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Identification of proteins whose expression is up- or down-regulated in the mushroom bodies in the honeybee brain using proteomics

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Abstract **To identify protein(s) with different expression patterns in the mushroom bodies (MBs) in the honeybee brain, we compared the protein profiles of MBs and optic lobes (OLs) using proteomics. Two-dimensional gel electrophoresis revealed that five and three spots were selectively expressed in the MBs or OLs, respectively. Liquid chromatography tandem mass spectrometry analysis identified juvenile hormone diol kinase and glyceraldehyde-3-phosphate dehydrogenase as MB- and OL-selective proteins, respectively. In situ hybridization revealed that *jhdk* expression was upregulated in MB neuron subsets, whereas *gapdh* expression was downregulated, indicating that MBs have a distinct gene and protein expression profile in the honeybee brain.**
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1. Introduction

The honeybee (*Apis mellifera* L.) is a social insect and the female adults differentiate into two castes, reproductive queens and sterile workers [1]. The workers exhibit various social behaviors, such as an age-dependent division of labor from nursing to guarding, and then foraging. In addition, foragers transmit information about the food source to their nestmates using symbolic dance communication [2]. The molecular and neural bases that underlie such honeybee social behaviors, however, remain largely unknown [3].

The mushroom bodies (MBs), which are a higher center in the insect brain, are prominently developed in the honeybee and their proportions change according to the division of labor [4], suggesting their association with honeybee social behavior. To identify candidate genes involved in honeybee social behavior, we used the differential display method and cDNA microarray to screen for genes expressed preferentially in the MBs [5–9]. A gene whose expression in the MBs differs depending on the division of labor has also been identified [10]. These findings indicate that the MBs have distinct gene expression profiles in the honeybee brain, which might underlie specific MB function.

Gene expression is regulated both at the transcriptional and post-transcriptional level, such as through translational regulation and protein stability against proteolysis [11]. The recent completion of the honeybee genome project [12] enables us to identify small amounts of proteins using mass spectrometry. Proteomics have not previously been used to search for proteins expressed in the honeybee brain. In the present study, we first used proteomics to search for protein(s) whose expression is up- or down-regulated in the MBs in the honeybee brain.

2. Materials and methods

2.1. Sample preparation and two-dimensional gel electrophoresis

European honeybee *Apis mellifera* L. colonies maintained at the University of Tokyo were used. Nurse bees, guards, attackers, and foragers were collected according to their behaviors, as described previously [9]. MBs and optic lobes (OLs), dissected from three workers that showed the same behavior, were homogenized in rehydration solution containing 8 M urea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.28% dithiothreitol, 0.5% IPG buffer (pH 3–10, GE Healthcare, UK), followed by centrifugation. Aliquots of supernatant containing 100 μg of protein were then applied to Immobiline Dry Strips (pH 3–10, 11 cm, GE Healthcare) and isoelectrically focused on a Multiphor II (GE Healthcare) according to the manufacturer's instructions. The second dimension of separation was performed on 12.5% SDS–polyacrylamide gels. The experiment was repeated 16 times with 48 workers (three workers for each experiment) to examine the reproducibility of the results. Gel images were acquired on a FMBIO III Image Scanner (Hitachi Software Engineering, Japan) after staining with SYPRO Ruby protein gel stain (Molecular Probes, Carlsbad, CA).

2.2. Identification of protein spots

In-gel tryptic digestion and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis were performed by the Environmental Research Center in Tsukuba, Japan. Proteins were identified using a Mascot server (Version: 2.0, Matrix Science, UK) and the NCBInr database (http://www.ncbi.nlm.nih.gov/).

2.3. Protein alignment and phylogenetic analysis

The amino acid sequences of juvenile hormone diol kinase (JHDK) homologs were aligned using the DNASIS Pro software (Hitachi Software Engineering). The phylogenetic analysis was performed with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amino acid sequences and the neighbor-joining method using the Clustal W program [13]. The statistical significance of branch order was estimated by performing 1000 bootstrap re-sampling iterations of the original aligned sequences.

2.4. In situ hybridization analysis

In situ hybridization was performed using frozen sections (10 μm) of forager brains, as described previously [10]. The digoxigenin (DIG)-labeled sense or antisense RNA probes corresponding to +52/+510

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of *jhdk* cDNA (GenBank Accession number XM_393500.3) and +73/+503 of *gapdh* cDNA (GenBank Accession number XM_393605.3) were prepared by in vitro transcription using a DIG RNA labeling kit (Roche, Switzerland).

3. Results

3.1. Screening and identification of brain-region selective proteins

First, to screen protein(s) whose expression is differently regulated in the MBs in the honeybee brain, protein profiles of MBs and OLs were compared using two-dimensional gel electrophoresis (Fig. 1A). Optic lobe-selective proteins were examined as candidate proteins whose expression is downregulated in the MBs. Five (spots 1, 2, 3, 4, and 8 in Fig. 1A and B) and three spots (spots 5, 6, and 7 in Fig. 1A and B) were detected reproducibly as MB- and OL-selective, respectively. Although we also compared protein profiles of MBs from workers engaged in different labors, differential expression depending on labor was barely detected (data not shown).

Next, by comparing the mass data obtained from LC-MS/MS with the NCBInr database, two of the MB-selective spots (spot 3: 42 kDa with an acidic pI, and spot 4: 22 kDa with an acidic pI) and one of the OL-selective spots (spot 7: 45 kDa with a basic pI) were identified. Their molecular weight and pI estimated from the gel image were consistent with those predicted from the database (Table 1).

Among them, spot 3 was identified as a cAMP-dependent protein kinase (PKA) type II regulatory chain (Table 1). Honeybee PKA is expressed preferentially in both the MBs and antennal lobes (ALs) [14], which is consistent with our results.

Spot 4 was identified as a predicted protein encoded by *GB10018* (gene number assigned by the honeybee genome project; Table 1). Database analysis revealed that GB10018 is highly homologous to *Anopheles gambiae* ENSANGP00000020907 (function unknown), *Manduca sexta* and *Bombyx mori* JHDK [15–17], and *Drosophila melanogaster* CG14904 (Fig. 2).

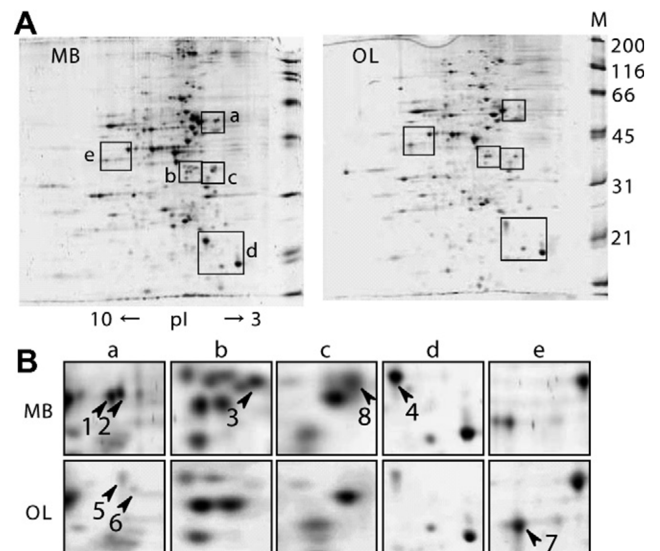

Fig. 1. Identification of proteins differently expressed between the MBs and OLs. (A) Comparison of the protein profiles of the MBs (left) and OLs (right) using two-dimensional gel electrophoresis. The numbers on the right indicate positions of molecular weight markers (M) in kDa. Squares (a–e) indicate regions corresponding to protein spots detected differentially between MBs and OLs. (B) Magnified images of the squares shown in (A). Upper and lower panels indicate proteins detected in the MBs and OLs, respectively. Arrowheads indicate MB- and OL-preferential spots, respectively.

CG14904 is also registered as JHDK, sarcoplasmic calcium-binding protein II (SCPII), and calexcitin identified in Cephalopodan [18]. The calcium-binding EF-hand and nucleotide-binding motifs as well as the overall helix-loop structure [16,17], are also conserved in GB10018 (Fig. 2), indicating that GB10018 is a honeybee homolog of JHDK/SCPII/calexcitin.

Finally, spot 7 was identified as a predicted protein encoded by *GB14798* (Table 1). The phylogenetic analysis revealed that GB14798 belongs to the GAPDH family, indicating that

Table 1
Summary of MB- or OL-selective proteins identified by two-dimensional gel electrophoresis and LC-MS/MS

Spot number	Brain region	2DE		LC-MS/MS			Predicted protein		
		MW (kDa)	pI	Gene number	Peptide sequences	Score	Protein	MW (kDa)	pI
3	MB	42	Acidic	GB14637	QGDDGDNFYVIER DQTDMESLIHTYDNR GAFGELALLYNMPR MNLADALVPK QGDSADGMYFVEDGIVR LGPCMELMK VPAGGYLGELALVTHKPR	166	cAMP-dependent protein kinase type II regulatory chain	43.9	4.9
4	MB	22	Acidic	GB10018	PLSEFR DLDLAIQR DTLLK	79	Juvenile hormone diol kinase	21.5	4.8
7	OL	45	Basic	GB14798	IGINGFGR IENDQLVVNGNK IAVFSER AGAEYIVESTGVYTTK GACQNIIPAATGAAK VIPALDGK VPVHNVSVVDLTVR AGIALNNNFVK	177	Glyceraldehyde-3-phosphate dehydrogenase	36.3	8.1

2DE, two-dimensional gel electrophoresis; MW, molecular weight; Score, total Mascot score of the spot.

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