus-mediated silencing in *Heliothis virescens* showed that the JH titer in the hemolymph was greatly reduced and

caused an aberrant morphogenesis of final-instar larvae [14]. Over expression of JHE in transgenic silkworms resulted in precocious metamorphosis after the third instar, two instar earlier than the wild-type worms [15]. These results indicated that JHE regulated the JH titer in hemolymph and played an important role in the growth and development of insects.

Several JHEH were cloned from insects such as *M. sexta* [16], *B. mori* [5], *Trichoplusia ni* [17], *Ctenocephalides felis* [18]. The JHEH was expressed in *M. sexta* through baculovirus expression system (BEVS), and compared to its wild type isolated from eggs of *M. sexta* [4]. The catalytic mechanism of JHEH in *M.* sexta might be similar to the mammalian epoxide hydrolases [19]. The JHEH gene cloned from *B. mori* was expressed in Sf9 cell using BEVS. This recombinant Bm-JHEH was membrane-bounded and showed high levels of enzyme activity [5].

JHDK is active as a homodimer with a subunit molecular mass of 20 kDa via a sequential Bi mechanism. *M. sexta* JHDK was found to have many similarities to GTP-binding proteins, because ATP and GTP were shown to be phosphate donors [6, 7]. JHDK is similar to G-proteins with three conserved sequence elements involved in purine nucleotide binding and contain two pairs of EF-hand motifs. Substrate docking to three dimensional models of JHDK has shown that the three conserved nucleotide-binding elements surround the putative substrate-binding site and align with conserved sequence elements of p21Ras and adenylate kinase (Maxwell et al. 2002). The recombinant JHDK from *B. mori* could catalyzed the conversion of 10S-JH diol into JH diol phosphate [8].

The physiological balance of juvenile hormone depends on its synthesis and degradation pathway. The activities of enzymes related to JH in the larvae of silkworm *B. mori* are important to understand the functional regulation of this hormone for silkworm growth and development. Therefore, in the present study, we reported the expression pattern of these three genes (JHE, JHEH, JHDK) in the early stages of larval development and in different tissues of *B. mori* L. in order to find out the relationship between these three genes, and relationship between the expression levels and JH degradation in insects.

Materials and methods

Insect

The silkworm, *B. mori* was inbred in our lab. Hybrid silkworm larvae (commercial name: Qingsong × Haoyue) were used for this study. Silkworms were reared on fresh mulberry leaves at 25 ± 2°C, 65 ± 5% relative humidity, under a 12 h light/12 h dark photoperiod.

DNA extraction and primer design

The cDNA sequence of JHE, JHEH and JHDK of silkworm were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/). The GenBank accession numbers of these genes were AF287267, AY377854 and AY363308, respectively. Three pairs of primers were designed based on their sequences: JHE-F: 5′-CAGAGCCTTTGGAGGTAATC-3′, JHE-R: 5′-GACTGGGAGAAGATAGGAGATG-3′; JHEH-F: 5′-CCTGGTTATGGCTTTTCCG-3′, JHEH-R: 5′-GCTCCCCAATCACCTCCTT-3′; JHDK-F: 5′-CGCAGAACATCGCTAAAC-3′, JHDK-R: 5′-CCATTCGTCCTGAGTAACC-3′. The constant expressed gene, RP49, was used as the internal control. Primers RP49-F: 5′-CAGGCGGTTCAAGGGTCAA-3′ and RP49-R: 5′-GCTGGGCTCTTTCCACGAT-3′, were designed on the basis of the sequence of the *B. mori* RP49 gene (Accession number: AY769302).

RT-PCR

The distribution of JHE, JHEH and JHDK in various tissues of the silkworm, *B. mori* was investigated. Total RNA was isolated from the midgut, fat bodies, silk gland, genitalia (ovary and testis), malpighian tubules, head, epidermis and hemocyte of the third day of fifth-instar larvae using RNeasy Mini Kit (QIAGEN) according to the user manual. 2 ug total RNA was used as a template in the first-strand cDNA synthesis. PCR was performed on the resulting cDNAs using the four pairs of primers mentioned above. PCR reaction was carried out with Taq polymerase for 35 amplification cycles (94°C/30 s, 60°C/30 s, and 72°C/30 s). PCR product was examined by electrophoresis in 1% agarose gel with ethidium bromide staining.

Quantitative real-time PCR and data analysis

Total RNA was extracted from the whole larvae of the second day of first-instar, first molter, the second day of second-instar, second molter, the second day of third-instar, third molter with Rneasy Mini Kit (QIAGEN). 2 μg of total RNA was used as a template in the first-strand cDNA synthesis of each enzyme. PCR was performed on the resulting cDNAs using four pairs of primers mentioned above. The constant expressed gene, RP49, was used as the internal control. This protocol was also used in the study of the expression level in different tissues. Quantitative real-time PCR (QRT-PCR) information was obtained using ABI7300 (Ambion, USA) for thermal cycling, real-time fluorescence detection and subsequent analysis. The two-step amplification protocol consisted of a 30 s at 95°C followed by target amplification via 40 cycles at 94°C for 5 s, 60°C for 31 s. After PCR, the absence of unwanted

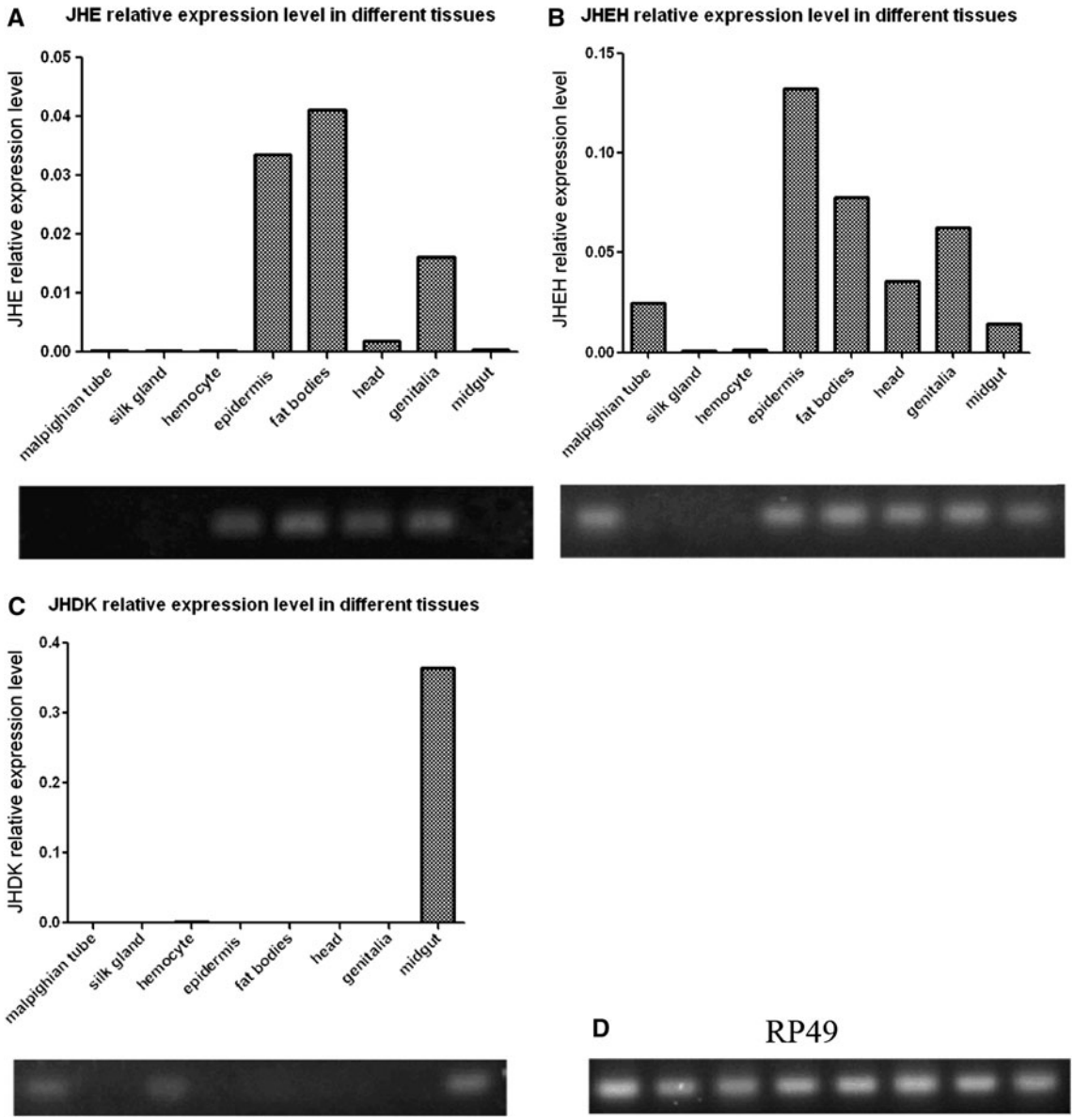

Fig. 1 Distribution and expression levels of JHE, JHEH and JHDK in differernt tissues. **a** distribution and expression levels of JHE in different tissues; **b** distribution and expression levels of JHEH in different tissues; **c** distribution and expression levels of JHDK in different tissues; **d** internal control and constant expressed gene, RP49. *Upper* is the expression levels in different tissues studied by quantitative real-time PCR. *Bottom* is the agarose gel electrophoresis of the RT-PCR products in different tissues

by-products was confirmed by automated melting curve analysis and agarose gel electrophoresis of the products. The transcript levels of the target fragment were normalized with RP49 transcript levels in the same samples. The comparative *C*t method is also known as the $2^{-\Delta Ct}$ method, where ΔCt is the *C*t value for any sample normalized to the RP49. Another $2^{-\Delta\Delta Ct}$ method also used, where $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$. Here, ΔCt_{sample} is the *C*t value for any sample normalized to the RP49 and $\Delta Ct_{reference}$ is the *C*t value for the calibrator also normalized to the RP49.

Result

RT-PCR and expression levels of JHE, JHEH and JHDK in different tissues

The expression levels of JHE, JHEH and JHDK in different tissues of fifth-instar larvae of *B. mori*, namely malpighian tubules, silk glands, hemocytes, epidermis, fat body, head, genitalia and midgut, were studied by quantitative real-time PCR. The results presented in Fig. 1 indicated that the JHE is present in the epidermis, fat body, head and

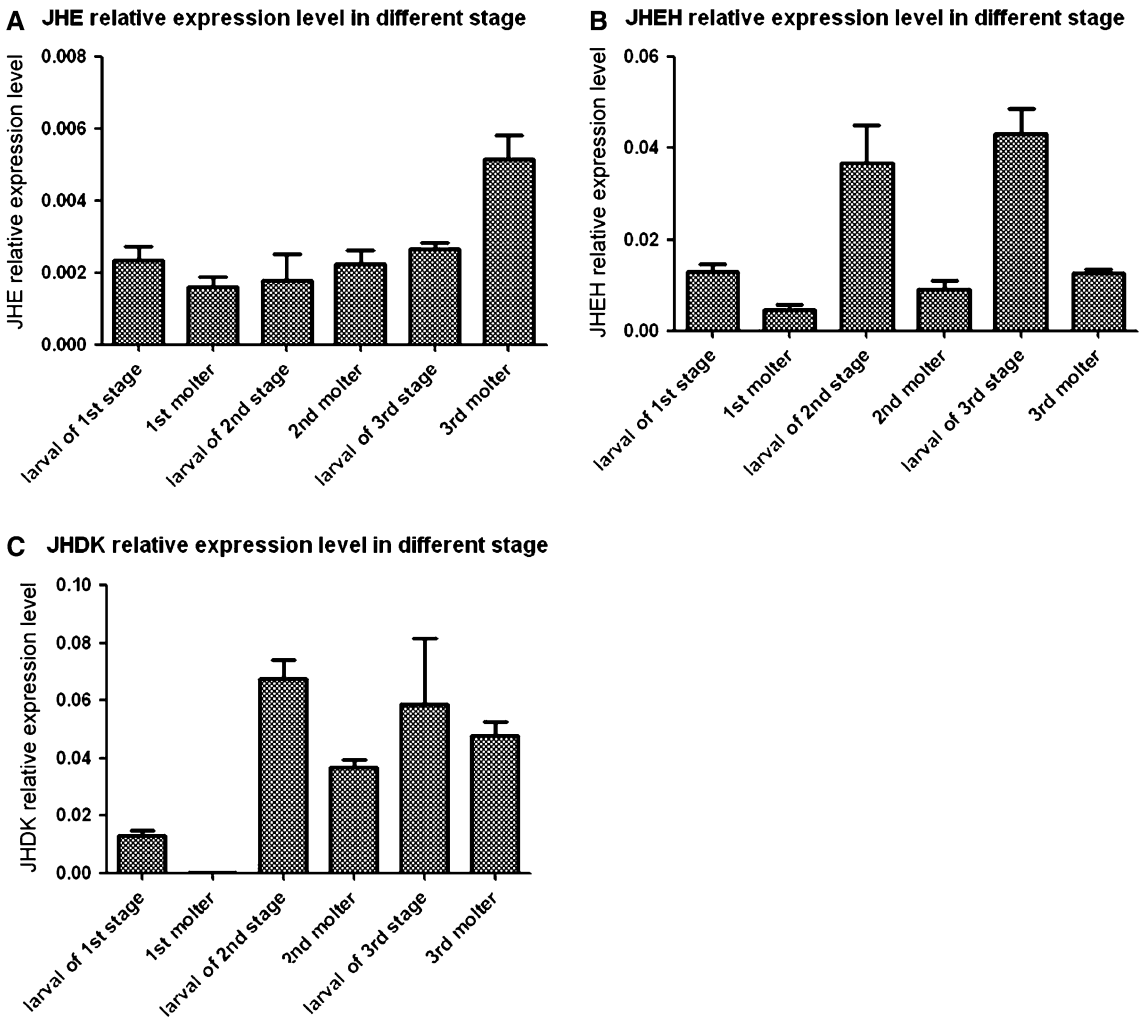

Fig. 2 JHE, JHEH and JHDK mRNA transcript levels in different stages. **a** JHE relative expression level. **b** JHEH relative expression level. **c** JHDK relative expression level. Real-time quantitative PCR data were evaluated using $2^{-\Delta Ct}$ method to calculate the comparative expression level

genitalia; JHEH is expressed in almost all tissues, except in the silk gland and hemocytes; while JHDK is mainly expressed in midgut and at relatively lower levels in the malpighian tubule and hemocytes.

Expression levels of JHE, JHEH and JHDK at different stages

The specificity of quantitative RT-PCR results was assessed by dissociation curve analysis, the melting curves showed specific product (data not shown). The Fig. 2a indicated that the expression level of JHE has not shown a higher difference at earlier stage until the third molter. Figure 2b showed that the expression level of JHEH was higher at the larval stages especially at the 2nd and 3rd stages, as compared with the molter stage. The graph for the expression pattern of JHDK was similar to JHEH (Fig. 2c), with the exception that no JHDK expression was detected at the 1st molter. Also, the expression levels of JHDK were higher in comparison to JHEH.

Comparative expression of JHE, JHEH and JHDK levels at different stages

The expression levels of JHE, JHEH and JHDK at different stages were compared with each other to study the relationship between them. From the results presented in Fig. 3, the expression level of JHE was relatively lower at all stages when compared to JHEH and JHDK. For JHEH, the expression levels were lower than JHDK at all stages, except the first larval stage where they were almost same or the first molter stage where no JHDK was detected. The JHDK expression was not prominent at the 1st larval or 1st molter stage but increased thereafter from the 2nd larval stage, indicating the importance of its expression in larval growth at these stages.

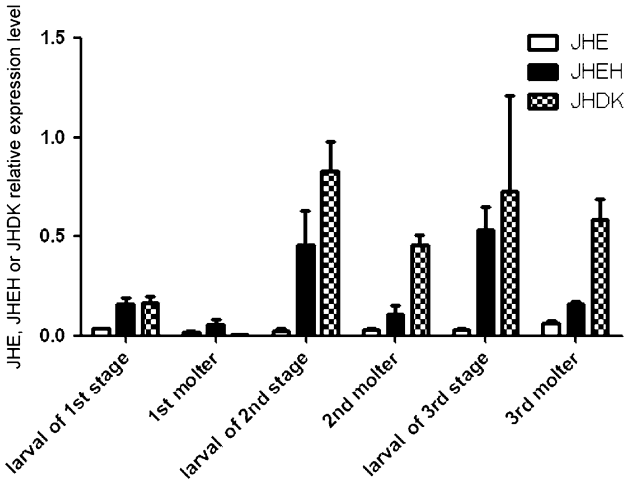

Fig. 3 Comparative expression levels of JHE, JHEH and JHDK to each other. Real-time quantitative PCR data were evaluated using the $2^{-\Delta\Delta Ct}$ method to calculate the comparative expression level

Discussion

Juvenile hormone (JH) plays a major role in insect development. JH acts in conjunction with ecdysone, also called molting hormone (MH), to regulate cellular commitment to pupation and adult development. JH allows larval molting in response to ecdysteroids but prevents the switching of gene expression necessary for metamorphosis. Its presence throughout late embryonic development and larval life ensures that ecdysteroid-induced molting yields another larval stage and thereby allows for continued growth of the insect larvae [1]. In order for metamorphosis to be initiated, the ecdysteroids must act in the absence of JH. After this initial ecdysteroid action, JH may then reappear to regulate the details of metamorphosis. Coincident with above physiological function, juvenile hormone is biosynthesized and secreted periodically from the corpora allata controlled by the brain, and often stored in the fat body, which then transfers it to its target tissues such as epidermis and genitalia through haemolymph. The titer of JH changes during the various larval developmental stages with higher levels being detected in the early instars. Then as the JH degradative enzymes become active, its activity reduces and its secretion is finally stopped towards the end of the larval period. Thus, the precise expression of JH biosynthesis and degradative enzymes in different tissues helps to maintain the physiological balance of hormone in insect body.

Different studies have implicated the three enzymes JHE, JHEH and JHDK to be involved in the degradation of JH. The Fig. 1a showed that JHE was mainly expressed in the epidermis, fat body, head and genitalia, which is similar to previous results [3]. The high expressions of JHE and JHEH observed in above tissues implied that these two enzymes are essential for regulating the JH level through degradation. In contrast, the expression of JHDK occurred only in the midgut with minute levels also being detected in malpighian tubule and hemocyte (Fig. 1c). The result presents a complex problem in that the midgut is a digestive organ and is not considered a target for JH mediated action or its storage for secretive purposes. However, it can be clarified, if we hypothesize that the JHd obtained from JHEH and JHE mediated hydrolysis of JH was transported to midgut and further hydrolyzed by JHDK which is then excreted out of the body.

In the fourth and fifth instar, the transcript of JHE in the fat body was changed dynamically, which was coincident with the JH titer pattern in vivo [3]. But, our result shows that the transcript of JHE was not parallel to the JH titer during the early stages (Fig. 2a). This may be the potential reason that JH is not critical for normal development of larvae in the early instars because of the fundamental difference between the early stage and later stage [15].

In conclusion, the physiological balance of JH in silkworm depends on its synthesis, timely secretion and degradation pathway. The activities of enzymes related to JH metabolism in the larvae of silkworm *B. mori* are important to understand the functional regulation of this hormone for silkworm growth and development. The present study investigated the expression patterns of JH degradation-related enzymes such as JHE, JHEH and JHDK. The results implied that the JH degradation is specific in different tissues and stages, which coincides with the JH functions.

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