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Molecular cloning and expression of a hexamerin cDNA from the honey bee, *Apis mellifera*

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Abstract

A cDNA encoding a hexamerin subunit of the Africanized honey bee (*Apis mellifera*) was isolated and completely sequenced. In the deduced translation product we identified the *N*-terminal sequence typical of the honey bee HEX 70b hexamerin. The genomic sequence consists of seven exons flanked by GT/AT exon/intron splicing sites, which encode a 683 amino acid polypeptide with an estimated molecular mass of 79.5 kDa, and pI value of 6.72. Semi-quantitative RT-PCR revealed high levels of *Hex 70b* message in larval stages, followed by an abrupt decrease during prepupal-pupal transition. This coincides with decaying titers of juvenile hormone (JH) and ecdysteroids that is the signal for the metamorphic molt. To verify whether the high *Hex 70b* expression is dependent on high hormone levels, we treated 5th instar larvae with JH or 20-hydroxyecdysone (20E). In treated larvae, *Hex 70b* expression was maintained at high levels for a prolonged period of time than in the respective controls, thus indicating a positive hormone regulation at the transcriptional level. Experiments designed to verify the influence of the diet on *Hex 70b* expression showed similar transcript amounts in adult workers fed on a protein-enriched diet or fed exclusively on sugar. However, sugar-fed workers responded to the lack of dietary proteins by diminishing significantly the amount of HEX 70b subunits in hemolymph. Apparently, they use HEX 70b to compensate the lack of dietary proteins.

Keywords: Hexamerins; Storage proteins; *Apis mellifera*; Honey bees; Juvenile hormone; Ecdysteroids

1. Introduction

Storage proteins represent an amino acid reserve that, in addition to other organic compounds, are essential for construction and differentiation of adult tissues in holometabolous insects during metamorphosis. Most of the storage proteins belong to the class of hexamerins, which are synthesized in large amounts by fat body cells during the larval growth phase, and are secreted into the hemolymph where they accumulate (Telfer and Kunkel, 1991). The larval-pupal transition triggers sequestration of hexamerins and storage in large granules into fat body in order to sustain pupal development and adult differentiation (Locke and Collins, 1968; Levenbook and Bauer, 1984; Haunerland, 1996; Burmester and Scheller, 1999).

Insect hexamerins belong to a phylogenetically old arthropod family of proteins that includes hemocyanins, prophenoloxidases and arylphorin-receptor proteins (Burmester, 2002). Consisting of six identical or similar subunits, hexamerins are large proteins with typical native molecular mass in the 500 kDa range. They have

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been characterized mainly in dipteran and lepidopteran species. In hymenopterans, hexamerins have been studied in ants (Wheeler and Martinez, 1995), in Vespidae wasps (Hunt et al., 2003) and also in the honey bee, *Apis mellifera*. In this species, Ryan et al. (1984) first described protein subunits in the range of 75–80 kDa, which specifically cross-reacted with an anti-*Manduca sexta* hexamerin serum. Later, several hexamerin subunits were identified in hemolymph samples subjected to SDS-PAGE (Danty et al., 1998). Analysis of their N-terminal sequences confirmed that these honey bee proteins are members of the hexamerin family. Three of these subunits presented molecular mass in the 70 kDa range (HEX 70a, HEX 70b, and HEX 70c) and distinct N-termini. The other subunits, which share a common N-terminal sequence, migrated at the range of 80/110 kDa (HEX 80/110). These data suggested the existence of at least four different hexamerin genes in honey bees.

Hexamerin genes provide excellent systems for understanding hormonally regulated expression on transcriptional and posttranscriptional levels. These proteins are of fundamental importance for insect development and, therefore, studies concerning their structure, biosynthesis, regulation and evolution have been conducted (Haunerland, 1996; Burmester, 1999, 2001). As an initial step to study the role of hexamerins in honey bee life cycle, we have isolated and characterized a cDNA encoding HEX 70b. Hence, to learn more about the physiological aspects that underlie *Hex 70b* gene transcription, we studied the mRNA stage-specific expression profile as well as the action of juvenile hormone (JH) and of 20-hydroxyecdysone (20E) in controlling *Hex 70b* expression. In addition, we examined *Hex 70b* gene expression and the abundance of the corresponding protein subunit in hemolymph, after manipulating the quality of the diet ministered to honey bee workers.

2. Material and methods

2.1. Apis mellifera

Developing honey bees were collected from colonies of Africanized stocks kept in the apiary of the University of São Paulo at Ribeirão Preto, Brazil. Worker larvae and pupae were staged according to Michelette and Soares (1993) criteria. Adult workers of defined age were obtained by paint marking newly emerged bees, returning them to experimental hives, and collecting 1, 3, 6, 9, 12 and 15 days later.

2.2. Electrophoresis of hemolymph samples

Hemolymph was rapidly collected and centrifuged at 10,000*g* for 5 min at 4–10 °C. Aliquots of the supernatants were separated for protein quantification using bovine serum albumin in standard curves (Bradford, 1976). To prevent oxidation (melanization), and protease action, a few crystals of phenylthiourea, and a cocktail of inhibitors (0.05 mg/10 μl Soybean Trypsin Inhibitor, Lima bean Trypsin Inhibitor and Leupeptin; 0.1 M Benzamidine) were added to the samples to be stocked. Aliquots of samples containing 1 μg of protein were mixed with sample buffer [0.25 M Tris-HCl pH 6.8 (1.25 ml), saccharose 70% (500 μl), 0.1 g SDS, 250 μl β-mercaptoethanol, 100 μl bromophenol blue, 3 ml water] and boiled for 2 min. Electrophoresis was performed according to Laemmli (1970), but using 7.5% or 4–10% polyacrylamide gels ($100 \times 120 \times 0.9\,mm^2$) prepared without SDS, which was added only to the running and sample buffers. Electrophoresis was carried out at 15 mA and 7–10 °C. Gels were stained with silver nitrate (Caetano-Anollés and Gresshoff, 1994) or 1% Coomassie blue dissolved in a solution of glacial acetic acid, ethanol and water (1:5:5 v/v) that was also used for gel destaining. Hexamerins were identified by their molecular mass in comparison with known protein standards, according to Danty et al. (1998).

2.3. Semi-quantitative reverse transcription PCR analysis

Hex 70b expression profile was evaluated by semi-quantitative RT-PCR, using specific primers (*Hex 70b* sense: 5′-ATC CGC TCT TCA AAT GTG GTC TAC–3′), and antisense: 5′-GTG TTG CTT CCG CTT TTC AGG-3′). The design of the primers was based on cDNA clone sequences isolated using Open Reading frame Expressed Sequence Tags strategy (Dias Neto et al., 2000; Nunes et al., 2004). Briefly, expressed gene transcripts from Africanized honey bees in different stages of development were amplified by reverse-transcription PCR using arbitrarily chosen primers. This strategy results in preferential amplification of the central portion (coding region) of transcripts, and was first defined with the objective of contributing new sequences to the ESTs (mainly 5′ESTs) available in public databases. Two cDNA clone sequences presenting high similarity with insect hexamerins were assembled to generate a contig that was used to construct sense and antisense primers. These sequences were subsequently deposited in a public database (GenBank accession numbers CK628855 and CK631851, respectively).

First-strand cDNA was synthesized by reverse transcription conducted with SuperScript II reverse transcriptase and an oligo (dT_{12-18}) primer (Invitrogen) using 2 μg of total RNA from entire larvae, prepupae and pupae, and from adult abdominal integument (cuticle, epidermis and adhered fat body). Larvae and adults, with the gut removed, were used for RNA extraction, using TRIzol (Invitrogen). Aliquots of first-