

Identification and partial characterization of juvenile hormone esterase from cotton pest *Dysdercus cingulatus*

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Juvenile hormone esterase (JHE), a selective enzyme that hydrolyzes the methyl ester of insect juvenile hormone plays an important role in regulating metamorphosis in nymphs as well as reproduction in adults. Studies on JH degradation provide insight into the possibilities of physiological disruption in the insects. In the present study, the JH degrading enzyme, JHE from the cotton pest *Dysdercus cingulatus* (Heteroptera) is characterized. Electrophoretic analysis of haemolymph during various developmental stages showed the JHE bands prominent only on the final day of 5th instar nymph, and the esterase substrate specificity confirmed the presence of JHE isoforms. In an attempt to clone cDNA of JHE gene from the final instar nymphs, mRNA isolated from fat bodies was coupled with JHE gene-specific primers and the cDNA was synthesized using RT-PCR. The PCR amplified cDNA showed the presence of JHE isoforms in *D. cingulatus*.

Keywords: *Dysdercus cingulatus*, Juvenile Hormone Esterase, isoforms, PCR, cDNA

Juvenile hormone (JH) produced in the *Corpora allata* (CA) of insect plays a pivotal role in the endocrine control of embryogenesis, molting, metamorphosis and reproduction. Metamorphosis being the most important among these adaptations, accurately timed changes of JH titer is essential for unfolding the cascade of events in insect development and reproduction. Juvenile hormone esterase (JHE), a member of the α/β -hydrolase family of enzymes is the key enzyme responsible for the formation of JH acid metabolite^{1,2}. The scavenging role of JHE is responsible for changes brought in the rate of degradation at critical times of insect development and metamorphosis. JHE has provided a suspect demonstration of feasibility of newer strategies for

pest management using recombinant viruses containing important endocrine genes for disrupting the endocrine targets. The rationale for this was to execute an anti-JH effect in early larval instars in order to disrupt normal development³.

JHE has been purified and characterized from several lepidopteran insect species like *Trichoplusia ni*⁴, *Heliothis virescens*⁵, *Manduca sexta*⁶, *Lymantria dispar*⁷, *Tenebrio molitor*⁸, *Bombyx mori*⁹, *Choristoneura fumiferana*¹⁰ and *Helicoverpa zea*¹¹. It has also been characterized from other insect orders such as, Coleoptera^{12,13}, Dictyoptera¹⁴, Diptera¹⁵⁻¹⁷ and Orthoptera¹⁸. Full or partial cDNA sequence of JHE has been made available from *H. virescens*^{5,19}, *L. decemlineata*⁷, *C. fumiferana*¹⁰, *M. sexta*²⁰, *T. molitor*²¹, *B. mori*²², *Apis mellifera*²³ and *Psacotha hilaris*²⁴. However, relatively little is known about similar or divergent mechanisms in the insect order, Heteroptera.

In the present study attempts have been made to characterize JHE from an insect evolutionarily distant from Lepidoptera. Accordingly, investigations have been carried out to explore the JHE gene in a heteropteran cotton pest *Dysdercus cingulatus* at the molecular level.

Materials and Methods

Dysdercus cingulatus (Heteroptera: Pyrrhocoridae) adults were reared in our laboratory under controlled conditions (temp 28±31°C, L/D cycle 12:12 and rh 90±3) and fed on soaked cotton seeds. Eggs laid in clusters among cotton seeds were collected at various time intervals, so that nymphs of known age-group were available for the present study.

Collection of haemolymph

Haemolymph was collected by cutting the antennae and draining hemolymph into an eppendorf tube. Tyrosinase activity was inhibited by the addition of a few crystals of phenylthiourea. The sample was then centrifuged at 10,000 rpm for 10 min at 4°C in a refrigerated centrifuge (Universal 16 R, Hettich, Zentrifugen, Germany) to remove the hemocytes and other debris. Supernatant was mixed with double the volume of sample buffer.

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Tris-glycine SDS-PAGE

The electrophoretic protein profile of haemolymph was determined by one-dimensional SDS-PAGE carried out under discontinuous and dissociating buffer systems²⁵. Gel electrophoresis was done using a vertical slab gel mini-electrophoresis unit (Genei, Bangalore) attached to the power supply (LKB-2297 Macrodrive). One mm thick stacking gel (0.5 M Tris HCl, pH 6.8) was laid over the resolving gel (1.5 M Tris HCl, pH 8.8). Protein samples were mixed with sample buffer [50 mM Tris-HCl, pH 6.8, 2 % (w/v) SDS, 10% (v/v) glycerol, 0.1% bromophenol blue and 5% 2-mercaptoethanol]. Standard molecular markers were also run along with the samples. Electrophoresis of the protein was carried out initially at 60V till the sample entered the resolving gel and at 120V till the end of the run.

Esterase substrate selectivity

Non-denaturing (native)-PAGE was used to detect esterase activity in the gel by staining. After electrophoresis, the gel was incubated in 50 ml of filtered 0.05 % fast blue RR salt in 0.05 M sodium phosphate buffer (pH 7.4) for 5 min at room temperature; 100 µl of 75mM 1-NA in ethanol was then added and the gel incubated at room temperature until well stained⁹.

RNA isolation

Tissue sample was homogenized (1 ml/100 mg) in RNA isolation buffer (4 M Guanidiumthiocyanate, 25 mM sodium citrate (pH 7.0) 0.5 % sarcosyl and 100 mM β-mercaptoethanol). To the homogenate, 100 µl of 2 M Sodium acetate, 1ml of 80 % water-saturated phenol, and 200 µl chloroform: isoamylalcohol (49:1) were added. After incubation on ice for 15 min, the sample was centrifuged. To aqueous phase, equal volume of ice-cold isopropanol was added and kept at -20°C for 1 h. The RNA was precipitated, washed with 80 % ethanol, dried briefly in vacuum and dissolved in sterile DEPC-treated MQ water. The amount of RNA was quantified spectrophotometrically²⁶.

Isolation and purification of poly (A+) RNA

The column was packed with Oligo dT cellulose powder swollen in 1 N NaOH, rinsed with water, equilibrated the column with loading buffer (0.5 M NaCl, 10 mM Tris Cl pH 7.5, 1 mM EDTA, 0.1 % SDS). RNA was heat-denatured at 70°C for 10 min, quick-chilled on ice; the salt concentration was adjusted to 0.5 M using NaCl and loaded on to the

column. The column was washed with loading buffer rinsed with middle wash buffer (0.15 M NaCl, 10 mM Tris Cl pH - 7.5, 1 mM EDTA, 0.1% SDS), and the RNA was eluted into a fresh tube with elution buffer (2 mM EDTA, 0.1% SDS). The eluted RNA was precipitated by adjusting the salt concentration to 0.3 M sodium acetate and precipitated with ethanol at 4°C. Pellet was dried briefly and then redissolved in 20 µl of TE buffer²⁷ (10 mM Tris Hcl, 1 mM EDTA pH 8.0).

Primers used for cDNA cloning

P23 RT 5' NACGATTCTTGAGTAIACAGTC 3' (gene specific primer for reverse transcriptase reaction. It was based on the JHE peptide sequence of C-terminal fragment VYQNVK of peptide 23 of *M. sexta*²⁰). N=A+C+G+T I=Inosine. LDJHE F (forward primer): 5'GATGGGTTGTTTCGAAGT TAA-3' and LDJHE R (reverse primer): 5'CAT ATCAGTAAGTGGGCCGAC-3' were used. Forward and reverse primers were selected on phylogenetic homology shown by *Leptinotarsa decemlineata*¹³ to *D. cingulatus* than any other known JHE sequences so far deduced. Primers used were synthesized by Sigma, Bangalore.

Synthesis of first strand cDNA using RT-PCR

mRNA was hybridized with 10 pmol of P23 RT Oligonucleotide in TE buffer (10 mM Tris -HCl, 1mM EDTA pH-8.0) in a total volume of 5 µl. The mixture was then mixed with Stratascript reverse transcriptase buffer (50 mM Tris-HCl pH- 8.3, 75 mM KCl, 3 mM MgCl₂, 2 µM DTT), 40 mM dNTP mix and made up the volume to 9.5 µl using RNAase free water. The reaction was incubated at 65°C for 5min, quick-chilled on ice, kept at 37°C for 5 min for the primer to anneal to mRNA. Then 0.5 µl of Strata script reverse transcriptase (20 U/µl) was added. Thereafter, the tubes were placed on a temperature-controlled block at 42°C and the reaction was incubated for 45 min for reverse transcriptase reaction and then brought down to 4°C.

PCR with degenerate primers

An aliquot of first strand cDNA was used for PCR amplification in a 50 µl reaction mix. The components of PCR reaction were 5 µl of 10 × PCR buffer, 1 µl of dNTP mix (40 mM), 1 µl of forward primer LDJHE F (10 pmol), 1 µl of reverse primer LDJHE R (10 pmol), and 1 µl of *pfu turbo* DNA polymerase (2.5 U/µl). The final volume was adjusted to 50 µl using RNAase free water. Thermo cycling

parameters were: initial denaturation of 95°C for 1 min, followed by 35 cycles of 95°C for 30, 55°C for 30, 68°C for 3 min. The last cycle was followed by final extension at 68°C for 10 min.

Results

Molecular mass determination

One band corresponding to molecular mass 66 kDa and another of 50 kDa were present in traces throughout 3rd, 4th and 5th instar (0-day old) but were prominent in 6-day old 5th instar nymph. The same bands again became feeble in 0-day old adult suggesting the possibility of this being the esterase band (Fig. 1)

Esterase substrate selectivity in haemolymph

Haemolymph esterase activity of 5th instar nymph was analyzed on native-PAGE. Two bands were observed showing esterase isoform pattern. These bands correspond to 66 and 50 kDa protein, as evident from the electrophoregram (Fig. 1). Only these bands showed positive reaction with substrate 1-naphthyl acetate and stained in similar intensity pattern, so confirming that these were esterase isoforms (Fig. 2).

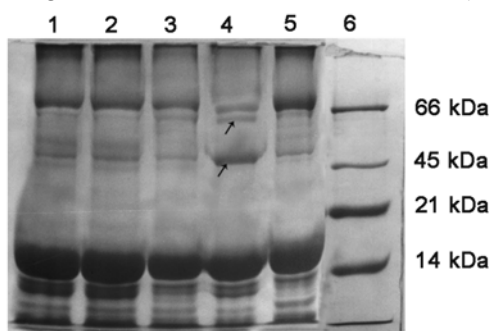


Fig. 1—Molecular mass determination of the putative JHE on 10% acrylamide gel from *D. cingulatus* haemolymph. [Lane 1, 3rd instar nymph; lane 2, 4th instar nymph; lane 3, 5th instar nymph (1st day); lane 4, 5th instar nymph (6th day); lane 5, adult; and lane 6, markers]

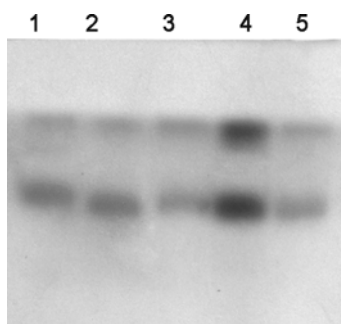


Fig. 2—Esterase substrate (α -naphthyl acetate) selectivity in *D. cingulatus* haemolymph. [Lanes 1 to 4, 5th instar nymphs of 0-, 2nd-, 4th- and 6th-day, respectively; and lane 5, adult]

Characterization of JHE

Using mRNA purified from the total RNA (Fig. 3) of late 5th instar nymphs of *D. cingulatus* as a template, first strand was synthesized using p23RT as a primer for reverse transcriptase and used as the template for two rounds of PCR using forward (LDJHEF) and reverse (LDJHER) primers. The products from this reaction were used for second round of PCR for enhanced amplification. PCR on fat body single stranded cDNA with different primer combinations showed 4 bands on an agarose gel stained with ethidium bromide. The bands lie within the range of known JHE cDNA from other insects (Fig. 4).

Discussion

Two JHE bands analogous to ~50 and 66 kDa were found in the haemolymph of late 5th instar nymphs when subjected to electrophoresis. All lepidopteran and dipteran JHEs reported so far are monomers

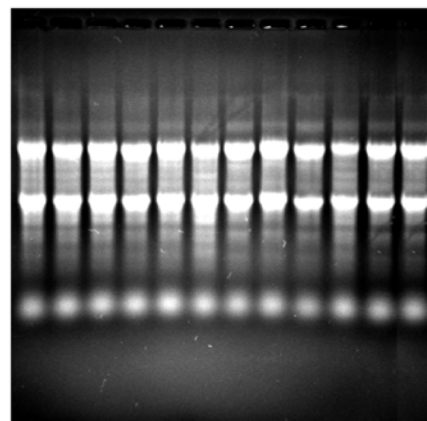


Fig. 3—Formaldehyde-Agarose gel showing total RNA isolated from the fat body of last day 5th instar nymph

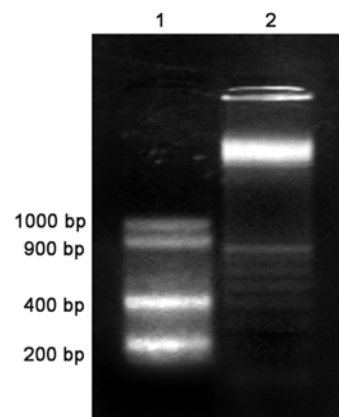


Fig. 4—1% Agarose gel showing PCR products. [Lane 1, JHE cDNA; and lane 2, marker (100 bp ladder)]

ranged from 50-68 kDa^{5,7,28,29}. The JHE from *H. zea* and *L. dispar* have shown with molecular mass of 66 kDa⁴ and 50 kDa⁷, respectively. Estimated molecular mass of JHE from *D. melanogaster* was 54 kDa⁴. In contrast, JHEs from two coleopterans exist as dimers with subunit molecular mass of 57 and 71 kDa^{8,13}. Molecular mass of native JHE from *G. assimilis* is found to be 92 kDa, and that of closely related *G. rubens*, 188 kDa¹⁸. These studies suggest that JHEs of different quaternary structures may exist even within the same species⁸ while it is already known that JHE can exist in an active state as dimer or monomer³⁰.

The pattern of the esterase isozymes in different lepidopteran species varied dramatically among developmental stages even within a species³¹. Four isozymes in *I. typographus*¹² and three isozyme patterns have been detected in the haemolymph of 5th instar larvae of *B. mori*⁹. Multiple JHEs have been reported in the haemolymph of *Hyalophora gloveri*³⁰, *M. sexta*³², *G. mellonella*³³ and the pyralid moths representing families Liparidae, Archidae, Gelechiidae and Saturniidae. But some pierids like *C. eurytheme*, *E. elutella* show only a single JHE form, illustrating the differences even possible within a single family^{31,34}.

In the present study, two isozymes having molecular mass of 66 kDa and 50 kDa have been observed. Differences of electrophoretic mobility of JHE isoforms from different insect species may be due to small regions of the proteins, which possess either potentially different sequence or different post translational modifications. Differential processing and/or post-translational modifications are an inherent property of JHE gene within an individual cell type²⁰. An attempt was made to clone the JHE gene from the late 5th instar nymphs of *D. cingulatus* using mRNA isolated from fat body as the starting material. The PCR products obtained after amplification of cDNA using degenerate primers showed four bands having molecular size varying from 1000, 900, 400 and 200 bp. These bands seem to be JHE isoforms since 1.3 kb PCR product obtained from *L. decemlineata*¹³ and 800 bp band generated after PCR in *T. molitor*⁸ show analogous base pair configuration.

Post-translational modification can give rise to different isoforms from the same gene product. If post-translational modification is the cause; elucidation of how a consistent percentage is converted to each isoform may reveal previously

unknown regulatory mechanism controlling isoform ratios. The significance of additional forms of JHE activity remains uncertain. However, further studies are needed to assess the role of each form and to examine their prominence during the development.

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