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Exoskeleton formation in *Apis mellifera*: Cuticular hydrocarbons profiles and expression of desaturase and elongase genes during pupal and adult development

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ABSTRACT

Cuticular hydrocarbons (CHCs) are abundant in the superficial cuticular layer (envelope) of insects where they play roles as structural, anti-desiccation and semiochemical compounds. Many studies have investigated the CHC composition in the adult insects. However, studies on the profiles of these compounds during cuticle formation and differentiation are scarce and restrict to specific stages of a few insect species. We characterized the CHCs developmental profiles in the honeybee workers during an entire molting cycle (from pupal-to-adult ecdyses) and in mature adults (forager bees). Gas chromatography/mass spectrometry (GC/MS) analysis revealed remarkable differences in the relative quantities of CHCs, thus discriminating pupae, developing and newly-ecdysed adults, and foragers from each other. In parallel, the honeybee genome database was searched for predicted gene models using known amino acid sequences of insect enzymes catalyzing lipid desaturation (desaturases) or elongation (elongases) as queries in BLASTP analysis. The expression levels of six desaturase genes and ten elongase genes potentially involved in CHC biosynthesis were determined by reverse transcription and real time polymerase chain reaction (RT-qPCR) in the developing integument (cuticle and subjacent epidermis). Aiming to predict roles for these genes in CHC biosynthesis, the developmental profiles of CHCs and desaturase/elongase transcript levels were evaluated using Spearman correlation coefficient. This analysis pointed to differential roles for these gene products in the biosynthesis of certain CHC classes. Based on the assumption that homologous proteins may share a similar function, phylogenetic trees were reconstructed as an additional strategy to predict functions and evolutionary relationships of the honeybee desaturases and elongases. Together, these approaches highlighted the molecular complexity underlying the formation of the lesser known layer of the cuticular exoskeleton, the envelope.

1. Introduction

The cuticular exoskeleton of insects is mainly formed by proteins, the polysaccharide chitin, and lipids arranged as a complex multilayered structure: the inner procuticle comprising the endocuticle and exocuticle, the epicuticle and an outermost envelope. These functional layers are sequentially produced and are secreted by the epidermis at each molt cycle, and differ from each other in biochemical composition and physiological properties. The envelope mainly consists of lipids that form a barrier against water loss and invading pathogens, and also serve as important cues for chemical communication, besides acting as sex pheromones (Wigglesworth, 1970; Blomquist and Dillwith, 1985; Gibbs, 2002; Châline et al., 2005). This lipid layer is largely enriched with hydrocarbons (Hepburn, 1985), which are synthesized in specialized cells called oenocytes (Piek, 1964; Diehl, 1973; Schal et al., 1998; Fan et al., 2003; Billeter et al., 2009). In honeybee workers the oenocytes are localized in close association with the epidermis and the parietal fat body that internally coat the exoskeleton, and are more frequently found closer to the sternites than the tergites (Ruvolo and Landim, 1993).

Intermediates and end-products of metabolic pathways, such as fatty acids, in addition to specific enzyme classes, are involved in

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CHC biosynthesis. Key enzymes in the CHCs biosynthetic pathways are the fatty acid synthases, elongases, desaturases, reductases and a P450 decarbonylase. Two forms of fatty acid synthases are likely involved in the synthesis of unbranched and methyl-branched fatty acids. Elongases catalyze the chain elongation of saturated and unsaturated fatty acids, which are converted to aldehydes by reductase enzymes. Aldehydes are substrates for the last step of hydrocarbons biosynthetic pathway, i.e., the oxidative decarbonylation catalyzed by a P450 enzyme. Desaturation, i.e., the insertion of carbon–carbon double bonds into the saturated fatty acid chain and consequent conversion to an unsaturated fatty acid is catalyzed by desaturases (Kolattukudy, 1965, 1968; Howard and Blomquist, 2005; Blomquist et al., 2012; Qiu et al., 2012). After being synthesized and released from the oenocytes, the CHCs are transported through the hemolymph by lipophorins (Chino and Kitazawa, 1981; Chino et al., 1981). CHCs reach the insect surface via the pore canals of the cuticular exoskeleton (Blomquist and Dillwith, 1985), where they integrate the envelope layer.

The multicomponent CHC blend shows a great variation among insect species. CHCs also display both quantitative and qualitative intraspecific variation depending on the developmental stage, environment changes and food availability. The majority of the information on CHC composition comes from studies on adult social insects and is focused on their roles in chemical communication for sex-, kin- and caste-recognition (Dallerac et al., 2000; Roelofs et al., 2003; Liu et al., 2004; Châline et al., 2005; Nunes et al., 2008; Ferreira-Caliman et al., 2010).

We have been studying the expression of genes, proteins and enzymes in the honeybee integument as part of a major project aiming to characterize the molecular elements involved in the exoskeleton formation and the regulation of this process (Bitondi et al., 1998; Santos et al., 2001; Zufelato et al., 2004; Soares et al., 2007, 2011, 2013; Elias-Neto et al., 2010). Such information generated using polyacrylamide gel electrophoresis (SDS-PAGE), western blot, RT-qPCR, gene sequencing, cDNA microarrays and fluorescence in situ hybridization (FISH), mainly highlighted the differential gene expression dynamics needed for the adult exoskeleton construction. These approaches, however, have neglected the outmost functional exoskeletal layer, i.e., the envelope, its formation and molecular composition. Trying to fill this gap in part we here used GC/MS to explore the composition as well as developmental profiles of CHCs during exoskeleton formation and maturation. Concomitantly, genes potentially encoding desaturases and elongases were searched in the honeybee genome and their expression patterns were characterized using RT-qPCR. To get an insight on the roles of the honeybee desaturases and elongases genes on cuticular envelope formation, the strength of the correlation between developmental profiles of transcripts and CHCs were estimated. In addition, we built molecular relationship trees for gene function prediction.

2. Material and methods

2.1. Sample collection

Africanized *Apis mellifera* workers were obtained from a single colony of the experimental apiary of the Faculty of Medicine, University of São Paulo in Ribeirão Preto, SP, Brazil. The samples were collected at successive developmental stages, from pupal ecdysis to a late adult stage, and included newly-ecdysed pupae (Pw phase: white eyes and unpigmented cuticle); pupae-in-apolysis (Pp phase: pink eyes and unpigmented cuticle); early, intermediate and late brown-eyed pharate adults showing unpigmented cuticle (Pb phase), partially pigmented cuticle (Pbm phase) and intensely pigmented cuticle (Pbd phase); newly-ecdysed adults (Ne) and foragers (Fg). These developmental time points include the sequential events of the pupal-to-adult molt and adult cuticle synthesis, deposition and differentiation/maturation. The pre-ecdysial phases were identified according Michelette and Soares (1993). Foragers carrying pollen were collected at the entrance of the hive. All these developmental phases were used to analyze CHCs profiles and the expression of genes encoding enzymes potentially involved in their biosynthetic pathways.

2.2. CHCs extraction, identification and statistical analysis

The samples (36 bees per developmental phase, except for Pw phase that comprised 35 bees) were individually added to 1.5 ml of 95% n-hexane (Mallinckrodt Chemicals) and bathed for 1 min to extract the CHCs. The extracts were then dried under N_2 flow, resuspended in 160 μl of 95% n-hexane and analyzed in a Gas Chromatograph/Mass Spectrometer (Shimadzu GCMS model QP2010), equipped with a 30 m DB-5MS column and helium as the carrier gas (1 ml/min), using the electronic ionization (EI) method. The injection volume was 1 μl at an initial temperature of 150 °C elevated at a rate of 3 °C/min to 280 °C and keeping this temperature for 15 min. Compounds identification was based on their diagnostic ions and in a standard solution containing different synthetic hydrocarbons (Fluka). To analyze the chromatograms we used the software GCMS solutions for Windows version 2.6 (Shimadzu Corporation). The positions of unsaturations in alkenes and alkadienes were identified according to the dimethyl disulfide derivatization technique (Carlson, 1989) and analyzed at the same GCMS system above mentioned. The initial temperature was 80 °C for 2 min, then increased to 180 °C at a rate of 30 °C/min and then to 300 °C at a rate of 3 °C/min, keeping 300 °C for 80 min. The identification of compounds and positions of unsaturations were done in splitless mode, which is recommended for low concentrated samples (Hübschmann, 2009), as those composed by a mixture of CHCs. Quantification of CHCs was based on their peak area in each chromatogram (Singer and Espelie, 1992). We adjusted the compounds percentage to 100% and each peak area was transformed according to the formula $Z = \ln[Ap/g(Ap)]$, where Ap is the peak area, g(Ap) is the geometric mean of the peak for each bee sample and Z is the transformed peak area (Aitchison, 1982). To discriminate developmental stages according to their CHCs profile, we performed a PerMANOVA test in R software (version 3.0.0; package vegan: version 2.0–7). This test allowed us to analyze the dispersion of the samples around each group (developmental phase) centroid giving us back a result based on permutation tests (here we utilized 10000 permutations). We plotted the results in a scatterplot with the two first Principal Coordinates as axes. The variation in the proportion of each compound during development was also verified using the One-Way ANOVA associated to the Tukey's Honestly Significance Difference (Tukey's HSD) *post hoc* test (R software). We used this same test to compare variations in the proportions of CHC groups: n-alkanes, methyl-alkanes, dimethyl-alkanes, alkenes, alkadienes and non-identified compounds.

2.3. Identification of desaturases and elongases potentially involved in hydrocarbons biosynthesis

Known desaturase and elongase amino acid sequences of *Drosophila melanogaster* and lepidopterans, available in the National Center for Biotechnology Information (NCBI) data bank (www.ncbi.nlm.nih.gov/), were used to search for homologous sequences in the honeybee genome (http://hymenopteragenome.org/beebase/) (version 4.5) using a BLASTP search tool (Altschul et al., 1990). The evolutionarily conserved functional motifs were confirmed in all the honeybee desaturase and elongase amino acid