Apidologie 39 (2008) 199–214
© INRA/DIB-AGIB/ EDP Sciences, 2008
DOI: 10.1051/apido:2007059

Available online at:
www.apidologie.org

Original article

Identification of the proteome complement of hypopharyngeal glands from two strains of honeybees (*Apis mellifera*)*

Jianke LI[a], Mao FENG[a], Zhaohui ZHANG[b], Yinghong PAN[b]

[a] Institute of Apicultural Research, Chinese Academy of Agricultural Science, Beijing 100093, China
[b] Institute of Crop Science, Chinese Academy of Agricultural Sciences, The National Key Facility for Crop Gene Resources and Genetic Improvement, 12 Zhongguancun Nandajie, Beijing 100081, China

Received 18 June 2007 – Revised 24 September 2007 – Accepted 26 September 2007

Abstract – We investigated the protein complement of the hypopharyngeal gland (HG) of winter worker bees from a strain of *Apis mellifera* artificially selected for increased royal jelly yield and *A. m. carnica* winter worker bees. Proteins were partially identified using two-dimensional gel electrophoresis (2-DE). MALDI-TOF MS and protein engine identification tools that were utilized for the honeybee genome. Most identified proteins in the two bees strains were assigned to major royal jelly (RJ) proteins (MRJPs). Marked differences were found in the heterogeneity of the MRJPs, in particular MRJP3. Two of the proteins, α-glucosidase and glucose oxidase, were related to carbohydrate metabolism and energy. For the first time in the HG of honeybees, two proteins, peroxiredoxin and thioredoxin peroxidase, which are related to antioxidation functions, and actin 5C, a major cytoskeletal actin protein which may supply enough actin for normal function of cells, have been identified. Results suggest that the HGs serve a storage function in winter and that during the winter period the HG of high RJ producing bees store more proteins than those of Carnica bees.

***Apis mellifera* / winter bees / hypopharyngeal gland / proteome**

1. INTRODUCTION

The hypopharyngeal gland (HG) is situated in an anterior position in the worker honeybees' head. It consists of many acini attached to a central duct which synthesize and secret a proteinaceous substance which is fed to larvae, queens and drones (Painter and Biesele, 1966; Huang et al., 1989). The age-dependent role of this secretion in workers is one of the most striking features in bee colonies (Ohashi et al., 1999). Nurse bees (young workers usually not more than 13 days past eclosion) feed and take care of the brood by synthesizing and secreting RJ. Middle-aged workers maintain the hive and store food, while old workers (foragers, older than 18 days) forage outside the hive and process the nectar into honey (Ohashi et al., 2000).

In parallel with this age-dependent shift in behavior, physiological changes occur in certain organs of the workers. The HG, which is fully developed and shows high rates of protein synthesis in nursing bees, regresses in foraging bees (Sasagawa et al., 1989; Ohashi et al., 2000). Although the HG is hypertrophied in the winter bees, it still has low secretion activities (Huang et al., 1989). Undeveloped or hypertrophied cells have less activity than glands of medium size (Deseyn and Billien, 2005).

Because of functions that are age-dependent, changes in the morphology of the worker HG can be expected. Acini size

Corresponding author: Y. Pan, apislijk@126.com
* Manuscript editor: Klaus Hartfelder

radically changes with age (Deseyn and Billien, 2005). Peak size is found around 6 days in summer bees when workers are known to feed the larvae with RJ (Hrassnigg and Crailsheim, 1998). Acini size begins to decrease after 15 days. The volume of the acini, as well as the number of secretory vesicles, decreases, and no vesicles are visible after 3 weeks of age. Gland size is positively correlated with gland activity (Deseyn and Billien, 2005). Amount of secretion in the secretory cells is also positively correlated with acini size. Secretory activity may be prolonged because secretory cell development is not synchronous within an individual bee or even within an acinus (Deseyn and Billien, 2005). There is no histological or cytochemical evidence for cell death occurring in the HG of young nursing bees. However, necrosis and apoptosis predominate in older foraging bees (Silva de Moraes and Bowen, 2000).

It is inferred that the HG exists in two distinct differentiation states, first producing RJ for nursing the brood followed by enzyme production (Ohashi et al., 1997). The HG can produce α-glucosidase (Kubo et al., 1996; Santos et al., 2005), glucose oxidase (Ohashi et al., 1999) and other enzymes such as galactosidase, esterase, lipase and leucine arilamidase (Deseyn and Billien, 2005). This would indicate that the gene for the 64 kDa protein/RJP57-1 is expressed specifically in the nurse bee glands while the gene for a-glucosidase is expressed in the forager bee glands only (Ohashi et al., 1996). In contrast, the gene coding for a 56 kDa protein is expressed in both the nurse bee and forager bee glands (Ohashi et al., 1997). The production of a-glucosidase increases with the age of workers (Deseyn and Billien, 2005). Expression of carbohydrate-metabolizing enzymes in the HG is age-dependent in worker honeybees (Ohashi et al., 1997). Dramatic changes of gene expression associated with age occur in bee brains between newly emerged bees and old foragers (Ohashi et al., 1999). Santos et al. (2005) investigated the protein composition of HG of Africanized nurse bees (*Apis mellifera* L.). The proteins identified were almost all related to the MRJP family and to the metabolism of carbohydrates and energy. This supports the hypothesis that most of the proteins in RJ are produced in the HG.

In dequeened colonies older workers began to synthesize brood food as nurse bees do. In other words, the HG of the foragers can be regenerated if not enough young bees are present (Huang and Robinson, 1996). This makes it clear that the function of the HG cells of the workers is flexible and depends on colony conditions (Ohashi et al., 2000).

Bees becoming wintering bees are affected by temperature conditions in the autumn and the size of the colony's pollen store (Amdam and Omholt, 2002). Previous life histories do not prohibit workers from becoming wintering bees so long as they get plentiful food and time to build up their protein reserves before they enter winter cluster conditions. Studies on the development of winter bees reveals that vitellogenin and arylphorin act as storage proteins, which may allow colonies to continue brood rearing in extremely cold periods (Otis et al., 2004). Research on the morphology and ultrastructure of the HG of wintering bees shows that secretions still occur, but production of RJ probably stops. The secretions are probably stored for brood rearing in early spring. These glands loaded with accumulated secretions can directly discharge their contents when circumstances change in the spring. (Deseyn and Billien, 2005).

The recent availability of the honeybee *Apis mellifera* genome and transcriptome of both female castes has provided new insights into the genetics of honeybees as well as comparisons with other species (The Honeybee Genome Sequencing Consortium, 2006). This has stimulated new efforts in investigating the proteome profile of the HG of the winter bees by proteomic approach. Due to lack of information about proteins of the HG, the aim of the present study is to do a large scale survey to obtain more information about the HG proteome profile.

2. MATERIALS AND METHODS

2.1. Chemical reagents

The immobilized pH gradient (IPG) strip (pH 3–10, Linear), the two-dimensional gel

electrophoresis (2-DE) marker, Bio-lyte (pH 3–10), and mineral oil were purchased from Bio-Rad Laboratories Ltd. Tris-base, ammonium persulfate (AP), sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylethylene diamine (TEMED) and glycin were from Sigma. Acrylamide, N, N'-methylenebisacrylamide, Bromophenol Blue, Coomassie Brilliant Blue (CBB) G-250, thiourea, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), glycerol, bovine serum albumin (BSA) were purchased from Amresco. Agarose and urea were from Solarbio. Dithiothreitol (DTT) and iodoacetamide were from Merck. Trypsin was from Roche; trifluoroacetic acid (TFA) and acetonitrile were from J.T. Baker.

2.2. HG samples

High RJ producing bees and *Apis mellifera. carnica* honeybees (further referred as Carnica bees) were maintained at the Institute of Apicultural Research, Chinese Academy of Agricultural Science. Sixty worker bees were collected from each strain in January, 2007, anesthetized on ice and the HG dissected using a binocular microscope.

2.3. Preparation of protein samples

Protein extraction was according to Li et al. (2007). Hypopharyngeal glands (1 mg HG/10 μL buffer) were added to phosphate buffer (PB) pH 7.6, containing 32.5 mMol/L K_2HPO_4, 2.6 mMol/L KH_2PO_4, 400 mMol/L NaCl. The glands were homogenized for 20 minutes on ice and sonicated for 2 minutes, then centrifuged at 12 000 *g* and 4 °C for 10 minutes, then further centrifuged at 15 000 *g* and 4 °C for 10 minutes. The supernatant was transferred to another tube for further use. The pellets (1 mg HG/2 μL buffer) were resuspended in PB and then centrifuged at 15 000 *g* and 4 °C for 10 minutes. The supernatant was removed and added to the tube containing supernatant as a PB-soluble proteins extract while the pellets (1 mg HG/10 μL buffer) of PB-insoluble proteins were mixed in lysis buffer (LB) (8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-base, 30 mM DTT, 2% Bio-lyte pH 3–10). The mixture was homogenized for 10 minutes on ice and sonicated for 2 minutes, centrifuged at 15 000 *g* and 4 °C for 10 minutes. The supernatant was removed and added to the tube containing PB-soluble protein extract, and the debris was discarded. Trichloroacetic (TCA) was added to the collected supernatants at a final concentration of 10%, and then the mixture was kept on ice for 10 minutes for protein precipitation and desalting. Subsequently, the mixture was centrifuged twice at 15 000 *g* and 4 °C for 10 minutes. The supernatant was discarded and the pellets (1 mg HG/4 μL buffer) were dissolved in LB. Then the mixture was homogenized for 5 minutes on ice, sonicated for 2 minutes and subsequently adjusted to pH 7.0 with 2 M NaOH. The HG protein extract mixture was stored at –70 °C for further use.

2.4. Protein determination

Protein concentration was determined according to the method developed by Bradford (1976) using BSA as the standard. The absorption was measured at 595 nm (Beckman, spectrophotometer DU800).

2.5. Two-dimensional gel electrophoresis (2-DE)

A 300 μg protein sample was suspended in LB and then mixed with rehydration buffer (8 M urea, 2% CHAPS, 0.001% bromophenol blue, 45 mMol/L DTT, 0.2% Bio-lyte pH 3–10). The mixture was loaded on a 17 cm IPG strip (pH 3–10, linear, Bio-Rad Hercules, CA, USA). IEF was performed at 18 °C (Protean IEF Cell. Bio-Rad Hercules, CA, USA) according to the following program: 14 h at 50 V, 250 V for 30 min × 4, 1000 V for 60 min, 9000 V for 5 h, and continuing at 9000 V until completing a total of 60 000 V·h. Before SDS-PAGE, the IPG strips were first equilibrated for 15 minutes in equilibration buffer 1 (6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, 2% DTT) and then placed in equilibration buffer 2 (6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, 2.5% iodoacetoamide) for 15 minutes. After equilibration, the strip was overlayed onto the SDS-PAGE gel, 12% T separating gel (1.00 mm). Meanwhile, 10 μL of 2-DE marker was placed on a piece of filter paper and then transferred adjacently to the acidic end of the strip when the filter paper was nearly dry. The second dimension electrophoresis, SDS-PAGE, was performed in a Protean II Xi Cell (Bio-Rad Hercules, CA, USA) at 25 mA/gel for 6.5 h. The gel was stained with CBB G-250. Each sample was replicated five times and the best three runs with good reproducibility

were subjected to analysis. To accurately compare samples with variations in spot size and intensity not due to differential protein expression, normalization was done by defining a quantity of a Carnica bee sample as the protein standard. Significance of the normalized volume in total density of identified proteins in all gels was tested by ANOVA (Version 6.12, SAS Institute, Cary, N.C., USA) using a Duncans parametric test. An error probability of $P < 0.05$ was considered to be statistically significant.

2.6. Image acquisition

Gels were scanned using a transparency mode scanner, connected to a PC system, at 32-bit red-green-blue colors and 300 dpi resolution for documentation. Images were analyzed using PDQuest V 7.3.0 (Bio-Rad Hercules, CA, USA).

2.7. Tryptic digestion

CBB stained spots were excised and destained for 30 minutes using 100 mL acetonitrile (50%) and 25 mM $(NH_4)HCO_3$ pH 8 (50%) until the gels were transparent. They were dried for 10 minutes with acetonitrile (100%) and then for 30 minutes using a Speed-Vac system. At this point 2.5 mL of 25 mM $(NH_4)HCO_3$ was added to 25 μg trypsin (final concentration 10 ng/μL); 10 μL of this solution was pipetted on each dried protein spot and incubated for 60 min at 4 °C. The supernatant was discarded to minimize auto digestion of trypsin. The Eppendorf tube was then turned upside down and the sample was incubated for 14 h at 37 °C. To extract the peptide fragments from the tryptic digests, 20 μL of 5% (v/v) TFA were added and incubated for 60 min at 37 °C. Then 20 μL of 50% (v/v) acetonitrile (containing 2.5% (v/v) TFA) was added to the gel and incubated for 60 min at 30 °C. After each step the supernatants were pooled and dried using a Speed-Vac system.

2.8. MALDI-TOF MS analysis and database search

Before obtaining the mass spectra of the peptide mixture, the digested peptides were desalted and cleaned with ZipTip C18 pipette tips (Millipore Corp., Bedford, MA, USA) according to the manufacturer's instructions. All analyses were performed using a Bruker Daltonics Autoflex system (Bruker Daltonics Billerica, Mass. USA) operated in the delayed extraction (190 ns) and reflector mode with an accelerating voltage of 20 KV. Peptide mixtures were analyzed using a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonics Billerica, Mass. USA) in 50% acetonitrile/0.1% TFA. External calibration was performed using a peptide calibration standard (Bruker Daltonics Billerica, Mass. USA, Part No. 206195) and internal calibration with trypsin autoproteolytic fragments. Masses of proteolytic peptide fragments were determined by peptide mass fingerprinting (PMF), which is a mass spectrometry based protein identification technique. To interpret the mass spectra of protein digests, the generated peak lists of the tryptic peptide masses were searched against MASCOT (http://www.matrixscience.com/search_form_select.html) and Xproteo (http://xproteo.com:2698).

3. RESULTS

Figure 1 is a representative 2-DE pattern from the HG of Carnica winter worker bees (Fig. 1A) and high RJ producing winter worker bees (Fig. 1B), respectively. The results showed that the amount of 157 protein spots detected in high RJ producing bees (Fig. 1B) were significantly higher than the 112 found in Carnica bees (Fig. 1A) ($P < 0.05$). Proteins of high abundance were subjected to MALDI-TOF MS analysis. Twenty-six different forms of proteins were identified in the HG of Carnica winter worker bees (Fig. 1A; Tab. I) and thirty-four in high RJ producing winter worker bees (Fig. 1B; Tab. II). Twenty (Carnica) and twenty-eight (high RJ bees) proteins were assigned as MRJPs, with MRJP 3 representing the largest group within the MRJP family (Fig. 1; Tabs. I and II). Three proteins, two glucose oxidases and one α-glucosidase, are related to the metabolism of carbohydrates. Three proteins were identified for the first time in this study: two proteins, peroxiredoxin and thioredoxin peroxidase, that are related to antioxidation functions, and an actin isoform, act5C isoform 1. The number of most frequently identified proteins in high RJ producing bees (Fig. 2) was significantly higher than those of Carnica bees,

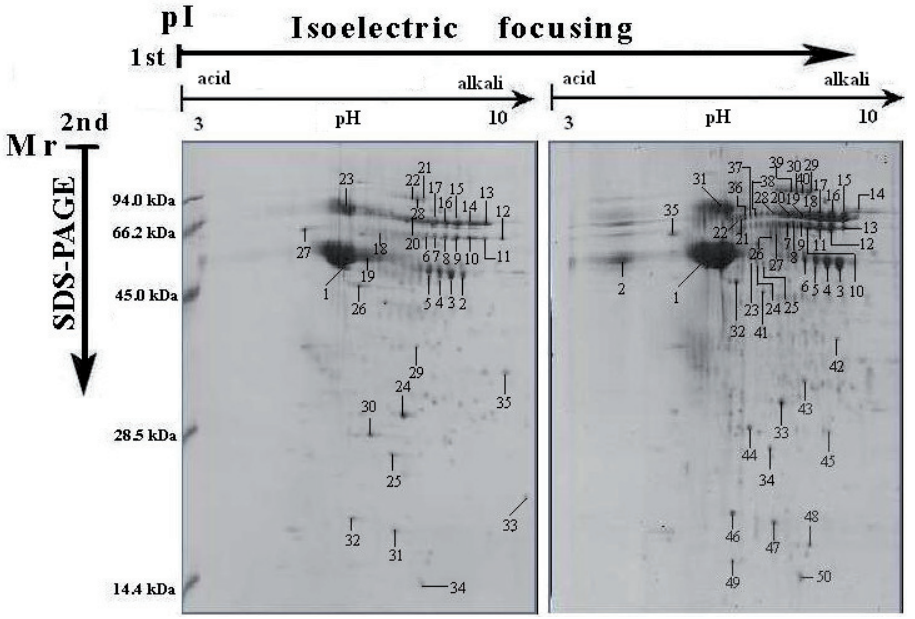


Figure 1. Representative 2-DE protein gel of hypopharyngeal gland proteome. Each sample (300 μg) was subjected to 2-DE and stained by CCB G-250. A and B represent 2-DE profiles of Carnica winter bees and RJ high-yield winter bees, respectively. Number-labeled spots were cut out and subjected to tryptic digestion for mass spectrometry analysis.

particularly in MRJPs. In contrast, three proteins involved in the metabolism of carbohydrates and three newly found proteins showed no significant differences between the two bee strains (Fig. 2B) ($P < 0.05$).

3.1. MRJPs identified in the HG of Carnica winter bees

The largest group of identified proteins was assigned as MRJPs. One protein (spot 1, Fig. 1A) was identified as MRJP1 with MW 48.86 kDa and pI 5.10 (Tab. I). Four different proteins (spots 2–5, Fig. 1A), presenting MW values 51.04 kDa and pI values 6.83, were identified as MRJP2 (Tab. I). Fourteen different forms of MRJP3 (spots 6–19, Fig. 1A) were identified with MW values varying from 61.62 to 65.66 kDa and pI values from 6.47 to 6.87 (Tab. I). No MRJP4 was identified in the HG of Carnica winter bees. One protein (spot 20, Fig. 1A) was identified as MRJP5 with MW 70.24 kDa and pI 5.90 (Tab. I).

3.2. MRJPs identified in the HG of high RJ producing winter bees

As in the Carnica bees, the largest group of identified proteins was classified as MRJPs. Spots 1 and 2 were identified to be MRJP1 with MW and pI values 48.86 kDa and 5.10 (Fig. 1B; Tab. II). Four different proteins (spots 3–6, Fig. 1B), with the MW values 51.04 kDa and pI values 6.83, were identified to be MRJP2 (Tab. II). Nineteen different forms of MRJP3 (spots 7–25, Fig. 1B) were identified with MW values from 61.62 to 65.70 kDa and with pI values ranging from 6.47 to 6.90 (Tab. II). Two different forms of MRJP4 (spots 26, 27, Fig. 1B) were identified with MW and pI values 52.88 kDa and 5.89 (Tab. II). Spot 28 (Fig. 1B) was identified to

Table I. Proteins identified in the HG of the Carnica winter bees (*Apis mellifera carnica*).

Spot number	pI	MW (kDa)	PMF Sequence coverage	PMF Matched peptides (Total signals)	Score	Protein name	Accession Number
1	5.10	48.86	21.00%	9/13	119	Major royal jelly protein 1 precursor (MRJP-1) [*Apis mellifera*]	MRJP1_APIME
2	6.83	51.04	28.00%	12/36	99	Major royal jelly protein 2 precursor (MRJP-2) [*Apis mellifera*]	MRJP2_APIME
3	6.83	51.04	23.00%	11/24	109	Major royal jelly protein 2 precursor (MRJP-2) [*Apis mellifera*]	MRJP2_APIME
4	6.83	51.04	19.00%	8/18	78	Major royal jelly protein 2 Precursor (MRJP-2)[*Apis mellifera*]	MRJP2_APIME
5	6.83	51.04	23.00%	10/24	93	Major royal jelly protein 2 precursor (MRJP-2) [*Apis mellifera*]	MRJP2_APIME
6	6.47	61.62	19.00%	12/34	78	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
7	6.47	61.62	18.00%	12/25	84	Major royal jelly protein 3 [*Apis mellifera*]	gi|58585142
8	6.47	61.62	19.00%	12/22	101	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
9	6.47	61.62	20.00%	13/47	76	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
10	6.47	61.62	19%	12/25	103	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
11	6.50	61.66	28.00%	16/35	137	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
12	6.50	61.66	18.00%	11/17	105	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
13	6.87	65.66	26.00%	15/57	82	Major royal jelly protein 3 [*Apis mellifera carnica*]	gi|56422035
14	6.51	61.66	28.3%	17/30	d' = 4.9	Major royal jelly protein 3 [*Apis mellifera*]	gi|58585142|
15	6.50	61.66	22.8%	16/28	d' = 4.0	Major royal jelly protein 3 [*Apis mellifera*]	gi|58585142|

Table I. Continued.

Spot number	pI	MW (kDa)	PMF Sequence coverage	PMF Matched peptides (Total signals)	Score	Protein name	Accession Number
16	6.50	61.66	18.70%	14/30	d' = 11.2	Major royal jelly protein 3 [*Apis mellifera*]	gi|58585142
17	6.47	61.62	19.00%	12/26	101	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
18	6.47	61.62	21.00%	11/56	73	Major royal jelly protein 3 precursor (MRJP-3)[*Apis mellifera*]	MRJP3_APIME
19	6.47	61.62	29.00%	14/29	129	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
20	5.90	70.24	26.40%	16/36	d' = 6.9	Major royal jelly protein 5 [*Apis mellifera*]	gi|58585108
21	6.48	67.90	23.00%	13/37	83	Glucose oxidase [*Apis mellifera*]	gi|58585090
22	6.48	67.90	32.00%	16/34	120	Glucose oxidase [*Apis mellifera*]	gi|58585090
23	5.06	65.52	34.00%	20/82	87	Alpha-glucosidase precursor (EC 3.2.1.20) (Maltase) - [*Apis mellifera*]	MAL1_APIME
24	5.88	25.12	46.00%	9/36	84	Peroxiredoxin 2540 CG11765-PA [*Apis mellifera*]	gi|66535082
25	5.65	21.77	34.00%	6/16	81	Thioredoxin peroxidase 1 CG1633-PA, isoform A isoform 1 [*Apis mellifera*]	gi|66548188
26	5.30	41.77	43.00%	17/54	113	Actin-5C isoform 1 [*Apis mellifera*]	gi|48137684

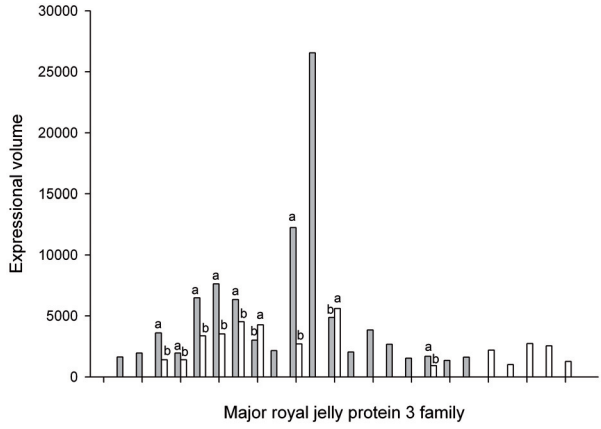
Note: Protein scores greater than 67, 79 and d' $\geqslant$ 4 are significant ($P < 0.05$) in Swissprot, NCBInr and Xproteo database, respectively. Spot Number corresponds to the number of a protein spot in Figure 2A. PMF represents the peptide mass fingerprinting result, a protein identification technique in which MS is used to measure the masses of proteolytic peptide fragments. MW (molecular weight) and pI (isoelectric point) in PMF are the theoretical results identified in Genbank or MSDB. Sequence coverage is the ratio of the number of amino acid in every peptide that matches with the mass spectrum divided by the total number of amino acid in the protein sequence. Matched peptide is the score of pairing of an experimental fragmentation spectrum to a theoretical segment of protein. Accession Number is the unique number given to mark the entry of a protein sequence to a primary or secondary database (i.e. GenBank).

Table II. Proteins identified in the HG of the high RJ producing winter bees (*Apis mellifera* L.).

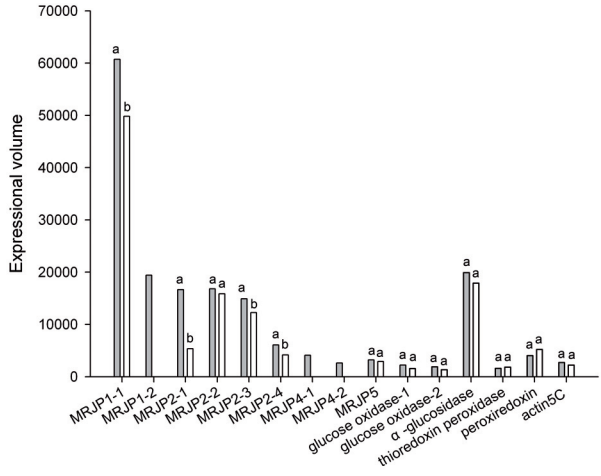
Spot number	pI	MW (kDa)	PMF Sequence coverage	PMF Matched peptides (Total signals)	Score	Protein name	Accession Number
1	5.10	48.86	21.00%	9/13	119	Major royal jelly protein 1 precursor (MRJP-1) [*Apis mellifera*]	MRJP1_APIME
2	5.10	48.86	25.00%	10/25	101	Major royal jelly protein 1 precursor (MRJP-1) [*Apis mellifera*]	MRJP1_APIME
3	6.83	51.04	28.00%	12/36	99	Major royal jelly protein 2 precursor (MRJP-2) [*Apis mellifera*]	MRJP2_APIME
4	6.83	51.04	23.00%	11/24	109	Major royal jelly protein 2 precursor (MRJP-2) [*Apis mellifera*]	MRJP2_APIME
5	6.83	51.04	19.00%	8/18	78	Major royal jelly protein 2 Precursor (MRJP-2) [*Apis mellifera*]	MRJP2_APIME
6	6.83	51.04	23.00%	10/24	93	Major royal jelly protein 2 precursor (MRJP-2) [*Apis mellifera*]	MRJP2_APIME
7	6.47	61.62	16.00%	9/36	68	Major royal jelly protein 3 precursor (MRJP-3)[*Apis mellifera*]	MRJP3_APIME
8	6.47	61.62	24.00%	13/33	103	Major royal jelly protein 3 [*Apis mellifera*]	gi|58585142
9	6.47	61.62	19.00%	12/34	78	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
10	6.47	61.62	18.00%	12/25	84	Major royal jelly protein 3 [*Apis mellifera*]	gi|58585142
11	6.47	61.62	19.00%	12/22	101	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
12	6.47	61.62	20.00%	13/47	76	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
13	6.47	61.62	19.00%	12/25	103	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
14	6.90	65.70	17.90%	11/25	d' = 4.1	Major royal jelly protein 3 [*Apis mellifera carnica*]	gi|56422035
15	6.51	61.66	28.30%	17/30	d' = 4.9	Major royal jelly protein 3 [*Apis mellifera*]	gi|58585142|ref|NP_001011601.1|
16	6.50	61.66	22.80%	16/28	d' = 4.0	Major royal jelly protein 3 [*Apis mellifera*]	gi|58585142|ref|NP_001011601.1|

Spot number	pI	PMF MW (kDa)	Sequence coverage	Matched peptides (Total signals)	Score	Protein name	Accession Number
17	6.50	61.66	18.70%	14/30	d' = 11.2	Major royal jelly protein 3 [*Apis mellifera*]	gi|58585142
18	6.47	61.62	19.00%	12/26	101	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
19	6.47	61.62	16.00%	11/25	88	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
20	6.47	61.62	20.00%	12/21	119	Major royal jelly protein 3 [*Apis mellifera*]	gi|58585142
21	6.47	61.62	42.00%	21/79	136	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
22	6.50	61.66	29.00%	15/49	115	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
23	6.47	61.62	21.00%	11/56	73	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
24	6.47	61.62	29.00%	14/29	129	Major royal jelly protein 3 precursor (MRJP-3) [*Apis mellifera*]	MRJP3_APIME
25	6.47	61.62	14.00%	8/23	70	Major royal jelly protein 3 precursor (mrjp-3) [*Apis mellifera*]	MRJP3_APIME
26	5.89	52.88	20.00%	9/23	85	Major royal jelly protein 4 precursor (MRJP-4) [*Apis mellifera*]	MRJP4_APIME
27	5.89	52.88	22.00%	10/28	92	Major royal jelly protein 4 precursor (MRJP-4) [*Apis mellifera*]	MRJP4_APIME
28	5.90	70.24	26.40%	16/36	d' = 6.9	Major royal jelly protein 5 [*Apis mellifera*]	gi|58585108
29	6.48	67.90	23.00%	13/37	83	Glucose oxidase [*Apis mellifera*]	gi|58585090
30	6.48	67.90	32.00%	16/34	120	Glucose oxidase [*Apis mellifera*]	gi|58585090
31	5.06	65.52	34.00%	20/82	87	Alpha-glucosidase precursor (EC 3.2.1.20) (Maltase) [*Apis mellifera*]	MAL1_APIME
32	5.30	41.77	43.00%	17/54	113	Actin-5C isoform 1 [*Apis mellifera*]	gi|48137684
33	5.88	25.12	46.00%	9/36	84	Peroxiredoxin 2540 CG11765-PA [*Apis mellifera*]	gi|66535082
34	5.65	21.77	34.00%	6/16	81	Thioredoxin peroxidase 1 CG1633-PA, isoform A isoform 1 [*Apis mellifera*]	gi|66548188

Note: Protein scores greater than 67, 79 and d' $\geqslant$ 4 are significant ($P < 0.05$) in Swissprot, NCBInr and Xproteo database, respectively. Spot Number corresponds to the number of a protein spot in Figure 2B. PMF represents the peptide mass fingerprinting result, a protein identification technique in which MS is used to measure the masses of proteolytic peptide fragments. MW (molecular weight) and pI (isoelectric point) in PMF are the theoretical results identified in Genbank or MSDB. Sequence coverage is the ratio of the number of amino acid in every peptide that matches with the mass spectrum divided by the total number of amino acid in the protein sequence. Matched peptide is the score of pairing an experimental fragmentation spectrum to a theoretical segment of protein. Accession Number is the unique number given to mark the entry of a protein sequence to a primary or secondary database (i.e. GenBank).



(a)



(b)

Figure 2. Quantitive analysis of the identified proteins. The gray and the white bars represent protein quantities found in the hypopharyngeal glands of winter worker bees from high royal jelly producing bees and Carnica bees, respectively. (a) represents all the MRJP 3 (Major Royal Jelly Protein 3) and (b) represent the other identified proteins. Identical letters at top right of the figures indicate no significant difference; different letters indicate significant differences. Paired bars indicate the proteins identified in both strains of honeybees. Unpaired bars are proteins identified in only one strain.

be MRJP5 with MW value 70.24 kDa and pI value 5.90 (Tab. II).

3.3. Additional identified proteins in the HG of winter bees from the two strains

Two forms of glucose oxidase (spots 21, 22, Fig. 1A; spots 29, 30, Fig. 1B) with MW values 67.90 kDa and pI values 6.48 (Tabs. I, II) and one α-glucosidase precursor (spot 23, Fig. 1A; spot 31, Fig. 1B) with MW values 65.52 kDa and pI values 5.06 (Tabs. I, II) were identified. These enzymes are thought to participate in the metabolism of carbohydrates and energy.

Three proteins were discovered in the HG of honeybees for the first time. Two of them, peroxiredoxin (spot 24, Fig. 1A; spot 33, Fig. 1B) with MW values 25.12 kDa and pI values 5.88 (Tabs. I, II) and one thioredoxin peroxidase 1 (spot 25, Fig. 1A; spot 34, Fig. 1B) with MW values 21.77 kDa and pI values 5.63 (Tabs. I, II) are thought to perform antioxidative functions. The third protein was identifdied as a member of the actin family, actin 5C isoform 1 (spot 26, Fig. 1A; spot 32, Fig. 1B) with MW values 41.77 kDa and pI values 5.30 (Tabs. I, II).

Some of the spots labeled in Figure 1 were not listed in Tables I and II because either their analysis resulted in poor spectral quality due to a noise background, or protein amounts too low to get reliable PMF data.

4. DISCUSSION

4.1. MRJPs

MRJPs play an important role in honeybee nutrition. MRJP1 may be involved in processing honeybee products besides its nutritive functions (Simuth, 2001). The previous study illustrates that MRJP1 may present different variant forms with MW values around 55 kDa and pI values ranging from 4.50 to 5.20 (Hanes and Simuth, 1992). Six different forms of MRJP1 were identified in the protein complement of the HG of Africanized nurse-bees with MW from 48.81 to 60.00 kDa and pI from 4.23 to 5.50 (Santos et al., 2005). The MW of MRJP1 identified in this study (Fig. 1; Tabs. I, II) differs from that estimated by Hanes and Simuth (1992), but was in accordance with Santos et al. (2005). That MRJP1 may present variant forms with different MW and pI is probably due to the presence of potential glycosylation sites as revealed by the NCBI protein databank (http://www.ncbi.nlm.nih.gov/) (Santos et al., 2005). Sano et al. (2004) identified only two different forms of this protein in the RJ of Africanized honeybees and one form in European honeybees. Six different forms were found in the HG of Africanized nurse honeybees (Santos et al., 2005). Due to the experimental design it could not be determined at present if the most frequent forms of MRJP1 are retained, degraded or even metabolically used by the winter bees.

MRJP2 and MRJP3 are thought to store processable forms of nitrogen (Albert et al., 1999). The MW and pI of MRJP2 identified in this study were in accordance with that of Santos et al. (2005) Using the NCBI protein databank (http://www.ncbi.nlm.nih.gov/), it is possible to identify two potential glycosylation sites which suggests the possible existence of different forms of MRJP 2, presenting different degrees of glycosylation (Santos et al., 2005). It is believed that all the forms of this protein observed in the secretion of HG were also present in the RJ as described by Sano et al. (2004). However, the MW (61.62–65.70 kDa) and pI (6.47–6.90) of MRJP3 in this study (Fig. 1; Tabs. I, II) were lower than those identified in the HG of Africanized nurse honeybees (MW 80.59–87.00 kDa and pI 7.05–8.04; Santos et al., 2005). This may be caused by the extensive repetitive regions in the C-terminal region and by various sugar chains attached to the protein (Sano et al., 2004). Ten and twenty-four different forms of MRJP3 are reported in RJ of Africanized honeybees and European honeybees, respectively (Santos et al., 2005; Sano et al., 2004). MRJP3 also showed the highest number of isoforms in the present study. This could be attributable to its polymorphism in a region with a variable number of tandem repeats (VNTR) located at the C-terminal part of the coding

region (Albert et al., 1999). PCR analyses have confirmed the presence of an extensive repetitive region that showed inter and intraspecific polymorphisms in size and sequence in four honeybee species (*Apis mellifera*, *Apis cerana*, *Apis dorsata*, and *Apis florea*). The repetition is suggested to be due to a selection for an increase in nitrogen storage which results in a more efficient nutrition of queens and larvae (Albertova et al., 2005).

MRJP4 and MRJP5 both supply essential amino acids. In particular MRJP5 stores biologically accessible nitrogen required for the fast developing organism (Albert et al., 1999). Five and two MRJP4 isoforms were identified in the RJ of Africanized and European honeybees, respectively (Sano et al., 2004). None of these were found in the HG of Africanized nurse-bees (Santos et al., 2005). Two different isoforms of MRJP4 were identified in the HG of high RJ producing winter bees (Fig. 1B, Tab. II), but not in those of Carnica winter bees (Fig. 1A, Tab. I). It is not clear whether this may be due to a genetic difference between high RJ producing honeybees and Carnica honeybees leading to a lack of MRJP4 production in the HG of Carnica winter bees.

Only one form of MRJP5 (MW 70.24 kDa and pI value 5.90) (Fig. 1; Tabs. I, II) was identified in the two lines of honeybees in this study, and its MW was lower than that of proteins in the HG of Africanized nurse honeybees (79.08 to 79.47 kDa and 6.34 to 6.80) (Santos et al., 2005). Taking into account a single copy of the gene of MRJP5, the heterogeneity in MW and pI may be a result of post-translational modifications. Three protein modifications in HG secretions from Africanized nurse-bees were found (Santos et al., 2005). In the RJ of Africanized and European honeybees, respectively seven and four different forms of this protein were identified (Sano et al., 2004).

The HGs in most of the Hymenoptera secrete food and enzymes for their nestmates (Cruz-Landim and Costa, 1998). Nurse bees secrete RJ for feeding brood with very high rates of metabolism (Costa and Cruz-Landim, 2005). The MRJPs in the HG of winter bees are probably produced and stored for brood rearing in early spring. Glands are loaded by such accumulations, and when circumstances change in spring they can directly discharge their stored secretions (Deseyn and Billien, 2005). This behavior reflects the functional flexibility of the HG of the workers (Ohashi et al., 2000). At the same time it provides supporting evidence that MRJPs are produced by the HG and then secreted into the RJ (Santos et al., 2005). The present study identified more MRJPs in HG of high RJ producing bees than that in Carnica bees (Figs. 1, 2; Tabs. I, II), indicating that there is more stored nitrogen in the HG of high RJ producing winter bees for subsequent brood rearing.

4.2. Glucose oxidase and α-glucosidase

The HG regresses after the nursing stage of worker bees but it still remains active (Costa and Cruz-Landim, 2000; Huang et al., 1989). Invertase and glucose oxidase are also produced (Takenaka et al., 1990; Ohashi et al., 1996, 1999; Santos et al., 2005). Glucose oxidase is needed to convert glucose to gluconic acid and hydrogen peroxide which have an antimicrobial action. In the HG of Africanized nurse-bees one and in their RJ, five different forms of glucose oxidase are presente, with MW values around 85 kDa (Sano et al., 2004; Santos et al., 2005). The lower MW of the two glucose oxidases identified in this study (Fig. 1; Tabs. I, II) may be due to previously observed variations in MW and pI (Sano et al., 2004).

An α-glucosidase was found in the HG extract of *A. mellifera* (Santos et al., 2005). The MW of α-glucosidase identified in the present study (Fig. 1; Tabs. I, II) was compatible with the 70 kDa previously observed (Ohashi et al., 1997). The HG is the only gland in the salivary system of *A. mellifera* that has α-glucosidase activity (Arnold and Delage-Darchen, 1978). It can catalyze the digestion of polysaccharides and perform the final steps in starch digestion. It is highly active in the HG of foragers of *A. mellifera* (Costa and Cruz-Landim, 2005), converting sucrose in nectar to glucose and fructose, which is essential for the conversion of nectar into honey.

These results constitute strong evidences that the HG has flexible functions. Winter bee HGs secrete enzymes for glucose metabolism and these enzymes may equally serve as a protection against oxidative stress and be connected with the longevity of winter bees. During the winter they may also be used for digesting the stored pollen bread being transfered to other bees during trophollaxis (Costa and Cruz-Landim, 2005), or deposited for honey processing in the coming spring.

4.3. New proteins identified in winter bee HG

To our knowledge, this study is the first to find peroxiredoxin, thioredoxin peroxidase 1 (TxP-1) and act5C in the HG of honeybees. Peroxiredoxin and TxP-1 may perform antioxidation functions in the honeybees' HG. Peroxiredoxins are a superfamily of nonheme and nonselenium peroxidases that are widely distributed throughout all phyla (Hofmann et al., 2002; Wood et al., 2003). They can play a major protective role in animal cells against reactive oxygen. In addition to their protective antioxidant role, it has been suggested that peroxiredoxins are involved in cell signaling, apoptosis, cell differentiation and other regulatory processes (Choi et al., 1998; Delaunay et al., 2002; Wood et al., 2003). Peroxiredoxin containing only one conserved cysteine (1-CysPrx) is expressed in all tissues, but at particularly high levels in brain, eyes and testes (Kim et al., 1998; Singh and Shichi, 1998). 1-cysPrx can reduce phospholipid and other hydroperoxides (Fisher et al., 1999) and protects against cellular membrane damage (Simuth et al., 2004). TxP-1 belongs to a highly efficient antioxidant family. It scavenges H_2O_2 using thioredoxin-1 (Trx-1) as a source of reducing equivalents, and plays an important role in eliminating peroxides generated during metabolism. These proteins may act as antioxidants to remove the metabolism waste of winter bees' HG and delay the aging process of winter bees. They have not been identified in RJ of Africanized and European nurse honeybees (Sano et al., 2004; Santos et al., 2005; Scarselli et al., 2005). This is probably due to differences in the strains, as well as ecological and seasonal conditions.

The actin family member act5C is one of the most abundant and highly conserved proteins in eukaryotes. It is very critical for a variety of cellular functions including determination of cell shape, cell motility, cytokinesis, intracellular transport and muscle contractility (Wagner et al., 2002). Act5C is one of the isforms of cytoplasmic actins. Act5c, including act42A, encodes the cytoskeletal actins of nonmuscle cells (Bond and Davidson, 1986). In *Drosophila*, the relative levels of transcripts for the two genes vary in particular tissues. Act5C mRNA levels are highest in the developing brain, whereas act42A mRNA levels are most abundant in the developing gonad and in portions of the intestine (Tobin et al., 1990). Act5C gene expression in certain cells supplies actin for normal functions (Sheterline et al., 1999). This abundant protein probably aids in maintaining the normal function of the HG during the winter season. It has not been found previously (Sano et al., 2004; Santos et al., 2005; Scarselli et al., 2005). The presently identified Act5C may have leaked from cells of glandular tissue during extract preparation since the whole HG was homogenized.

In conclusion, winter bees secrete a complex of proteins that are very similar to those found regularly in RJ. MRJP3 seems overrepresented, whereas MRJP5 is underrepresented in the HG of winter bees. We noted a significantly higher numbers of proteins to be present in the HG of high RJ producing winter bees when compared to Carnica winter bees. Quantities of most MRJPs were also significantly higher in high RJ producing winter bees than in Carnica winter bees. The HG of high RJ producing winter bees store more MRJPs for brood rearing and show higher activity than Carnica winter bees. Secretions are produced but not released. The present study provides evidence for the functional flexibility of the workers and for HG protein storage during the winter season. Three novel proteins, two peroxiredoxins and thioredoxin peroxidase 1 related to antioxidation and one highly conserved actin 5C isoform 1 related to maintainance of the normal function for the HG,

were found in the HG of honeybees for the first time.

ACKNOWLEDGEMENTS

This work was funded by the National 11th five-year development program (2006-10). Key projects were 2006BAD06B04 (Research and demonstration of bee-products security and applications), 2006BAD12B08-06 (Integration and demonstration of balanced feed for ruminant and aquatic animals) and JBKY0032007016CAAS (Basic scientific research found of Chinese academy of agricultural science) and special scientific and research fund for public welfare industy (nyhyzx07-041). We thank John Kefuss, Toulouse, France; Denis Walsh, Pocatello, Idaho, USA; and Joel Kefuss, Flagstaff, Arizona, USA, for language editing.

Identification du protéome des glandes hypopharyngiennes de deux lignées d'abeilles domestiques (*Apis mellifera*).

***Apis mellifera* / abeille d'hiver / glande hypopharyngienne / protéome**

Zusammenfassung – Analyse des Proteoms der Hypopharynxdrüsen von zwei Zuchtlinien der Honigbiene (*Apis mellifera*). Mit der Verfügbarkeit eines komplett sequenzierten Genoms ergeben sich jetzt neue Einsichten sowohl für die Honigbiene selbst, als auch für Vergleiche mit anderen Arten. Obwohl einzelne Gene, Proteine und Enzyme des Metabolismus bereits seit einiger Zeit intensiv untersucht werden, stecken generelle Proteomanalysen, z.B. zum Proteom der Hypopharynxdrüsen, noch in den Anfängen. Ziel dieser Studie war eine systematische Untersuchung der Proteinzusammensetzung der Hypopharynxdrüse (HD) von Winterbienen. Der experimentelle Ansatz bestand in einer zweidimensionalen elektrophoretischen Auftrennung gefolgt von MALDI-TOF Analysen der einzelnen Proteinspots.
Aus der Gesamtzahl der Proteine, die in den HD von Winterbienen zu finden waren, identifizierten wir 26 Spots für die HD von Carnica-Arbeiterinnen (Abb. 1A; Tab. I) und 34 Spots für die HD einer chinesischen Zuchtlinie, die in hohen Mengen Gelée royale produziert (viel-Gelée-royal-Linie) (Abb. 1B; Tab. II). Die meisten dieser Proteine (20 für Carnica und 34 für die viel-Gelée-royal-Linie) waren Gelée royal Proteine (GRPs) und dies überwiegend Isoformen von GRP 3. Mit Bezug zum Kohlenhydratstoffwechsel fanden wir drei Proteine, zwei Glucoseoxidasen und eine alpha-Glucosidase. Drei Proteine wurden in dieser Studie zum ersten Mal für Honigbienen beschrieben. Zwei dieser Proteine, Peroxiredoxin und Thioredoxinperoxidase haben eine antioxidante Funktion. Das dritte Protein war ein Actin, die Isoform 1 von Act5C. Die Gesamtzahl der für die viel-Gelée-royal-Linie identifizierten Proteine lag deutlich über der für Carnica Winterbienen, vor allem was die GRPs angeht. Keine Unterschiede zwischen den beiden Linien fanden wir für drei der Kohlenhydratstoffwechselproteine und die drei neubeschriebenen Proteine (Abb. 2B). Diese vorläufigen Ergebnisse zum Proteom der Hypoharynxdrüse deuten darauf hin, dass diese Drüse bei Winterbienen eine Speicherfunktion haben könnte. Ausserdem konnten wir zeigen, dass die HD der viel-Gelée-royal-Linie mehr Proteine enthält als die der Carnica Arbeiterinnen. Da diese Studie nur einen Teil der Proteine des Gesamproteoms der HD identifizieren konnte, sind weitere Untersuchungen erforderlich.

***Apis mellifera* / Winterbienen / Hypopharynxdrüse / Proteom**

REFERENCES

Albert S., Klaudiny J., Simuth J. (1999) Molecular characterization of MRJP3, highly polymorphic protein of honeybee (*Apis mellifera*) royal jelly, Insect. Biochem. Mol. Biol. 29, 427–434.

Albertova V., Su S.K., Brockmann A., Gadau J., Albert S. (2005) Organization and potential function of the mrjp3 locus in four honeybee species, J. Agric. Food Chem. 53, 8075–8081.

Amdam G.V., Omholt S.T. (2002) The regulatory anatomy of honeybee lifespan, J. Theor. Biol. 216, 209–228. A. K.

Arnold G., Delage-Darchen B. (1978) Nouvelles données sur l'équipement enzymatique des glandes salivaires de l'ouvrière d'*Apis mellifica* (Hyménoptère, Apidé), Ann. Sci. Nat. Zool. Biol. Anim. 12, 401–422.

Bond B.J., Davidson N. (1986) The *Drosophila melanogaster* actin 5C gene uses two transcription initiation sites and three polyadenylation sites to express multiple mRNA species, Mol. Cell. Biol. 6, 2080–2088.

Bradford M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding, Anal. Biochem. 72, 248–254.

Choi H.J., Kang S.W., Yang C.H., Rhee S.G., Ryu S.E. (1998) Crystal structure of a novel human peroxidase enzyme at 2.0 Å, Nat. Struct. Biol. 5, 400–406.

Costa R.A.C., Cruz-Landim C. (2000) Comparative study of the ultrastructure and secretory dynamic

of hypopharyngeal glands in queens, workers and males of *Scaptotrigona postica* Latreille (Hymenoptera, Apidae, Meliponinae), Biocell 24, 39–48.

Costa R.A.C., Cruz-Landim C. (2005) Hydrolases in the hypopharyngeal glands of workers of *Scaptotrigona postica* and *Apis mellifera* (Hymenoptera, Apinae), Genet. Mol. Res. 4, 616–623.

Cruz-Landim C., Costa R.A.C. (1998) Structure and function of the hypopharyngeal glands of Hymenoptera: a comparative approach, J. Comp. Biol. 3, 151–163.

Delaunay A., Pflieger D., Barrault M.B., Vinh J., Toledano M.B. (2002) A thiol peroxidase is an H_2O_2 receptor and redox- transducer in gene activation, Cell 111, 471–81.

Deseyn J., Billen J. (2005) Age-dependent morphology and ultrastructure of the hypopharyngeal gland of *Apis mellifera* workers (Hymenoptera, Apidae), Apidologie 36, 49–57.

Fisher A.B., Dodia C., Manevich Y., Chen J.W., Feinstein S.I. (1999) Phospholipid hydroperoxides are substrates for non-selenium glutathione peroxidase, J. Biol. Chem. 274, 21326–21334.

Hanes J., Simuth J. (1992) Identification and partial characterization of the major royal jelly protein of the honey bee (*Apis mellifera* L.), J. Apic. Res. 31, 22–26.

Hofmann B., Hecht H.J., Flohe L. (2002) Peroxiredoxins, Biol. Chem. 383, 347–364.

Hrassnigg N., Crailsheim K. (1998) Adaptation of hypopharyngeal gland development to the brood status of honeybee (*Apis mellifera* L.) colonies, J. Insect Physiol. 44, 929–939.

Huang Z.Y., Otis G.M., Teal P.E.A. (1989) Nature of brood signal activating the protein synthesis of hypopharyngeal gland in honey bees, *Apis mellifera* (Apidae: Hymenoptera), Apidologie 20, 455–464.

Huang Z.Y., Robinson G.E. (1996) Regulation of honey bee division of labor by colony age demography, Behav. Ecol. Sociobiol. 39, 147–158.

Kim T.S., Dodia C., Chen X., Hennigan B.B., Jain M., Feinstein S.I., Fisher A.B. (1998) Cloning and expression of rat lung acidic Ca^{2+}-independent PLA2 and its organ distribution, Am. J. Physiol. Lung Cell Mol. Physiol. 274, L750–L761.

Kubo T., Sasaki M., Namura J., Sasagawa H., Ohashi K., Takeuchi H., Natori S. (1996) Change in the expression of hypopharingeal-gland proteins of the worker honeybees (*Apis mellifera* L.) with the age and/or role, J. Biochem. 119, 291–295.

Li J.K., Wang T., Peng W.J. (2007) Comparative analysis of the effects of different storage conditions on major royal jelly proteins, J. Apic. Res. 46, 73–81.

Ohashi K., Sawata M., Takeuchi H., Natori S., Kubo T. (1996) Molecular cloning of cDNA and analysis of expression of the gene for a-glucosidase from the hypopharyngeal gland of the honeybee *Apis mellifera* L., Biochem. Biophys. Res. Commun. 221, 380–385.

Ohashi K., Natori S., Kubo T. (1997) Change in the mode of gene expression of the hypopharyngeal gland cells with an age-dependent role change of the worker honeybee *Apis mellifera* L., Eur. J. Biochem. 249, 797–802.

Ohashi K., Natori S., Kubo T. (1999) Expression of amylase and glucose oxidase in the hypopharyngeal gland with an age-dependent role change of the worker honeybee (*Apis mellifera* L.), Eur. J. Biochem. 265, 127–133.

Ohashi K., Sasaki M., Sasagawa H., Nakamura J., Natori S., Kubo T. (2000) Functional flexibility of the honey bee hypopharyngeal gland in a dequeened colony, Zool. Sci. 17, 1089–1094.

Otis G.W., Wheeler D.E., Buck N., Mattila H.R. (2004) Storage proteins in winter honey bees, Apiacata 38, 352–357.

Painter T.S., Biesele J.J. (1966) The fine structure of the hypopharyngeal gland cell of the honey bee during development and secretion, Zoology 55, 1414–1419.

Sano O., Kunikata T., Kohno K., Iwaki K., Ikeda M., Kurimoto M. (2004) Characterizaton of royal jelly proteins in both Africanized and European honeybees (*Apis mellifera*) by two-dimensional gel electrophoresis, J. Agric. Food Chem. 52, 15–20.

Santos K.S., Santos L.D., Mendes M.A., Souza B.M., Malaspina O., Palma M.S. (2005) Profiling the proteome complement of the secretion from hypopharyngeal gland of Africanized nurse-honeybees (*Apis mellifera* L.), Insect Biochem. Mol. Biol. 35, 85–91.

Sasagawa H., Sasaki M., Okada I. (1989) Hormonal control of the division of labor in adult honeybees (*Apis mellifera* L.). I. Effect of methoprene on corpora allata and hypopharyngeal gland, and its α-glucosidase activity, Appl. Entomol. Zool. 24, 66–77.

Scarselli R., Donadio E., Giuffrida M.G., Fortunato D., Conti A., Balestreri E., Felicioli R., Pinzauti M., Sabatini A.G., Felicioli A. (2005) Towards royal jelly proteome, Proteomics 5, 769–776.

Sheterline P., Clayton J., Sparrow J.C. (1999) Protein Profile: Actin, Oxford University Press, Oxford, UK.

Silva de Moraes R.L., Bowen I.D. (2000) Modes of cell death in the hypopharyngeal gland of honey bee (*Apis mellifera* L), Cell Biol. Int. 24, 737–743.

Simuth J. (2001) Some properties of the main protein of honey bee (*Apis mellifera*) royal jelly, Apidologie 32, 69–80.

Simuth J., Bilikova K., Kovacova E., Kuzmoya Z., Schroder W. (2004) Immunochemical approach to detection of adulteration in honey: physiologically active royal jelly protein stimulating TNF-alpha

release is a regular component of honey, J. Agric. Food Chem. 52, 2154–2158.

Singh A.K., Shichi H. (1998) A novel glutathione peroxidase in bovine eye: sequence analysis, mRNA level, and translation, J. Biol. Chem. 273, 26171–26178.

Takenaka T., Ito H., Yatsumami K., Echigo T. (1990) Changes of glucose oxidase activity and amount of gluconic acid formation in the hypopharyngeal glands during lifespan of honey bee workers (*Apis mellifera* L.), Agric. Biol. Chem. 54, 2133–2134.

The Honeybee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee, *Apis mellifera*, Nature 443, 931–949.

Tobin S.L., Cook P.J., Burn T.C. (1990) Transcripts of individual Drosophila actin genes are differentially distributed during embryogenesis, Dev. Genet. 11, 15–26.

Wagner C.R., Mahonald A.P., Miller K.G. (2002) One of the two cytoplasmic actin isoforms in *Drosophila* is essential, Proc. Natl. Acad. Sci. USA 99, 8037–8042.

Wood Z.A., Schroder E., Robin Harris J., Poole L.B. (2003) Structure, mechanism and regulation of peroxiredoxins, Trends Biochem. Sci. 28, 32–40.