# Biochemical characterization, molecular cloning and localization of a putative odorant-binding protein in the honey bee Apis mellifera L. (Hymenoptera: Apidea)

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# Biochemical characterization, molecular cloning and localization of a putative odorant-binding protein in the honey bee *Apis mellifera* L. (Hymenoptera: Apidea)

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**Abstract A honey bee antennal water-soluble protein, APS2, was purified and characterized as the first Hymenoptera putative odorant-binding protein. Comparison of its measured $M_r$ (13 695.2 ± 1.6) to that of the corresponding cDNA clone shows it does not undergo any post-translational modification other than a 19-residue signal peptide cleavage and formation of three disulfide bridges. These biochemical features are close to those of Lepidoptera odorant-binding proteins. In situ hybridization experiments demonstrated its specific expression in olfactory areas. Based on its higher expression in the worker than in the drone, ASP2 might be more involved in general odorant than in sex pheromone detection.**



*Key words:* Odorant-binding protein; Post-translational modification; Sequence; Sexual dimorphism; Antenna; *Apis mellifera* L.

## 1. Introduction

In insects, hydrophobic odorant molecules are thought to be translocated from the air to the receptor proteins of the sensory dendrites by soluble proteins, the so-called odorant-binding proteins (OBPs), major protein components of the sensillum liquor [1]. The pioneer work of binding experiments in Lepidoptera species [1] led to the distinction of a category of such proteins involved in sex pheromone detection, namely the pheromone-binding proteins (PBPs), which are largely male-specific [2]. The differential location of PBPs in diverse categories of olfactory sensilla together with their binding specificity supports the hypothesis of a functional implication in ligand transport and/or selection [3–6]. Other moth proteins homologous to the PBPs and commonly found in both sexes were called general odorant-binding proteins (GOBP), since there is no direct evidence of odorant-binding properties [2]. PBPs and GOBPs share common biochemical properties such as high hydrophilicity, low molecular weight (15–21 kDa) and acidic isoelectric point (4–5) [5,6].

The behavioral and neurobiological mechanisms underlying the striking odor discrimination ability of the worker honey bee have been thoroughly investigated [7,8]. The related molecular mechanisms have been considered only recently [9]. In this context the characterization of OBP-like proteins is of interest. Considered from their respective odor repertoires, foragers and drones are largely different [10]. We took advantage of these sexual differences to characterize a putative OBP which was more abundant in workers than in drones. Its cDNA was cloned and sequenced and its antennal localization determined by in situ hybridization.

## 2. Materials and methods

### 2.1. Tissue preparation and protein extraction

Honey bees *Apis mellifera* L., workers and drones, were reared in natural conditions (hive). Antennae, legs, brain, thorax and hemolymph were isolated from adults, homogenized in PBS, EDTA 5 mM, pH 7.4, PMSF 1 mM, chimostatin (Boehringer Mannheim) 1 μg/ml, antipain (Boehringer Mannheim) 1 μg/ml, leupeptin (Boehringer Mannheim) 1 μg/ml, pepstatin (Boehringer Mannheim) 1 μg/ml, *N*-tosyl-L-phenylalanine chloromethyl ketone (Sigma) 1 μg/ml, *N*-tosyl-L-alanine chloromethyl ketone (Sigma) 1 μg/ml. Sediments were removed by centrifugation for 5 min at 4°C, 12 000 × *g*. Water-soluble

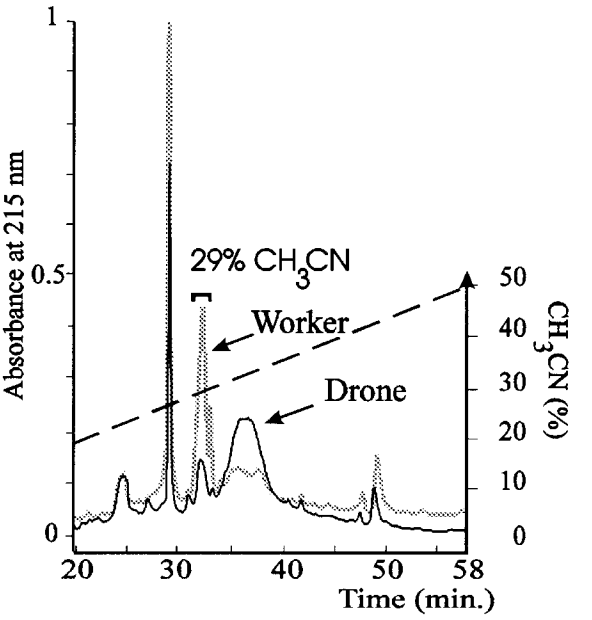


Fig. 1. Compared chromatographic profiles of worker and drone water-soluble antenna-specific protein. RPLC fractionation of water-soluble proteins from 100 antennae of workers or drones on a 2.1 × 100 mm Aquapore column in a 25 mM $CH_3COONH_4$, pH 6.8/$CH_3CN$ system. A linear gradient of 9.5–50% $CH_3CN$ was applied in 50 min at a flow rate of 200 μl/min. Dashed line indicates the $CH_3CN$ gradient.

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*Abbreviations:* DIG, digoxigenin; GOBP, general odorant-binding protein; IS-MS, ion-spray mass spectrometry; OBP, odorant-binding protein; PBP, pheromone-binding protein; RPLC, reverse-phase high performance liquid chromatography


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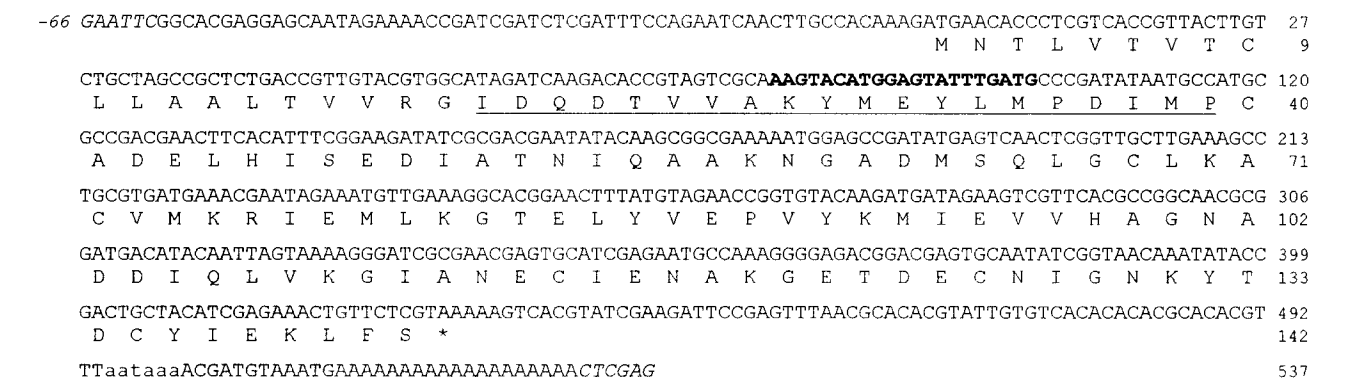


Fig. 2. Nucleotide and deduced amino acid sequences of O12.7 antennal cDNA clone from *Apis mellifera* L. workers. *Eco*RI and *Xho*I cloning sites used for cDNA library synthesis are indicated in italic characters. The 5′ RACE primer position is indicated in bold-faced characters and the presumed polyadenylation site AATAAA in lower-case characters. Asterisk marks the termination codon. The nucleotides and amino acids are numbered at the right end of each line, with the starting codon as a reference. The N-terminal sequence of the purified mature protein ASP2, determined by Edman sequencing, is underlined.

and membrane-associated proteins were separated by ultracentrifugation for 45 min, 140 000×*g* (average) at 4°C (Beckman L5, rotor Ti 60).

*2.2. Protein purification and characterization*

Water-soluble proteins from antennae were separated by RPLC online coupled with both IS-MS (Sciex API100, Perkin-Elmer) and a Perkin Elmer model 173 microblotter. RPLC was run using a Perkin-Elmer device (Applied Biosystems Inc. pump 140C and detector 785) on a 2.1×100 mm C8 Aquapore column (300 Å) in a 25 mM $CH_3COONH_4$ pH 6.8 using $CH_3CN$ linear gradients (9.5% for 5 min, from 9.5% to 50% in 50 min at a flow rate of 200 μl/min). After being monitored for absorbance at 215 nm, the flow was split between the microblotter (160 μl/min) equipped with a Problot Perkin Elmer PVDF membrane and the ion-spray source (40 μl/min). IS-MS experiments were controlled with the Sample Control 1.1 software using a positive mode from 600 to 3000 amu with 0.2 amu steps and a 0.4 ms dwell-time. The ion spray needle voltage was +5000 V and the orifice plate voltage +40 V. Mass spectrometry data were analyzed with the Perkin Elmer Sciex Bio-Multi-View 1.2 software. The blotted PVDF membranes were cut according to the eluted peak positions and submitted to Edman microsequencing using a Perkin Elmer Applied Biosystems Procise 494A sequencer with reagents and methods of the manufacturer. The average molar masses were calculated from the sequence using the Perkin Elmer Sciex Peptide Map 2.2 software and the isoelectric point using the Perkin Elmer Sciex Peptide Mac Bio Spec 1.0.1 software.

*2.3. RACE 3′ amplification*

Approximately 1 μg poly($A^+$) mRNA from 600 worker antennae was purified with the Quick mRNA Prep Kit (Pharmacia). Total first strand cDNA synthesis was performed with Mu-MV reverse transcriptase (Stratagene), using the polyT primer from λZAP II cDNA Library Synthesis Kit (Stratagene). The 5′ extension primer CGAAGCTTA(A/G)TA(T/C)ATGGA(A/G)TA(T/C)(C/T)TIATG was deduced from the amino acid sequence KYMEYLM and the 3′ primer was GAGAGAACTAGCTCGAGTT.

*2.4. Worker antennal cDNA library synthesis and screening*

Antennae were sectioned from 7500 adult workers and poly($A^+$) mRNA isolated using the Quick mRNA Purification Kit (Pharmacia). A cDNA library of $10^7$ primary recombinants was generated from 14 μg poly($A^+$) mRNA using the λZAP II cDNA cloning system (Stratagene). PCR amplification product labeled with the DIG Random Priming Kit (Boehringer Mannheim) was used for library screening under high stringency conditions according to the DIG-DNA Detection Kit procedure (Boehringer Mannheim). Nucleotide sequence of both strands was performed by Eurogentec custom service.

*2.5. In situ hybridization procedure*

T3 and T7 polymerases were used to generate sense and antisense DIG-labeled 11UTP RNA probes (Boehringer Mannheim) from linearized cDNA template. The procedure of Byrd et al. [11] was adapted for in toto hybridization. Antennae were prehybridized for 20 h and hybridized with the full-length DIG-labeled RNA for 18 h at 55°C in the 50% formamide hybridization solution. Detection was performed in standard conditions with the anti-DIG AP conjugate antibody (1:100) and colorimetric substrates from Boehringer Mannheim were applied for 20 min at 37°C. Antennae were then embedded in Tissue-Tek OCT, rapidly frozen in nitrogen, and 7 μm cryosections were cut and mounted on polylysine coated slides.

## 3. Results and discussion

As shown in Fig. 1, an antenna-specific protein, ASP2, eluting at 29.0% $CH_3CN$, was separated by HPLC and characterized by an identical $M_r$ in workers (13 695.2 ± 1.6) and drones (13 694.9 ± 0.9) and a same N-terminal sequence (IDQDTVVAKYMEYLMPDIMP). The total ion counts and UV detection (Fig. 1) indicated that this protein was about 5 times more abundant in workers than in drones. It exhibited the biochemical criteria of OBPs, i.e. acidic isoelec-

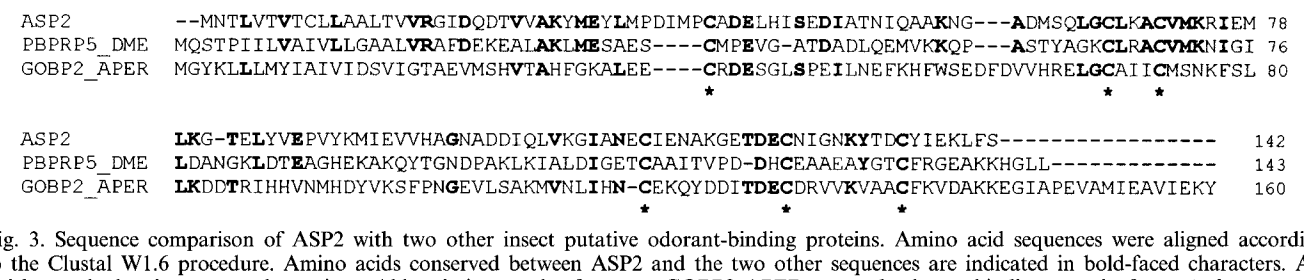


Fig. 3. Sequence comparison of ASP2 with two other insect putative odorant-binding proteins. Amino acid sequences were aligned according to the Clustal W1.6 procedure. Amino acids conserved between ASP2 and the two other sequences are indicated in bold-faced characters. Asterisks mark the six conserved cysteines. Abbreviations and references: GOBP2 APER, general odorant-binding protein from *Antherea pernyi* [20], PBPRP5 DME, pheromone-binding protein related protein from *Drosophila melanogaster* [21].

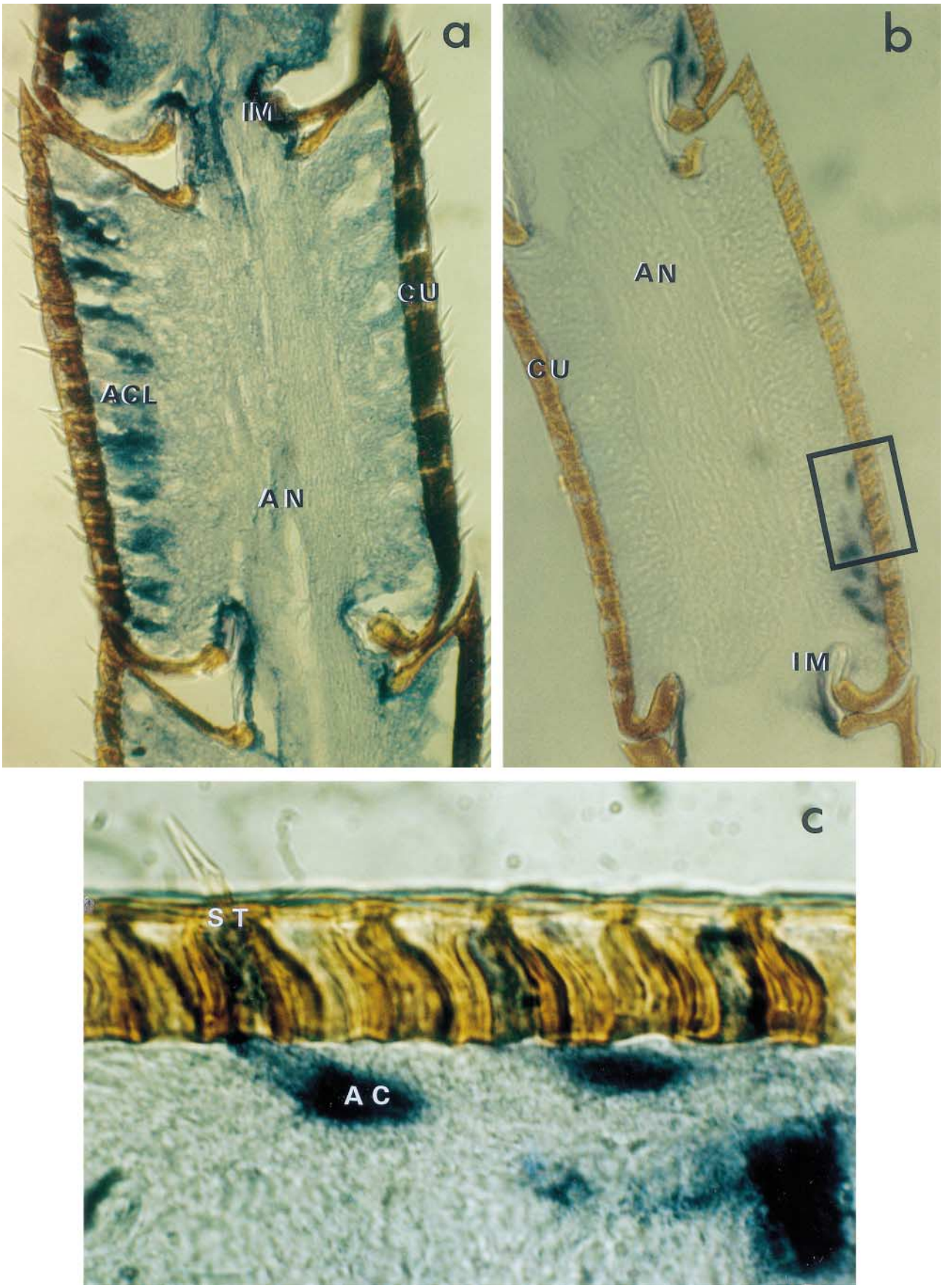


Fig. 4. Expression pattern of ASP2 mRNA in adult bee antennae. a: worker (×400), b: drone (×300), c: drone (×2000). Longitudinal sections were hybridized with antisense riboprobe from the O12.7 clone. The nonspecific labeling in the intersegmental membrane is due to anti-DIG AP conjugate antibody. Abbreviations: ac, auxiliary cell; acl, auxiliary cell layer; an, antennal nerve; cu, cuticle; in, intersegmental membrane; st, sensilla trichodea.

tric point and hydrophobicity. Its apparent $M_r$ determined by SDS-PAGE was in the range of 18.5 kDa (data not shown), in accordance with those reported for PBPs and GOBPs. The disagreement with mass spectrometry determined $M_r$ could be assumed to be due to non-standard interactions with SDS [12]. We did not find any known protein homologous to the N-terminal sequence of ASP2 in the EMBL, Genbank, Swissprot and PIR sequence libraries.

The complete O12.7 cDNA sequence corresponds to ASP2 (Fig. 2). It comprises 603 nucleotides, including cloning sites, an open reading frame of 429 nucleotides starting at the ATG codon in position 1 and ending at the TAA codon at positions 427–429. A 142-amino acid polypeptide is encoded by the open reading frame. The comparison of the amino acid sequence deduced from O12.7 with the N-terminal sequence of ASP2 shows that a 19-residue N-terminal sequence is cleaved after translation (Fig. 2). Comparison of the measured $M_r$ of ASP2 ($13\,695.2 \pm 1.6$) to the calculated $M_r$ of the mature protein deduced from O12.7 (13 700.8) shows a discrepancy of $5.6 \pm 1.6$ amu, which allows us to conclude that the protein encoded by O12.7 is indeed ASP2 and that the 6 amu $M_r$ difference originates in the formation of three disulfide bridges between all six cysteines. This protein does not therefore undergo any post-translational modification in addition to signal peptide cleavage and disulfide bridge formation. This is the first evidence of total cysteine pairing in an insect OBP-like protein. The isoelectric point of ASP2 has been calculated to be 4.8, in agreement with those reported for OBPs.

The deduced amino acid sequence was compared with those of Lepidoptera binding proteins and related proteins isolated in Diptera. Whereas PBPs present at least 90% amino acid identity within Lepidoptera and GOBPs up to 40% [2], the honey bee ASP2 protein only exhibits 8–15% identity with Lepidoptera or Diptera. As illustrated in Fig. 3 the six cysteines are found to be perfectly aligned in all known sequences, except for *Limantria dispar* PBP2 [3] which has lost the first one (data not shown). It is therefore likely that these conserved cysteines are involved in disulfide bonds whatever the species concerned.

The ASP2 protein precursor was analyzed for local hydrophobicity according to Kyte and Doolittle [13]. In addition to the putative signal peptide found to be highly hydrophobic, other short hydrophobic regions can be observed, but are neither long nor hydrophobic enough to be membrane-spanning domains. In *Antherea polyphemus* PBP3 [14], a pheromone analogue has been shown to specifically interact with a hydrophobic domain ranging from $D^{27}$ to $R^{46}$ [15], which is not conserved either in the ASP2 amino acid sequence and hydrophobicity profile or in the fruit fly OBP-like proteins, suggesting that the ligand binding sites may differ upon such divergent proteins.

The ASP2 protein was purified by RPLC and compared through non-SDS-PAGE to the crude extract of worker water-soluble proteins from different tissues, i.e. antennae, legs, thorax, brain and hemolymph, to ensure its antennal specificity (not shown). In situ hybridization revealed a striking density of the O12.7 mRNA specifically associated with the antennal olfactory regions (articles 3 to 10 in workers, to 11 in drones). In drones, the labeled cells or cell clusters were very few, generally located on the proximal and/or distal borders of each article, in the right place where sensilla trichodea A are restricted [16]. In workers, the labeled cells or cell clusters were numerous and uniformly distributed in the layer of auxiliary and epithelial cells situated under the cuticular evaginations of chemosensory sensilla (Fig. 4). In Lepidoptera, GOBPs and PBPs have been proposed to be secreted by sensilla auxiliary cells into the extracellular cavity continuous with the sensory process-surrounding space within the sensory sensillum [17–19]. In situ hybridization experiments suggest a similar conclusion for honey bee ASP2.

In conclusion, in order to select proteins preferentially involved in worker bee olfaction, water-soluble proteins from various tissues from workers and drones were compared and led to the identification of an antenna-specific protein which was most abundant in workers. In situ hybridization indicates that this sexual difference is also related to the number of cells expressing ASP2. Both its distribution pattern of expression confined to olfactory sensilla and its biochemical features close to those of Lepidoptera odorant-binding proteins are consistent with its involvement in olfaction.

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## References

[1] Vogt, R.G. and Riddiford, L.M. (1981) Nature 293, 161–163.
[2] Vogt, R.G., Prestwich, G. and Lerner, M. (1991) J. Neurobiol. 22, 74–84.
[3] Prestwich, G., Du, G. and Laforest, S. (1995) Chem. Senses 20, 461–469.
[4] Laue, M., Steinbrecht, R. and Ziegelberger, G. (1994) Naturwissenschaften 81, 178–180.
[5] Pelosi, P. (1994) Crit. Rev. Biochem. Mol. Biol. 29, 199–228.
[6] Pelosi, P. and Maida, R. (1995) Comp. Biochem. Physiol 111B, 503–514.
[7] Masson, C., Pham-Delègue, M.-H., Fonta, C., Gascuel, J., Arnold, G., Nicolas, G. and Kerzberg, M. (1993) Apidologie 24, 169–194.
[8] Menzel, R. (1993) Apidologie 24, 157–168.
[9] Danty, E., Cornuet, J.-M. and Masson, C. (1994) C.R. Acad. Sci. Paris (Life Sci.) 307, 1073–1079.
[10] Free, J. (1987) Pheromones of Social Bees. Chapman and Hall, London.
[11] Byrd, C., Jones, J., Quattro, J., Rogers, M., Brunjes, P. and Vogt, R.G. (1996) J. Neurobiol. 29, 445–458.
[12] Weber, K., Pringle, J.R. and Osborn, M. (1972) Methods Enzymol. 26, 3–27.
[13] Kyte, J. and Doolitle, R. (1982) J. Mol. Biol. 157, 105–132.
[14] Raming, K., Krieger, J. and Breer, H. (1989) FEBS Lett. 256, 215–218.
[15] Du, G., Ng, C.-S. and Prestwich, G. (1994) Biochemistry 33, 4812–4819.
[16] Esslen, J. and Kaissling, K. (1976) Zoomorphology 83, 227–251.
[17] Steinbrecht, R. (1992) Microsc. Res. Tec. 22, 336–350.
[18] Steinbrecht, R., Ozaki, M. and Ziegelberger, G. (1992) Cell Tissue Res. 270, 287–302.
[19] Maida, R., Steinbrecht, A., Ziegelberger, G. and Pelosi, P. (1993) Insect Biochem. Mol. Biol. 23, 243–253.
[20] Breer, H., Krieger, J. and Raming, K. (1990) Insect Biochem. 20, 735–740.
[21] Pikielny, C., Hasan, G., Rouyer, F. and Rosbash, M. (1994) Neuron 12, 35–49.