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Proteome Analysis of Hemolymph Changes during the Larval to Pupal Development Stages of Honeybee Workers (*Apis mellifera ligustica*)

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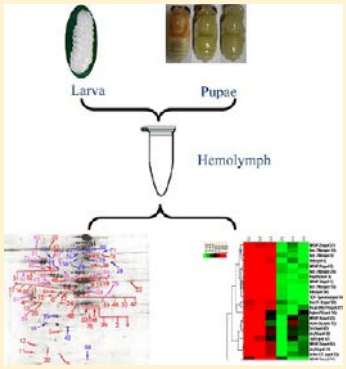
Supporting Information

ABSTRACT: Hemolymph is vital for the flow and transportation of nutrients, ions, and hormones in the honey bee and plays role in innate immune defense. The proteome of the hemolymph changes over the life of a honey bee, but many of these changes are not well characterized, including changes during the life cycle transition from the larval to pupal stages of workers. We used two-dimensional gel electrophoresis, mass spectrometry, bioinformatics, and Western blot to analyze the proteome changes of the honeybee hemolymph during the transition from newly hatched larvae to five-day-old pupae. Of the 49 nonredundant proteins that changed in abundance (identified by 80 protein spots), 29 (59.2%) and 20 (40.8%) were strongly expressed in the larvae and the pupae, respectively. The larval hemolymph had high expressions of major royal jelly proteins and proteins related to metabolism of carbohydrates and energy, folding activities, development, and the cytoskeleton and antioxidant systems. Proteins involved in food storage and the metabolism of fatty acids and amino acids were abundantly expressed during the late larval to pupal development stages. The proteins expressed by the young larvae are used to enhance their development process and as a temporal innate immune protection mechanism until they gain immunity with age development. The pupae use more energy storage related proteins as they prepare for their non-diet-driven pupation. Our data provide new evidence that changes in the hemolymph at the proteome level match the processes during life transitions in the honeybee.



KEYWORDS: *honeybee, Apis mellifera, larvae, pupae, hemolymph, proteome*

1. INTRODUCTION

Like other arthropods, the honeybee has an open circulatory system containing hemolymph that surrounds its tissues.[1,2] This hemolymph is a secretion containing numerous proteins from the fat body, which is similar to the adipose tissue and liver of mammals, and other proteins that are secreted from the hemocytes, epidermis, and midgut epithelium, with high concentrations of inorganic ions, amino acids, sugars, and proteins.[5] The contents of the hemolymph vary considerably according to developmental stage, sex, and season.[6,7]

The hemolymph acts as connective tissue responsible for transporting various molecules throughout the body, controlling systemic changes in other pathways.[12,13] The main function of the hemolymph is to transport nutrients, ions, and hormones, but it can also aid in the response to invading pathogens such as bacteria, fungi, and other parasites.[3,4] The innate immune system existing in the hemolymph,[8] such as macrophage-like cells (hemocytes),[9] antimicrobial peptides,[10] and prophenoloxidase for the encapsulation of pathogens,[11] make this fluid vital for the recognition and defense against microorganisms. Cellular and humoral immune responses against invading agents are elicited by the infection or introduction of foreign material into the hemolymph of insects.[13] Because of their roles in immunity, wound healing, and the regulation of those processes, and as a result of variations in the content of hemolymph following developmental stages or in relation to physiological changes, many proteins from insect hemolymph are of interest, leading to a need for more thorough studies of insect proteomes.[14,15]

Proteomics has recently become an important platform to study the changes in protein expression in tissues and body fluids during different developmental stages or under various environmental effects.[16,17] In the study of honey bee biology, proteomics has been widely used to examine worker and drone embryogenesis[18,19] and larval development,[20,21] and some studies of the hemolymph proteome have been done on adult honeybee workers[1,22−25] and during the larval development stages.[12,25]

Large differences in hemolymph protein composition have been reported between the larval and adult stages of honeybees, indicating changes in life processes.[1] The development of the honeybee from larvae to pupae is a crucial life transitional stage, but knowledge regarding changes of the hemolymph proteome during this key life cycle transition is lacking. Therefore, the

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main objective of this work is to analyze how the changes in the hemolymph proteome fulfill its biological mission as nutrition carrier and innate immune defense to ensure the normal life transition from the honeybee larvae to pupae.

2. MATERIALS AND METHODS

2.1. Chemical Reagents

Chemicals used for the two-dimensional gel electrophoresis (2-DE), such as urea, Tris-base, sodium dodecyl sulfate (SDS), ammonium bicarbonate (NH_4HCO_3), dithiothreitol (DTT), iodoacetamide, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA), whereas Biolyte and immobilized pH gradient (IPG) strips were purchased from Bio-Rad (Hercules, CA, USA). The modified sequencing grade trypsin was from Roche (Mannheim, Germany).

2.2. Biological Samples

The larvae and pupae of the honeybee worker (*A. mellifera ligustica*) were collected from five bee colonies maintained in the Apiary of the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing. To acquire larvae of known ages, the queen was confined to a single wax comb frame containing worker cells in each colony for 6 h, with a cage made of a queen excluder, through which workers but not the queen can pass. Subsequently, the queen was removed, and the eggs contained in the frame were maintained in the honeybee colony for further development.

Before the hemolymph collection, all the larvae and pupae were washed twice with phosphate-buffered saline (PBS) followed by wipe with soft paper to remove the potential of royal jelly contamination from feeding by nurse bees in the comb cells. For the larvae aged between 1 and 3 days, their skins were pierced with great care to avoid damaging their organs, and the hemolymph was collected following a previously used protocol.[2] For the larvae aged between 4 and 5 days and all the pupae aged between 1 and 5 days, the hemolymph was collected by inserting a disposable glass microcapillary pipet (5 μL) into one side (two-thirds down from head to the body) of the larva and pupa, avoiding deep cuts and drawing hemolymph liquid by capillary action. Over 150 larvae and 120 pupae were sampled in each replication (three replications for each sample) and an average of 250 μL of hemolymph was collected per sample. All of the collected samples were stored at −80 °C. Larvae aged 1, 3, and 5 days and pupae aged 1, 3, and 5 days were used for the following analysis.

2.3. Two-Dimensional Gel Electrophoresis (2-DE) and Image Statistical Analysis

Hemolymph protein extraction was carried out according to a previously described method.[26] A 558 μg protein sample from each larval and pupal sample with three biological replicates (which were pooled together from five honeybee colonies) was suspended in 90 μL of lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 20 mM tris-base, 30 mM DTT, and 1.25% Biolyte, pH 3−10) and mixed with 360 μL of rehydration buffer (8 M urea, 2% CHAPS, 0.001% bromophenol blue, 45 mM DTT, 0.2% Biolyte pH 3−10). The first dimension isoelectric focusing (IEF) and second dimension electrophoresis were performed according to Feng et al.[26]

Three independent and reproducible 2-DE gel images from the samples of each time point (day 1 larva to day 5 pupa) were digitized at 16 bit and 300 dpi resolution (GE Healthcare, Piscataway, NJ, USA). Image quality control, spot alignment, filtration, normalization, and analysis of quantitative intensity were subjected to Progenesis Samespot software (version 4, nonlinear Dynamics, UK). Using selected reference gels, automatic matching analysis was done for all gels, with further manual editing to correct the mismatched and unmatched spots. The expression level of each protein spot was calculated in terms of its volume. The log-normalized volume data for a spot was calculated as the mean of the log-normalized volume values of its match series within the averaged gel/replicate group subgels. The normalization was to fix the data of one sample and then to calibrate all other sample data to this reference. This reference sample was regarded as having a gain factor of 1. A reference gel was chosen and then gain factors for all other samples were calculated to implement normalization.

The means and standard deviations from the triplicate experiments were calculated, and the differences in protein expression levels between each time point throughout the selected development stage was determined by one-way ANOVA (Samespot, version 4, nonlinear Dynamics, UK) using a q-value for multiple tests. The differences of protein spots were taken as statistically significant when they contained at least 2-fold changes and $p < 0.05$. The q-value, which determines adjusted p-values for each test, was calculated by the Samespot software to estimate false positive results.

2.4. Trypsin Digestion and Protein Identification by Mass Spectrometry (MS)

All differentially expressed protein spots in the hemolymph samples were manually excised from the Coomassie Bright Blue (CBB) stained gels. The following protein digestion and peptide extractions were undertaken according to our previously established protocol.[26] Analysis of the digested protein spots was done by liquid chromatography-chip/electrospray ionization quadruple time-of-flight mass spectrometry (LC-Chip/ESI-QTOF-MS) (QTOF G6520, Agilent Technologies), equipped with a capillary pump G1382A, a nanopump G2225A, an autosampler G1377D, and the Chip Cube G4240A according to our previously described protocol.[27] Mass Hunter software (version B. 03. 01, Agilent Technologies) was used to retrieve tandem mass spectra. A peak list was generated by Mascot Distiller software (version 3.2.1.0, Matrix Science) prior to MS/MS data search. The data were stored in a combined mgf file and searched against sequence database generated from protein sequences of *A. mellifera ligustica* (downloaded May 2011, version 4.5 of the honeybee genome) augmented with sequences from other honeybee species, *Drosophila melanogaster* (downloaded May 7, 2011), *Sacharomyces cerevisiae* (downloaded May 7, 2011), and a common repository of adventitious proteins (cRAP, from The Global Proteome Machine Organization, downloaded May 2011), totaling 72672 entries, using in-house Mascot (version 2.3, Matrix Science, UK). Search parameters were as follows: carboxymethyl (C) and oxidation (M) were selected as fixed and variable modifications, respectively, taxonomy = all; enzyme = trypsin; missed cleavages = 2; peptide tolerance = ±50 ppm, MS/MS tolerance = ±0.05 Da.

When an identified protein was matched to multiple members of a protein family or a protein appeared under the same names and accession number, the match was made in terms of differential patterns of protein spots on 2-DE gels. Protein identification was accepted if it contained at least two

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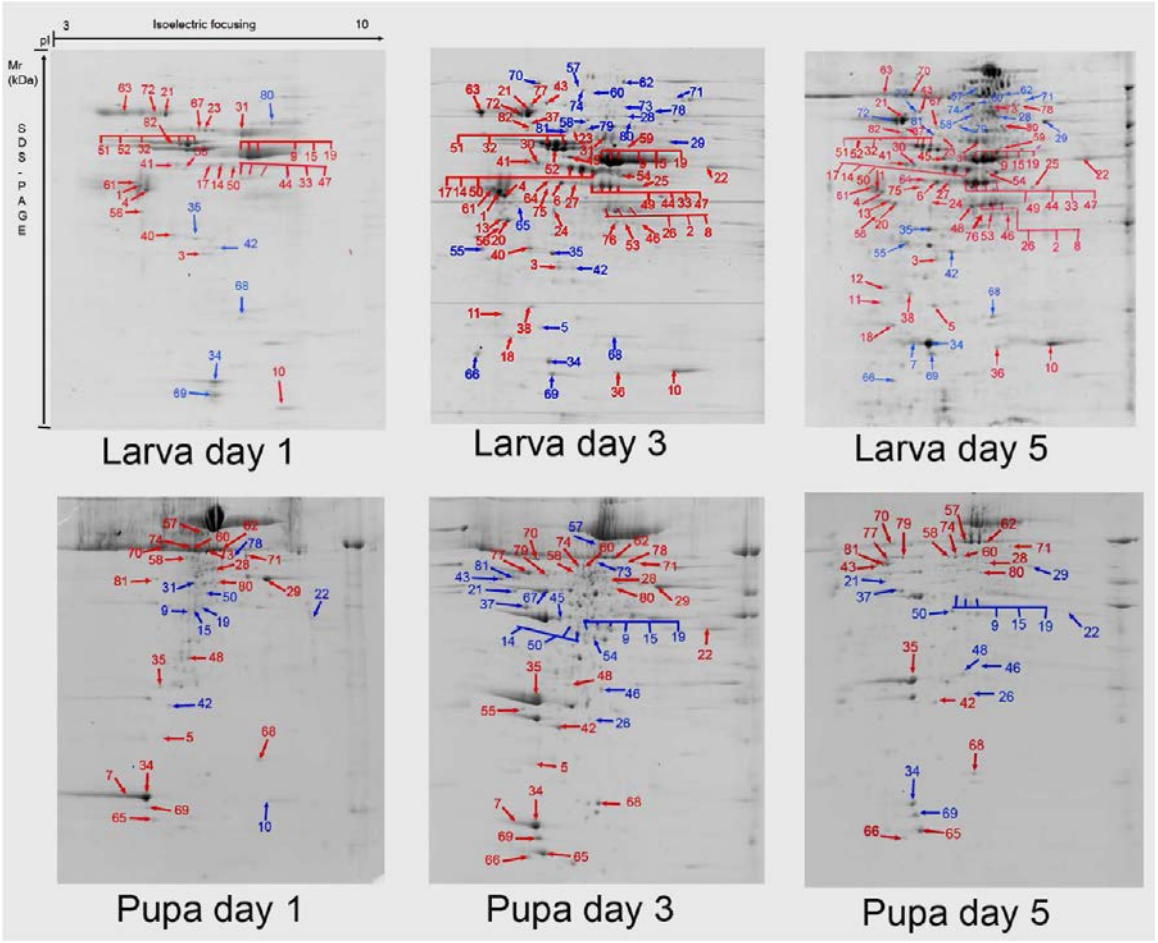

Figure 1. Representative 2-DE gel images of hemolymph proteome of honeybee worker (*Apis mellifera ligustica*) larvae (day 1, 3, and 5) and pupae (day 1, 3, and 5). Proteins were separated on IPG gel strips (17 cm, 3−10 L) with 558 μg of sample loaded, followed by SDS-PAGE on a vertical slab gel (12.5%), and detected by Coomassie Brilliant Blue G-250. Differentially expressed protein spots of known identity are marked with color codes. The red and blue colors indicate the proteins that were up-regulated and down-regulated, respectively. The protein abundances of each age class are compared with the average of all six age classes (following the cluster analysis of Figure 4).

unique peptides and was above the ion cutoff score of 24 with 95% confidence.

2.5. Bioinformatics Analysis

Annotation of the identified proteins was done with a search against the Uniprot database (http://www.uniprot.org/) and Flybase (http://flybase.org/), and categorization was made based on their biological process of Gene Ontology (GO) terms that were determined according to the information of UniProt. Hierarchical clustering of the expression of the identified proteins was undertaken using the expression values of the protein spots according to their average distance calculated by cluster software (Gene cluster, version 3.0). The biological interaction network (BIN) of the identified proteins was predicted using Pathway Studio (http://www.ariadnegenomics.com). The *Drosophila* database, which is equipped with functional relationships from other scientific literature, was used to make the protein lists. The applied filters included "all shortest paths between selected entities," and the received information was narrowed down to our proteins of interest. At least three publications were used as evidence to build each link. Based on the default settings of the software, protein entities belonging to different functional categories were represented by different shapes as shown in Figure 5.

Analysis of the identified proteins' GO functional term enrichment against their specific functional terms was done by ClueGo[28] applied to the *Drosophila* database, downloaded from the GO database (release date, May 10, 2011). The biological process was selected and the enrichment analysis was conducted by a right-side hypergeometric statistic test. The Bonferroni method was used to correct the probability value, and the results were represented visually in a graph.

2.6. Western Blot

To further verify the expression results of our identified proteins in 2-DE analysis, major royal jelly protein 1 (MRJP1), MRJP2, and MRJP3 were subjected to three replication runs, and 6 μg of protein sample was loaded on each lane separated by stacking (4%) and separating (12%) SDS-PAGE gels. The following experiment was performed according to Feng et al.[26] Enhanced chemiluminescence detection (ECL, Pierce, Rochford, IL, USA) reagents were used to visualize the immunoreactive protein bands, and the bands were quantified by densitometry using a Quantity-one image analysis system (Bio-Rad). Differences were determined by ANOVA analysis (SPSS version16.0, SPSS, Inc.) using Duncan's multiple-range test. An error probability $p < 0.05$ was considered statistically significant.

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3. RESULTS

3.1. Proteome Expression Profiling

The main goal of this study was to explore changes in the hemolymph proteome of the honeybee worker through the larval to pupal development transition. Figure 1 represents proteome of larval and pupal hemolymph. Consequently, over 460 protein spots were detected in the hemolymph at each time point of larval and pupal development stages. A total of 82 protein spots showed differential expression (>2-fold change, $p < 0.05$, false positive <0.51 spots). Of these, 49 nonredundant proteins (derived from 80 protein spots) were identified by MS (Table S1, Supporting Information). Two other spots were not identified; this might be due to being too low in abundance to generate adequate spectra or to a lack of unambiguous results in the databases of the search scores. Two-dimensional gel electrophoresis based proteomics usually visualizes the same proteins as multiprotein spots (protein species).[29] Therefore, the number of identified proteins is less than the spot number (Table S1, Supporting Information). These protein species may derive from alternative splicing, proteolytic cleavage, or post-translational modifications.[29]

3.2. Qualitative Comparisons of Differentially Expressed Proteins

Qualitative comparison is important to understand the biological significance of the differential expression of functional groups of hemolymph proteins at the developmental stages of larvae and pupae. The 49 differentially expressed proteins were annotated into 10 functional categories on the basis of their biological activities at the six time points of development (larvae on day 1, 3, and 5 and pupae on day 1, 3 and 5). The most represented categories were proteins related to protein folding (22.4%, 11 proteins) and those related to carbohydrate metabolism and energy production (20.4%, 10 proteins), followed by proteins related to the cytoskeleton (12.2%, 6 proteins) and development (12.2%, 6 proteins). Proteins involved in food storage were the next largest group (8.2%). Proteins associated with MRJPs, molecular transporters, and the antioxidant system were the following largest groups with equally representation (6.2%, 3 proteins). The others were implicated with metabolism of amino acids, nucleotides, and fatty acids (Figure 2). Interestingly, the functional classes had a higher representation in the larval than in the pupal hemolymph, except for proteins involved in food (energy) storage, molecular transporters, and the metabolism of amino acids and fatty acids. In particular, proteins related to MRJPs and development were specifically found in the larvae, whereas proteins associated with the metabolism of amino acids, nucleotides, and fatty acids were specifically expressed in the pupal hemolymph (Figure 3). Six of the ten functional categories had proteins that were upregulated in both the larval and pupal stages. Of these, two functional categories had equal numbers of upregulated proteins in both age groups - those related to carbohydrate metabolism or energy production and molecular transporter proteins; one group had a higher number of upregulated proteins in the pupae compared with the larvae, proteins involved in food storage; and three categories had higher numbers of proteins upregulated in the larval stages, protein folding, antioxidant system, and cytoskeleton (Figure 3).

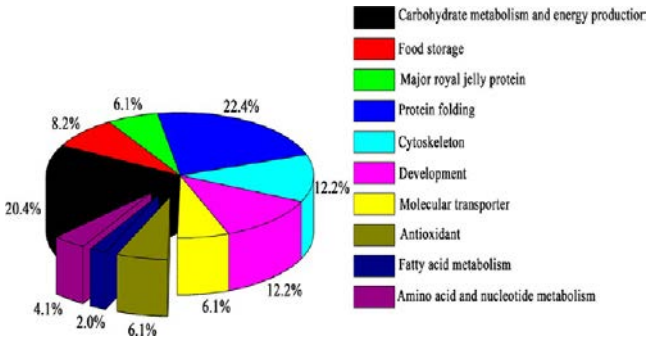

Figure 2. Categorization of proteins altered in their expression in the hemolymph of honeybee worker larvae and pupae. Colors indicate functional groups of differentially expressed protein spots.

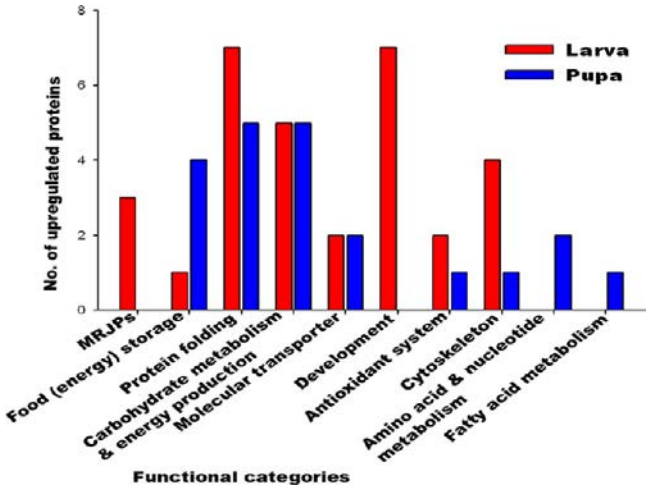

Figure 3. Comparisons of the number of up-regulated proteins in the hemolymph of honeybee worker larvae and pupae.

3.3. Quantitative Analysis of Identified Proteins

A remarkable change of the hemolymph proteome throughout the shift from larvae to pupae was observed. Hierarchical clustering is an efficient way to reveal the degree of similarity among different proteomics data sets, indicating intrinsic relations between them. Figure 4 clearly shows two major clusters of all 49 proteins (80 spots); 29 abundantly expressed proteins were clustered in the larvae (59.2%, 50 spots), and the other 20 highly expressed proteins were clustered in the pupae (40.8%, 30 spots).

Of the 29 proteins clustered in the larva, the young larva (<day 3) displayed stronger expression of the proteins involved in MRJPs, folding activities, cytoskeleton, carbohydrate metabolism, and energy production, food (energy) storage, development and antioxidant activities, and molecular transporters. In the mid to late aged larva (day 3−5), the more strongly expressed proteins were mainly associated with MRJPs, development, carbohydrate metabolism, energy production, protein folding, and molecular transporters. Proteins with constant higher expressions during the whole larval period (day 1−5) were associated with the metabolism of carbohydrates and energy production, food storage (hexamerin), protein folding, the cytoskeleton, and MRJPs (Figures 3 and 4).

Of the 20 proteins with higher abundance in the pupal hemolymph, most of them (12) were highly expressed at the early/prepupal stage (≥day 5 larva to day 1 pupa). They were mainly related to food storage (hexamerins), molecular

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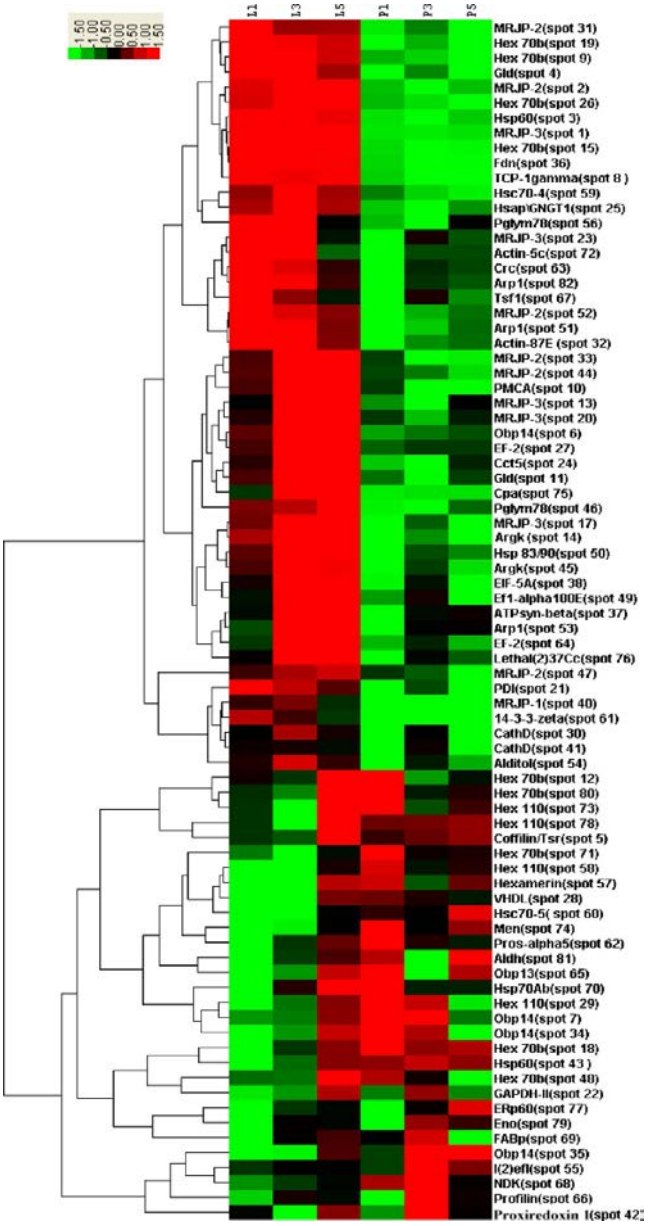

Figure 4. Hierarchical clustering analysis of 80 differentially expressed hemolymph protein spots of honeybee worker larvae and pupae at development time points on days 1, 3, and 5 for each stage. The protein expression value at each developmental stage is averaged from three replicates. Similarity between the protein expression values is computed based on Pearson correlation. Hierarchical clustering organizes proteins in a tree structure (left), based on their similarity. Protein name is indicated on the right. The columns represent the worker bee larvae on day 1 (L1), 3 (L3), and 5 (L5) and pupae on day 1 (P1), 3 (P3), and 5 (P5). The rows represent the individual protein spots. The up- or down-regulated proteins are indicated in red and green, respectively. The color intensity changes with increasing expression differences as noted on the key bar on the top left side. The value indicated on the legend (1.5) denotes the average distance between each protein, calculated by the algorithms in the agglomerative hierarchical cluster analysis.

transporters, protein folding, carbohydrate metabolism, energy production, cytoskeletal functions, and metabolism of amino acids.

At the end of the prepupal stage (day 3), seven highly expressed protein were involved in carbohydrate metabolism, energy production, molecular transporters, metabolism of fatty acids, protein folding, cytoskeleton, antioxidant activities, and metabolism of amino acids. Four proteins were highly expressed in the 5-day-old pupae that were involved in protein folding, molecular transporters, carbohydrate metabolism, and energy production (Figures 3 and 4).

3.4. Bioinformatics Analysis

A total of 29 proteins were identified as key nodes in the constructed BIN (Figure 5). Sixty-two percent (18 of 29) of these proteins were abundantly expressed in the larva, while 38% (11 of 29) were expressed in higher abundance in the pupae. Proteins involved in folding activities had an abundant representation in the networks (38% or 11 proteins): hsc70-4, hsp83, cct5, PDI, crc, cct-gamma, hsp60, hsc70-5, hsp70Ab, l(2) ef1, and ERP60. The second most abundant group (27.6% or 8 proteins) was proteins associated with metabolism of carbohydrates and energy production, that is, argk, pglym78, gld, cap60a, GAPDH-II, men, eno, and ATP-syn-beta. Proteins involved in developmental regulation of were the third most represented group (14% or 4 proteins): including 14-3-3-zeta, ef-2b, l (2)37Cc, and ef1 alpha100E. Additionally, three cytoskeletal proteins (cathD, actin-87E/Ptx1, and actin-5c), two proteins involved in the metabolism of amino acids (prosma5 and awd), one molecular transporter (OBP 99a), and one antioxidant protein (peroxiredoxin 1) were also linked in the network.

Analysis of the over-represented GO terms helps to interpret the biological significance behind large proteomic data sets. The identified proteins were therefore significantly enriched into five major functional terms: (1) carbohydrate metabolism or energy production, (2) cytoskeletal functions, (3) protein folding, (4) development, and (5) antioxidant activities (Figure 6). Over 35% (8 of 22) of these proteins were abundantly expressed in the early larval stage, 30% (7 proteins) in the mid to late larval stage, about 12% (3 proteins) in the early pupal stage, and the rest were expressed in the pupa at day 3 and 5 (Table S2, Supporting Information). Protein folding was the leading functional term (the term with the lowest statistically significant p-value), and proteins involved in folding activities were the most overrepresented, comprising >46.2% of the total enriched proteome. These folding proteins include Cct gamma (spot 8), PDI (spot 21), hsp 60 (spot 43), hsc70-4 (spot 59), Crc (spot 63), Cct5 (spot 24), hsp 60 (spot 3), hsp70Ab (spot 70), hsp 83/90 (spot 50), hsc70-5 (spot 60), and ERP60 (spot 77). Similarly, with glucose metabolic processes as the leading term, the functional group of carbohydrate metabolism/energy production was the second (23%) highly enriched group, containing gld (spot 4 and 11), pglym78 (spot 46 and 56), GAPDH-II (spot 22), and eno (spot 79). Proteins associated with the cytoskelton were enriched by tsr (spot 5), elf-5A (spot 38), and Cpa (spot 75). Proteins related to antioxidant activities were significantly enriched to redox homeostasis with PDI (spot 21), peroxiredoxin 1 (spot 42), and ER60 (spot 77), whereas development function was significantly enriched by transitional elongation processes with ef2b (spot 27), elf-5A (spot 38), and ef1-alpha100E (spot 49). Taken together, proteins involved in the folding activities, carbohydrate metabolism or energy production, development, and antioxidation had major representation in both the protein interaction networks and the enrichment groups.

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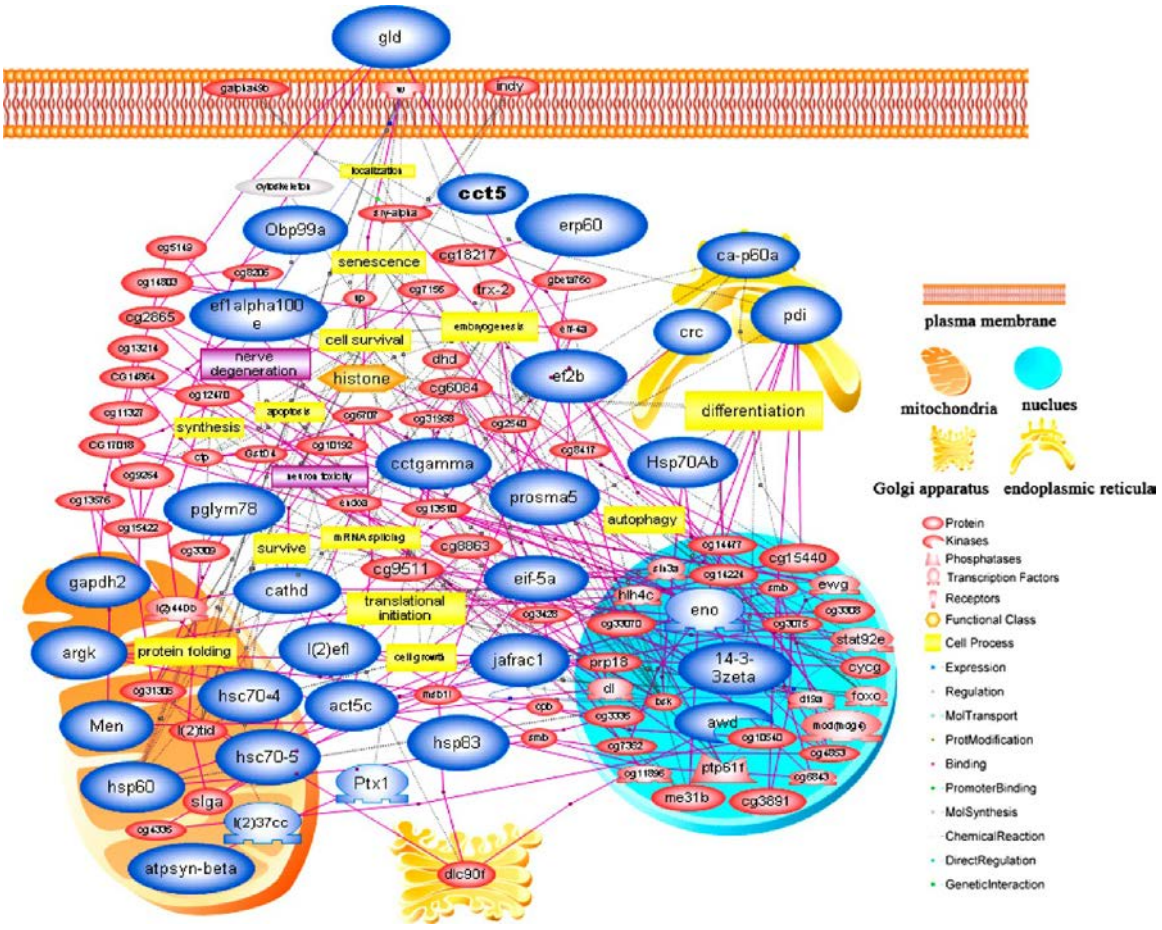

Figure 5. Biological interaction network of the identified differentially expressed proteins from honeybee worker larval and pupal hemolymph at the different developmental stages. The big blue ellipse represents identified up-regulated proteins in this experiment. Protein entities of the distinct category were described in the legend according to the default setting of the software.

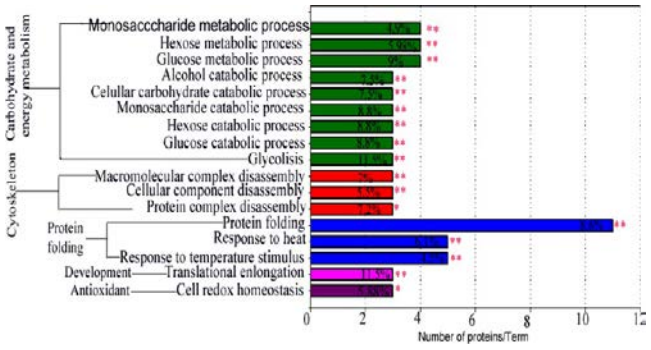

Figure 6. GO functional term enrichment analysis of the differentially expressed proteins using ClueGO. The one and two asterisks indicate significant enrichment at the $p < 0.05$ and $p < 0.01$ statistical levels, respectively. Numbers on the horizontal axis indicate number of proteins per term. The percentages in the bars indicate the ratio of associated proteins to GO Process in each term.

3.5. Western Blot Analysis

To further validate the changed hemolymph protein abundance at different developmental stages of larva and pupae, Western blot analysis was conducted (Figure 7). Significantly higher abundances of MRJP1 ($p = 0.0052$), MRJP2 ($p = 0.0006$), and MRJP3 ($p = 0.0011$) were found in the larvae compared with in the pupae and were confirmed at the corresponding protein level. Generally, this result is in agreement with the results of 2-DE analysis.

4. DISCUSSION

We examined time-resolved changes of the honeybee hemolymph proteome in worker larvae and pupae to gain insight into how molecular mechanisms match with physiological life transitions. Generally, to support the amazing growth of the honeybee larva (the weight of last day is 1600 times more than that of the first day) and to boost their immature immune systems, the larval hemolymph has stronger expression of proteins involved in the metabolism of carbohydrates, MRJPs, protein folding, cytoskeletal proteins, development, molecular transporters, and energy storage/hexamerins. In the pupal hemolymph, most of these proteins are down-regulated, and some new protein families are recruited (including metabolism of amino acids, nucleotides, and fatty acids) as the bee prepares for the nonfeeding pupal stage.[30]

4.1. MRJPs

Royal jelly proteins are a highly nutritive source of essential amino acids for honeybee larva that accounts for 82−90% of the larval jelly.[31] MRJPs have repeatedly been identified in several hemolymph studies of honeybee and play roles in the innate immunity of the honeybee.[1,12,22,25] The identified MRJPs (MRJP1, -2, and -3) in the larvae indicate that the

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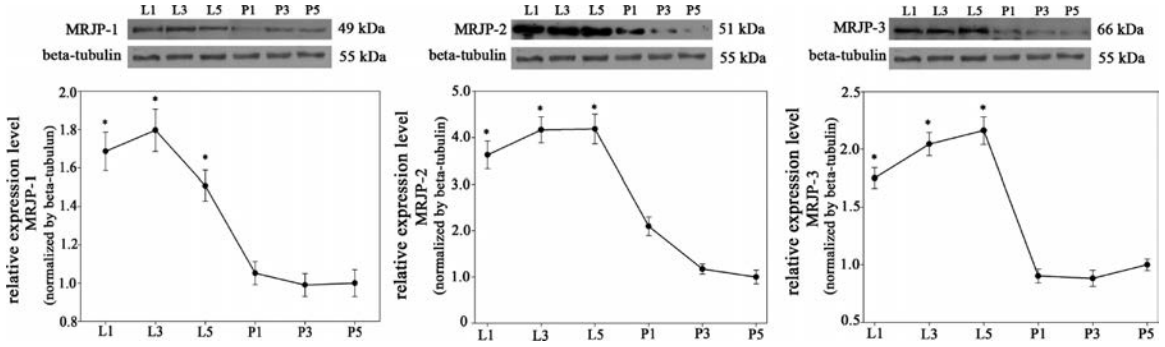

Figure 7. Western blot analysis of major royal jelly protein1 (MRJP1), MRJP2, and MRJP3 in the hemolymph proteome of honeybee worker (*Apis mellifera ligustica*) larvae on day 1, 3, and 5 and pupae on day 1, 3, and 5. Upper panel, Western blot analysis; lower panel, relative densitometry analysis normalized by β-tubulin. *Y*-axis represents relative expression level normalized by β-tublin; *x*-axis represents different development stages on day 1, 3, and 5 of both larvae and pupae. The asterisks show significant differences ($p < 0.05$) between the larvae and pupae in protein expression abundances.

hemolymph is a nutrition reservoir for the young larvae. The specifically high abundance of MRJPs in the young larvae hemolymph (<day 3), confirmed by a Western blot, suggests that MRJPs at this stage are key for boosting their development and immune abilities.[32] Duo to a weak immune system in the young larvae (the first 48 h),[12] the MRJPs in the hemolymph of young larvae are supposed to play a role in supporting their immune systems and maintaining normal larval growth.[33] The down regulation of the MRJPs beyond day 3 is likely related to shifts taking place in their feeding system from royal jelly to honey, pollen, and water mixtures.[34]

4.2. Food (Energy) Storage Proteins

Storage proteins are largely synthesized in the fat body of the insect larvae and are stored in the hemolymph as a source of amino acids required for the subsequent development toward the adult life.[35,36] We found that the expression of storage proteins started in the hemolymph of larvae at day 3 and became more abundant in correlation with their age. This suggests that the brood hemolymph of the honeybee acts as a nutritive resource to ensure the successful development[37] as it does in ants[38] and in *Drosophila*.[39]

4.3. Carbohydrate Metabolism and Energy Production

It is known that metabolic fuel generated by carbohydrate metabolism is key for honeybee development. The strong expression of most proteins related to carbohydrate metabolism during the larval stage (6 of 10) suggests not only a high metabolic energy demand to ensure the formation of important organs and metamorphosis, but also an inherent mechanism to manage the discerning demands on its enormously carbohydrate-rich diet.[40] Even though fewer numbers of these proteins were abundantly expressed in the older larvae, some continuous expression indicates their key role for various respective activities in the process of larval and pupal development. Specifically, overexpression of gld, argk, pglym78, and ATP synthase beta subunit in the larvae, and Men, Aldh, and Enolase in the pupae, suggest that larvae and pupae have diverse levels of metabolic activities at each point of their developmental stages and in response to various internal brood conditions. This is similar to the involvement of large numbers of metabolic-associated cellular proteins that respond as part of an immune response to oxidative harm or to changes in energy/biosynthetic pathways in the hemolymph of *Drosophila*.[15] In honeybees, the high expression of Aldh as a mitochondrial metabolic enzyme in the larvae infected with *Paenibacillus larvae* indicates a role in innate immune responses.[12] The later period of larvae down-regulation of some of these proteins suggest that the aged larvae require less intensity of metabolic energy to support their development and immunity, which coincides well with *Drosophila*.[41]

4.4. Fatty Acid Metabolism

Fatty acid metabolism proteins perform central roles in the transportation of energy from mitochondria to tissue for honeybee development. The abundant expression of this protein family, particularly fatty acid binding protein (FABP), at the pupal stage is mostly related to the demand for stored energy during this nonfeeding period. Family members of FABP have important roles in the control of fatty acid uptake and transportation of intercellular proteins,[42] as well as in signal transduction and regulation of gene expression in lipid transport and metabolism.[43] The abundant expression of FABP in the pupae also reflects different defense mechanisms against oxidative stresses through a reduction in the generation of free radicals and removal of fatty acids.[44]

4.5. Metabolism of Amino Acids and Nucleotides

Proteins metabolizing the amino acids and nucleotides are vital for rapid larval growth and tissue construction.[45] The abundant expressions of pros-alpha5 and Ndk/Awd at the early pupal stage suggest that they function to supply energy and enhance the metamorphosis process.[46] This protein family participates significantly in the degradation pathway that delivers amino acids for the synthesis of new proteins and also takes part in the processes of development of some organs, glands, and secretions.[18,47]

4.6. Protein Folding

Heat shock proteins (HSPs) are expressed at high levels when exposed to a sudden temperature fluctuation or other stress as a defense mechanism[11] and are present in cells under normal conditions as molecular chaperones.[19] Hence, the abundant expression of HSPs, such as HSP6, HSP 83/90, and HSC70-4 in the day 3−5 larvae, as well as expression of HSP70Ab, ERP60, and HSC70-5 in the pupae, suggests that they are significant for cell maintenance and immune activity.[49] Since our study was conducted under normal colony temperature, up-regulation of numerous HSP proteins at different developmental stages of larvae and pupae indicates their important roles in protein folding to accomplish their functional shape or conformation through polypeptide folding.[50] This is important

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for providing the building blocks of organ and tissue formation from the newly synthesized proteins for the developing honeybee brood.

4.7. Antioxidant Activities

Antioxidant systems are important in preventing cellular components from oxidative damage by removing free radicals and subsequently inhibiting other oxidative reactions.[51] High expression of considerable numbers of proteins related to antioxidant activities at the larval stage indicates that the antioxidant metabolites and the enzymes' multifaceted network are required to carry out self-oxidation, avoid other oxidation reactions, and halt oxidative harm to other cellular components as they function in the development of the honeybee embryo[20] and hypopharyngal gland.[26] The higher demand for oxygen in the actively growing larvae compared with the pupae is to deal with their active production of more reactive oxygen species (ROS).[51,52] Specifically, abundant expression of cathepsin (cathD) in the larval hemolymph indicates its role in the protection of larvae from ROS-mediated organ damage.[52] The abundant expressions of FDH in the larvae and peroxiredoxin 1 in the early pupae has great importance for the antioxidant system in its respective developmental stages.[53] Some of the proteins abundantly expressed here are similar to enzymes with protective roles against oxidative stress as in the hemolymph of infected larvae.[44,54]

4.8. Cytoskeletal Proteins

The cytoskeletal proteins help maintain cell shape and participate in cellular division and intracellular transport. Their high expression in the larval hemolymph suggests a role in supporting cell shape maintenance, motility, intracellular transportation, and cellular division during the active larval development stage.[19,55] For instance, the stronger expression of actin may contribute to actin filament assembly and intracellular vesicular transport during dorsal closure throughout the embryonic development as in *Drosophila*[56] Tsr, restricting the actin polymerization, controls actin-based motility processes and enhances removal of ADP bound actin monomers from the pointed end of an actin filament.[57] Hence, the abundant expressions of cytoskeletal proteins here indicates their vital roles in supplying skeletal elements to keep up cell scaffolding and support organ formation as in *Drosophila*.[58] Their changed expression such as actin-binding, myosin, and cuticle has also reported in honeybee pupal heads after a bacterial challenge.[14] In addition to their role in maintaining cell shape and division, cytoskeletal proteins may act as regulators in immune cells.[19,25] For instance, the expression of profilin at the early pupal stage may be important in actin polymerization and depolymerization and directly involved in phagocytosis as in *Drosophila*.[59] The abundant expression of actin5c at the early larval stage suggests its role as an immune protein being released into the hemolymph in response to infection by all types of microorganisms.[60]

4.9. Development Related Proteins

Regular organism development is often guaranteed by growth factor or development regulating proteins. The synthesis of these proteins has been reported throughout all developmental stages in the honeybee.[1] In this study, the larval hemolymph has a significantly increased number of proteins involved in development. For instance, abundantly expressed lethal (2) 37Cc at the mid to late larval stage suggests it is required for larval metabolism as in *Drosophila*.[61] The 14-3-3 zeta (Leonardo protein) abundantly expressed at the larval stage may be related to the role of 14-3-3 proteins (as one of the highly conserved families of molecules) in regulating intracellular signal transduction and the cell cycle and preventing apoptosis.[62] Abundant expression of an elongation factor 2 (EF-2) in the larval hemolymph likely implies rapid growth of tissue. The strongly expressed Elf-5A at the mid to late larval stage is probably involved in the peptide bond formation in translation and is essential for cell proliferation and cell-cycle regulation.[18,63] Overall, the abundant expression of development related proteins at the larval stage is believed to regulate the process of transcription and translation for the regular larval to pupal development of the honeybees as in the *Drosophila*.[64]

4.10. Molecular Transporter Proteins

Transport proteins serve the function of moving other materials within an organism and are vital to the growth and life of all living things. The expression of proteins involved in this group at late larval and pupal stages indicates their significance in enhancing developmental and physiological processes. Some of the identified odorant-binding proteins (OBPs) are expressed as one of the three major chemosensory organs on the larval cephalic lobes in the dorsal organs of larvae,[65] and some of them are induced upon viral and bacterial challenges.[60] Their expression presumably indicates the increasing ability of larvae through time to respond to external stimuli and internal regulatory signals.[2] This agrees with reports of the odorant-based communication in old larvae.[66] The abundant expression of transferrin1 (TSF1) during the larval developmental stage suggests it is one of the iron transporting proteins that circulates in the hemolymph and has an additional role in immunity.[67−69] Hence, it may be involved in the innate immune response in honey bees, either as an antimicrobial peptides or as an inducer as in *Drosophila*.[70]

The high representation of proteins involved in protein folding, metabolism of carbohydrates, development, and antioxidant activities, both in the BIN and functional GO term enrichment, indicates their central roles for the achieving biological activities of the honeybee hemolymph. This helps us to identify the important pathways and key node proteins for the further functional study of the honeybee hemolymph.

5. CONCLUSION

To accomplish this biological duty, the young larvae (≤3 day old) require a wild cascade of hemolymph proteins to boost their fast growth and to build an innate immunity, such as MRJPs and proteins associated with carbohydrate and energy metabolism, protein folding, cytoskeletal function, development, and the antioxidant system. With age development, proteome changes are recognized by the strong demand for stored energy during the transition from a larva to nonfeeding pupa, which is supported by abundant expression of proteins involved in hexamerin, and metabolism of fatty acids, amino acids, and other proteins in the hemolymph. Our data contribute vital new knowledge on how changes in the hemolymph proteome guarantee the normal development of the worker honeybee larvae and pupae. It also helps us to identify some important pathways and key node proteins for future functional studies of insect hemolymph.

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■ ASSOCIATED CONTENT

Supporting Information

Identification of deferentially expressed hemolymph proteins during larval and pupal development stages of honeybee worker (*Apis mellifera* L.) by 2-DE analysis and peptide sequence assigned to the identified protein. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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