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Separation, Characterization and Sexual Heterogeneity of Multiple Putative Odorant-binding Proteins in the Honeybee *Apis mellifera* L. (Hymenoptera: Apidea)

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Abstract

According to precise molar mass determined by mass spectrometry and N-terminal sequence, some 25 odorant-binding-like proteins were characterized from the antennae and legs of worker and drone honeybees. Antennal specific proteins, composed of six different molecules, were classified into three subclasses according to N-terminal sequence homology. The major sexual difference was shown to lie in the relative abundance of these antennal specific proteins and in the occurrence of a drone-specific isoform. At least 19 other related proteins were found to occur in antennae and legs, forming another class showing homology with insect OBP. Genotype comparison of two honeybee races revealed a variability limited to this second class. Provided that these odorant-binding-like proteins are indeed able to bind odorants or pheromones, the question of whether their peculiar multiplicity contributes to the remarkable capacity of the honeybee to discriminate among a wide range of odor molecules is raised.

Introduction

How odor ligand and odor receptor proteins interact represents an important step in deciphering the mechanisms responsible for the large capability of the olfactory system to discriminate between a wide range of odor molecules. This requires the elucidation of how the airborne hydrophobic odor molecules are translocated through the aqueous medium (i.e. vertebrate olfactory mucus, insect sensillum lymph) surrounding the olfactory dendrites, in the membrane of which the receptor proteins are distributed. In such a context, very abundant, small, soluble extracellular proteins are of interest. Such proteins, the odorant-binding proteins (OBP) have been characterized independently in insects and in vertebrates (for review see Pelosi, 1994). Their presumed function is to play the passive roles of carrier and solubilizer, or a more specific function such as codification and recognition of the odor message (Pelosi, 1994, 1996; Pelosi and Maida, 1995).

In insects, OBP were first described in the antennae of male Lepidoptera and named pheromone-binding proteins (PBP) after their ability to bind pheromonal components (Vogt and Riddiford, 1981; Du *et al.*, 1994; Feixas *et al.*, 1995; Prestwich *et al.*, 1995). They are observed in large amounts in males (10 mM in Lepidoptera), occurring as several acidic proteins of 15–20 kDa (for reviews see Pelosi and Maida, 1995; Vogt, 1995) or isoforms in the same species (Nagnan-Le Meillour *et al.*, 1996). Less homologous proteins have been characterized in both sexes (Vogt *et al.*, 1989, 1991); these were shown to be associated to sensilla involved in the detection of general odor molecules (Laue *et al.*, 1994; Vogt, 1995) and were named general odorant-binding proteins (GOBP), although their odorant-binding properties have not yet been demonstrated. Related proteins, presenting low sequence conservation, have also been identified in Heteroptera (Dickens *et al.*, 1995) and Diptera (McKenna *et al.*, 1994; Pikielny *et al.*, 1994; Ozaki *et al.*, 1995).

Although these proteins were generally found to be specifically associated with olfactory sensilla, recent data report a possible role of related proteins in contact chemoreception, as illustrated in flies (Pikielny *et al.*, 1994; Ozaki *et al.*, 1995) and phasmids (Tuccini *et al.*, 1996). A novel protein family represented by Os-D in *Drosophila melanogaster* has also been proposed to be an OBP on the basis of its specific expression in chemosensory sensilla, although its ability to bind odorants is still to be shown (McKenna *et al.*, 1994; Pikielny *et al.*, 1994).

The overall goal of the present study was to characterize putative OBP in the honeybee. In this insect, sexes express

different odor repertoires: there is a wide spectrum of general odors together with different pheromones for workers, which are sterile females, and a more restricted specificity for queen pheromone as a sex attractant for the male drones (Kaissling and Renner, 1968; Vareschi, 1971; Free, 1987). This allows a comparative analysis of putative OBP respectively involved in food and pheromonal odor detection in workers, and queen pheromone detection in drones. Here we report the biochemical isolation and physicochemical characterization of numerous proteins specifically associated with worker and/or drone antennae. Moreover, we have identified another family of proteins commonly found in antennae and legs.

Materials and methods

Materials

Adult workers and drones were reared in natural conditions (hive). The insects originated from hybridization between two *Apis mellifera* races, *A. m. mellifera* and *A. m. ligustica*. Insects of selected age were marked at emergence and collected 6–17 days later. A comparative analysis was performed with *A. m. mellifera* and *A. m. ligustica* workers.

Tissue preparation

Antennae, legs, brains, hemolymph, thorax and genital organs were isolated from insects which had been anesthetized by cooling to –20°C for a few minutes. Legs and antennae were homogenized in liquid nitrogen, the others on ice, and dissolved in PBS, 5 mM EDTA, pH 7.4, 1 mM PMSF, 1 μg/ml chimostatin (Boehringer Mannheim, Meylan, France), 1 μg/ml antipain (Boehringer Mannheim), 1 μg/ml leupeptin (Boehringer Mannheim), 1 μg/ml pepstatin (Boehringer Mannheim), 1 μg/ml *N*-tosyl-L-phenylalanin chloromethyl ketone (Sigma, Saint Quentin Fallavier, France) and 1 μg/ml *N*-tosyl-L-alanine chloromethyl ketone (Sigma). Sediments were removed by centrifugation at 12 000 *g* for 5 min at 4°C. Soluble and insoluble proteins were separated by ultracentrifugation at 140 000 *g* for 45 min at 4°C (Beckman L5, rotor Ti 60; Beckman, Gagny, France). Supernatants containing soluble proteins were stored at –80°C until use.

Electrophoresis and electroblotting

Protein analysis was performed on non-denaturing gel electrophoresis according to the procedure of Laemmli (1970) in which SDS was omitted. Soluble protein extracts, ~25 μg for each tissue (corresponding to 20 antennae or 10 legs), estimated using the Pierce (Interchim, Montluçon, France) BCA protein assay kit, for other tissues were subjected to analytic electrophoresis in a 16.8% acrylamide gel and separated at 250 V for 2.5 h. Proteins were stained with a colloidal Coomassie Blue-R (Serva, Boehringer Ingelheim, Gagny, France) 0.035% solution in 12% trichloroacetic acid, 5% ethanol and destained in water. Electrophoretic bands from worker or drone antennae and legs were electroblotted onto ProBlott membranes (Applied Biosystems, Courtaboeuf, Les Ulis, France) according to the procedure of Rassmussen *et al.* (1991).

High performance liquid chromatography coupled with ion-spray mass spectrometry

Soluble proteins from antennae were separated by reverse phase high performance liquid chromatography (RPLC) on-line-coupled with an ion spray mass spectrometer (IS-MS) (Sciex API100, Perkin-Elmer, Courtaboeuf, Les Ulis, France). RPLC was run using a Perkin-Elmer device (Applied Biosystems pump 140C and UV detector 785) on a 2.1×100 mm C8 Aquapore column (300 Å) in 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 ion-spray source (40 μl/min) and a fraction collector device. IS-MS experiments were controlled with 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 Perkin-Elmer Sciex Bio-Multi-View 1.2 software. The average molar masses were calculated from the sequence using Perkin-Elmer Sciex Peptide Map 2.2 software. The collected fractions were submitted to Edman microsequencing.

N-terminal sequencing

Automated Edman sequencing of the N-terminal end was performed using a Perkin-Elmer Applied Biosystems Procise 494A sequencer with the reagents and methods of the manufacturer (Applied Biosystems). N-terminal sequencing was applied both to proteins electroblotted onto a PVDF membrane (Problott) and RPLC fractions spotted onto glass-fiber filters. Cysteine determination was performed after reduction and alkylation of electroblotted proteins, according to the procedure of Moritz *et al.* (1996). In the case of mixtures, analysis of the quantitative sequencing data, cycle by cycle, allowed the determination of each of the sequences, provided their amounts were sufficiently distinct. Protein amounts were estimated through sequencing and electroblotting yields. Sequence homologies were analyzed by comparison with the sequence databases (GenBank, PDB, SwissProt, SPupdate and PIR) included in BLASTP 1.4.9 MP (Altschul *et al.*, 1990).

Results

In order to identify OBP-like proteins, soluble proteins were independently separated in non-denaturing gel electrophoresis and RPLC. In the first step, the soluble proteins of various body parts from workers and drones were analyzed

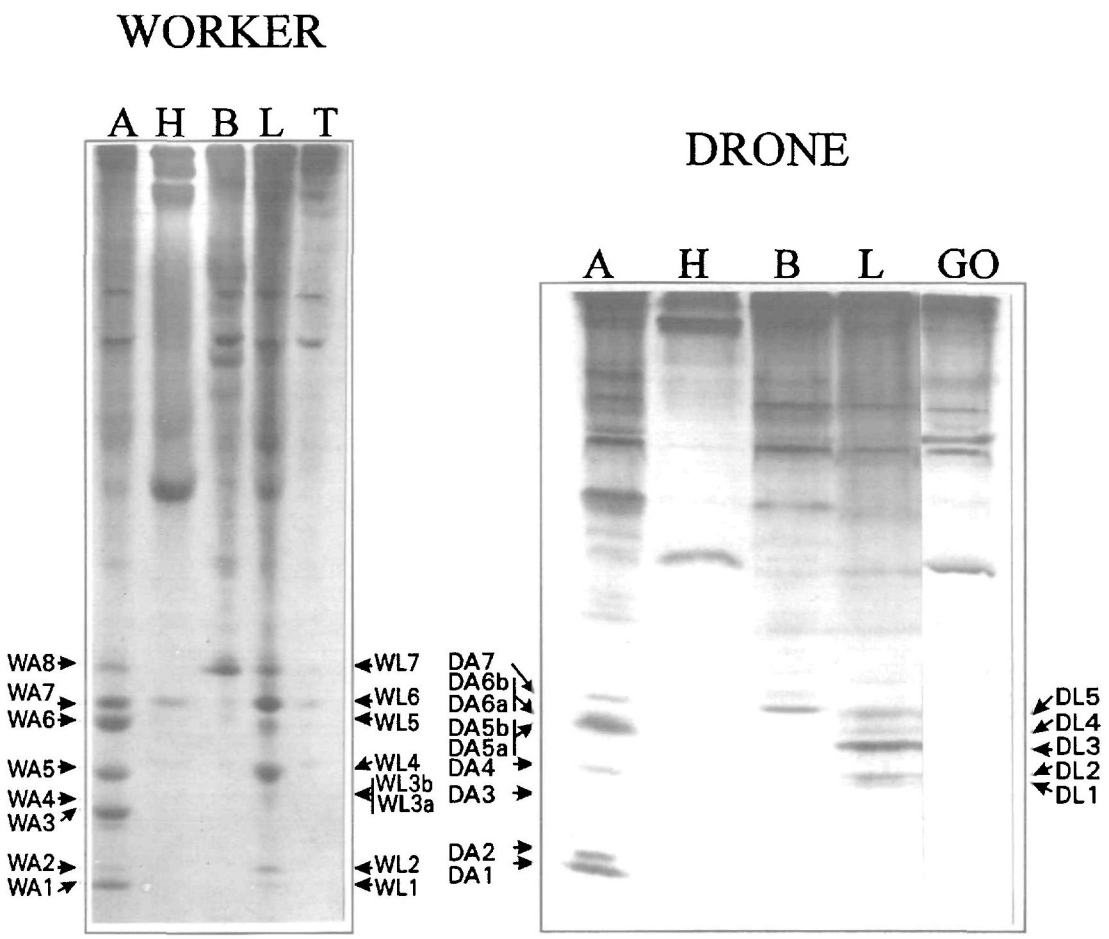

Figure 1 Non-denaturing PAGE of soluble proteins of adult worker and drone honeybees. Experiments were performed on soluble proteins (~25 µg, i.e. 20 antennae or 10 legs) in 16.8% polyacrylamide gels which were Coomassie Blue-R stained. Arrows indicate antenna and leg bands selected for N-terminal microsequencing after electroblotting; A, antenna; B, brain; DA, drone antenna; DL, drone leg; GO, genital organ; H, hemolymph; L, leg; T, thorax; WA, worker antenna; WL, worker leg.

by non-SDS–PAGE. Antennal proteins were compared with hemolymph, brain, legs, thorax and drone genital organ soluble extracts (Figure 1). The soluble proteins were named after the sex, the body part and the decreasing electrophoretic mobility. Proteins from the antennae and legs of workers and drones were electroblotted from gels onto Problott membranes prior to being submitted to Edman microsequencing. We performed a second approach to separate these proteins by using RPLC, allowing protein discrimination according to their hydrophobicity (Figure 2). This liquid chromatography system was on-line-coupled with both IS-MS and a fraction collector for further N-terminal sequencing. Since some peaks contained several proteins, they were submitted to a second RPLC run in 0.1% trifluoroacetic acid (results not shown) before sequencing as previously described (Nagnan-Le Meillour *et al.*, 1996), in order to verify the assignments. Sequence determination of the chromatographic peaks allowed pairing with the corresponding electrophoretic bands. The relative amounts of WA1 and WA2, and of DA1 and DA2, derived from sequencing data are not in perfect agree- ment with the Coomassie stain of the corresponding electrophoretic bands. This can be explained by the different methods used, one depending on the electroblotting and the initial sequencing yields and the other on the ability to bind Coomassie blue, methods based on different protein properties. Precise molar masses determined by IS-MS and N-terminal sequences are reported in Table 1 together with the protein identification and RPLC elution acetonitrile percentage. As shown by sequencing, it is noteworthy that the electrophoretic bands DA5, DA6 and WL3 were composed of several proteins separated by RPLC. They were numbered DA5a and b, DA6a and b, and WL3a and b. By combining electrophoretic and chromatographic separations, we could identify 11 different proteins in worker antennae, of which only eight were observed through electrophoresis, and eight in their legs. Ten different proteins were found in the drone antennae and five in their legs. The less acidic proteins from antennae (WA8 in workers, DA7 in drones) exhibited a blocked N-terminus while the worker leg

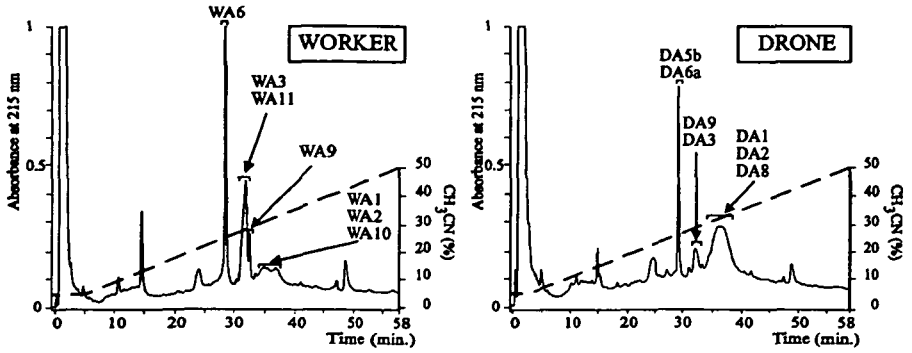

Figure 2 Comparison of soluble proteins from drone and worker antennae by RPLC. Soluble proteins from 100 antennae were separated on a C8 Aquapore column in 25 mM CH_3COONH_4, pH 6.8, using CH_3CN. Linear gradients (dashed line) (9.5% for 5 min, from 9.5 to 50% in 50 min) were applied at a flow rate of 200 µl/min. The elution flow was on-line analyzed by IS-MS and collected for N-terminal microsequencing. DA, drone antenna; WA, worker antenna. Peaks are named after electrophoretic bands correspondence determined by sequence comparison.

Table 1 Characterization of soluble acidic proteins from worker and drone antennae and legs

Name	N-terminal sequence	Q (pmol)	CH_3CN (%)	M_r (Da)
WA1*	APDWVPPEVFDLVAEDKAR	0.08	31–33	13 182.5 ± 2.3
WA2*	APDWVPPEVF	0.18	31–33	13 182.5 ± 2.3
WA3	IDQDTVVAKYMEYLMPDIMP	0.67	29.0	13 695.2 ± 1.6
WA4	MTIEELKIQL	0.04	nd	nd
WA5*	MTHEELKTGIQTLQPICVGE	0.36	nd	nd
WA6	EELYSDKYDY	0.67	26	11 537.6 ± 1.6
WA7	LTLEELQIGLRAVIPVXXID	0.33	nd	nd
WA8	blocked N-terminus	nd	nd	nd
WA9	MTIEELKIQLHDVQEIXKTE	nd	29.5	13 894.4 ± 2.2
WA10	DESYTSKFDNINVDEIL	nd	31–33	12,758.3 ± 1.7
WA11	LTLEELKTRLHTVQSVXKTE	nd	29.0	13,515.1 ± 1.1
DA1	APDWVPPEVFDLVAEDKARXMSEHGTTQAQIDDVD	0.6	31–33	13 181.4 ± 0.6
DA2	APDWVPPEVFDLVAEDKARXMSEHGTTQAQIDDVD	0.6	31–33	13 181.4 ± 0.6
DA3	IDQDTVVAKYMEYLMPDIMP	0.07	29.0	13 694.9 ± 0.9
DA4	MTHEELKTGIQTLQPICVGE	0.22	nd	nd
DA5a	LTLEELQIGL	0.02	nd	nd
DA5b	EELYSDKYDYVNIDEILAND	0.06	26	11 537.4 ± 1.0
DA6a	EELYSDKYDYVNIDEILANDRLRNQYYDXFIDAGSXLTPDXSVF	0.75	26	11 537.4 ± 1.0
DA6b	LTLEELQIGLRA	0.07	nd	nd
DA7	blocked N-terminus	nd	nd	nd
DA8	nd	nd	31–33	12 757.1 ± 0.3
DA9	LTIEELKTRLHTEQSVXKTE	nd	29.5	13 514.6 ± 0.6
WL1	nd	nd		
WL2	DTVAILQXXKAGFDXXDLK	0.03		
WL3a	MTLDELKSGLHTVQSVCMKEIGTAQQIIDD	0.63		
WL3b	MTHEELKTGIQTLQPICMGETGTAQKIIDD	0.42		
WL4	LTLEEFQIGLRAVVPICRIETGIDQQKEDD	0.32		
WL5	LTLEELQIGLRAVIPVCRIDSGIDEKKEDD	1.37		
WL6	LTLEEFQIGLRAVVPICRIETSIDQQKEDD	0.2		
WL7	nd	nd		
DL1	MTHEELKTGIQTLQPICMGETGTAQKIIDD	0.34		
DL2	MTLDELKSGLHTVQSVCMKEIGTAQQIIDD	1.00		
DL3	LTLEEFQIGLRAVVPICRIETSIDQQKEDD	2.24		
DL4	LTLEEFQIGLRAVVP	0.69		
DL5	LTLEEFQIGLRAVVPICRIETS	0.43		

Proteins are named according to Figure 1. N-terminal sequences were determined after electrophoresis and electroblotting of 200-antenna or 200-leg extracts of 6- to 14-day-old workers and 200-antenna or 75-leg extracts of 6- to 17-day-old drones, and also from RPLC fractions of 100-antenna extracts. Q, protein amount per antenna or leg estimated from microsequencing and electroblotting yields; M_r, molar mass determined from IS-MS; CH_3CN%, the elution acetonitrile percentage; *, major protein in the fraction; X, undetermined residue; nd, not determined.

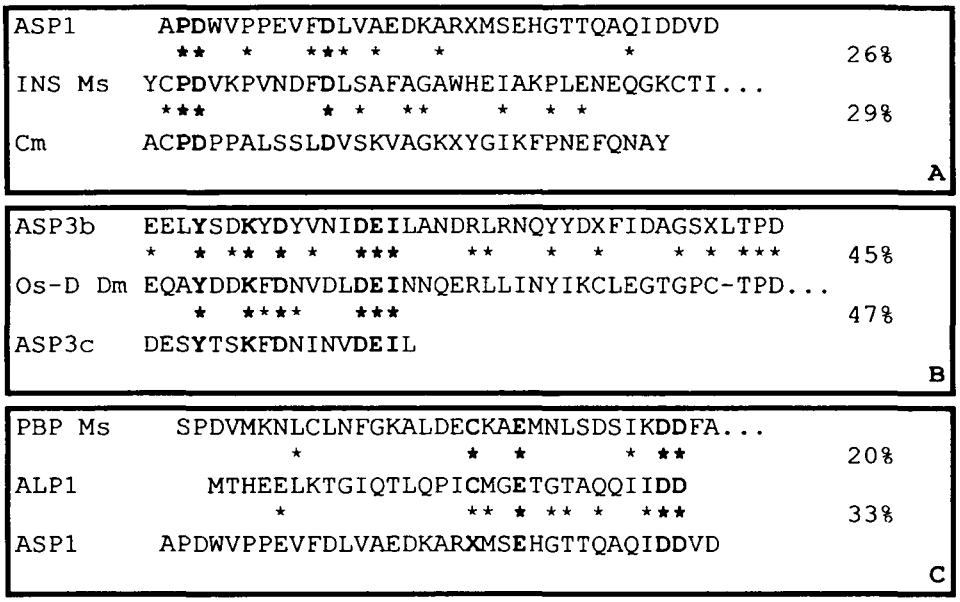

Figure 3 Comparison of the N-terminal sequences of honeybee soluble proteins with lipophilic-carrier proteins. **(A)** ASP1 compared with INS Ms, insecticyanin from *Manduca sexta* (Riley *et al.*, 1984), and Cm, a putative OBP from *Carausus morosus* (Tuccini *et al.*, 1996); **(B)** ASP3b compared with Os-D Dm from *Drosophila melanogaster* (McKenna *et al.*, 1994; Pikielny *et al.*, 1994) and ASP3c; **(C)** ALP1 compared with ASP1 and PBP Ms from *M. sexta* (Györgyi *et al.*, 1988). Bold characters indicate identities between all sequences; *, line-to-line conserved amino acids; X, undetermined residues, assumed to be C when homology was possible with such a residue; sequence identity percentages are indicated on the right.

Table 2 Classification of honeybee antenna and leg soluble proteins according to their molar mass, localization, sex-distribution and N-terminal sequence homology with OBP and related proteins

Class	Name	M_r (Da)	Q (ng) workers	Q (ng) drones	% ASP workers	% ASP drones	Related proteins
ASP1a	WA1–DA1	13 182	1.0	7.9	5	30	insecticyanin?
ASP1b	WA2–DA2	13 182	2.4	7.9	12	30	
ASP2	WA3–DA3	13 695	9.2	1.0	45	4	insect OBP
ASP3a	WA6–DA5b	11 537	7.7	0.7	38	3	Os-D Dm
ASP3b	DA6a	11 537	0	8.7	0	33	
ASP3c	WA10-DA8	12 757	traces	traces			
ASP			20.3	26.2			
ALP	19 isoforms	13 000–13 900	A: 9.8	A: 4.0			insect OBP
	see Figure 5		L: 41.7	L: 63.2			

A, antenna; L, leg; ALP, proteins specific for antennae and legs or specific for legs alone; ASP, antenna-specific protein; % ASP, percentage of ASP, relative to the sum of each ASP amount estimated from N-terminal sequencing; Q, protein amount per organ (an M_r mean value of 13 450 was attributed to calculate Q for ALP).

proteins WL1 and WL7 could not be sequenced due to insufficient amounts (Table 1 and Figure 1). The worker leg protein WL2 was not observed in RPLC and was revealed as a novel protein without any sequence homology in the databases, whereas all other antenna and leg proteins could be related to known insect proteins.

The question of whether the polymorphism of the acidic soluble proteins arises through genetic variability led to the comparison of some bee genotypes, i.e. *A. m. mellifera* and *A. m. ligustica* races and hybrids. No obvious quantitative difference was found in the worker antennal proteins, and only one qualitative difference was observed by RPLC corresponding to WA9. It was characterized in the hybrids by a molar mass of 13 894.4 ± 2.2, identical to the *A. m. mellifera* protein, but was different in *A. m. ligustica*, where it exhibited a molar mass of 13 917.0 ± 1.9 and less hydrophobic behaviour, eluting at 29.2% acetonitrile instead of 29.5%.

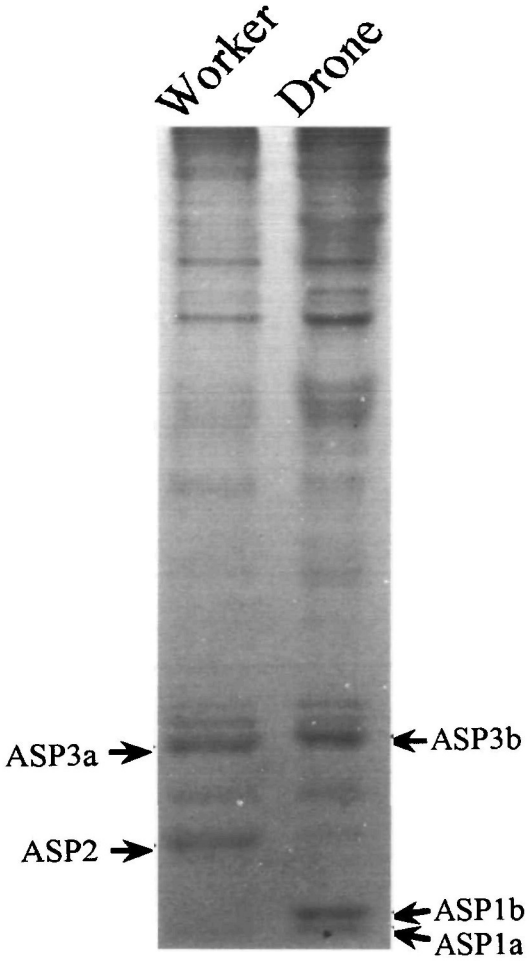

Figure 4 Non-denaturing PAGE of worker and drone antennal soluble proteins. Experiments were performed as described in Figure 1. Arrows indicate the antennal specific proteins (ASP). The worker-specific ASP3a was systematically found to move faster than the drone-specific ASP3b.

Discussion

Acidic honeybee soluble proteins can be classified according to their tissue origin and sequence homology into two main classes. The first one comprises the antennal specific proteins (ASP) only found in antennae; the other class (called ALP, for antenna and leg proteins) includes those found either in legs or in legs and antennae (Figure 3 and Table 2).

Antennal specific proteins

The ASP proteins can be subdivided in three subclasses, ASP1 (two different isoforms), ASP2 (one protein) and ASP3 (two isoforms and one different protein), according to their molar mass and sequence similarity. Figure 4 shows the comparison of antennae drone and worker soluble proteins, among which those specific to the antennae were marked ASP. Resulting from another experiment, the migration and staining look different from that reported in Figure 1, so that some bands are not observed as clearly.

The ASP1 subclass contains two isoforms in each sex (WA1 and DA1, WA2 and DA2) that have been distinguished on the basis of their electrophoretic mobility in non-SDS–PAGE. Nevertheless, they exhibited identical molar mass and N-terminal sequence. This suggests that they differ by the amidation of an acidic side-chain or the C-terminal function, the only alteration undetectable by IS-MS. We cannot decide whether this difference originated in post-translational modifications, i.e. deamidation, or in the co-expression of two alleles. It is worth noticing that examples of one-site replacement have previously been reported in databases (access number in NCBI: 129675) to occur in moth PBP (Györgyi *et al.*, 1988; Vogt *et al.*, 1991). The insecticyanin isolated from *Manduca sexta* hemolymph (Riley *et al.*, 1984) was found to be the protein closest to ASP1 (Figure 3A). A 26% identity among only 35 N-terminal residues is nevertheless insufficient to conclude that ASP1 are indeed insecticyanins. Moreover, the antennal specificity of ASP1 and their absence from hemolymph indicate that they are likely not insecticyanins. ASP1 also showed some homology with a putative OBP found both in legs and antennae of *Carausus morosus* (Tuccini *et al.*, 1996).

The ASP2 subclass is composed of a single protein and is identical in both sexes (WA3 and DA3). We did not found any N-terminal sequence significantly related to that of ASP2 in databases. Its complete sequence was recently deduced from cloned cDNA, showing poor homology with insect OBP, except the location of six cysteines involved in disulfide bonds (Danty *et al.*, 1997).

The ASP3 subclass contains two isoforms (ASP3a and b) and another protein (ASP3c). ASP3a, corresponding to WA6 and DA5b, and ASP3c, corresponding to WA10 and DA8, were commonly found in drones and workers. This is in contrast to ASP3b, composed of DA6a, which was specific to drones. ASP3a and ASP3b were found to be identical in their N-terminal sequence and molar mass, and presented 41% amino-acid identity with ASP3c. They correspond to two electrophoretically distinct isoforms (Figure 1), suggesting a similar difference as previously described for the two ASP1 isoforms. ASP3 proteins are homologous to the *Drosophila* Os-D protein located in sensory sensilla (Figure 3B), the function of which is still unknown (McKenna *et al.*, 1994; Pikielny *et al.*, 1994). The comparison of the two genotypes with the hybrids dismisses the hypothesis that the occurrence of many ASP isoforms arises in a mixture of honeybee genotypes in our samples.

Antenna and leg proteins

We also isolated a considerable amount of acidic proteins in the soluble fractions that were not found specifically in the antennae but also in the legs (Figure 1), called ALP. N-terminal sequencing of the corresponding electroblotted

Name	N-terminal sequence
ALP1	
WA5	*MT HE*EL KTG *IQT LQPI* C *VGE*
DA4	*MT HE*EL KTG *IQT LQPI* C *VGE*
DL1	*MT HE*EL KTG *IQT LQPI* C *MGETG TAQK II*DD
WL3b	*MT HE*EL KTG *IQT LQPI* C *MGETG TAQK II*DD
WA9	*MT* IE*E* LKI *QLHDVQEIXKTE*
WA4	*MT* IE*E* LKI *QL*
WL3a	*MT LD*E LK *SGLHTVQSV* C *MKE IG TAQQ II*DD
DL2	*MT LD*E LK *SGLHTVQSV* C *MKE IG TAQQ II*DD
ALP2	
WA11	*LT* LE*E* LKT *RLHTVQSVXKTE*
DA9	*LT* IE*E* LKT *RLHT EQSVXKTE*
DA5a	*LT* LE*E* L *QIGL*
WA7	*LT* LE*E* L *QIGL RAV IPVXXID*
DA6b	*LT* LE*E* L *QIGL RA*
WL5	*LT* LE*E* L *QIGL RAV IPV* C *RIDSG IDEKKE*DD
WL4	*LT* LE*E FQIGL RAV VPI* C *RIET SIDQQKE*DD
DL4	*LT* LE*E FQIGL RAV VP*
DL3	*LT* LE*E FQIGL RAV VPI* C *RIET SIDQQKE*DD
DL5	*LT* LE*E FQIGL RAV VPI* C *RIETS*
WL6	*LT* LE*E FQIGL RAV VPI* C *RIET SIDQQKE*DD
Consensus sequences	
ALP	LTLE*E*LQIGLRAVQPI C RIETGTAQQII**DD**
	M ID FKTQIQTLVSV VGDSSIDEKKE
	H SR HDEIE MT I
	KK
ALP1	**MT** *H*E**ELK**TG *IQT LQPI* C *MGE*T**G TAQQ II**DD
	ID SQLHDV SV VK I K
	L I *E* KT
ALP2	**LT**LE**E**LQIG**L** *RAV VPI* C *RIET SID*QQ**KE**DD
	I *FKTR* HT *EISV* KT *DSG EK*
	Q

Figure 5 N-terminal sequence classification of honeybee ALP. Amino acids common to a whole class or subclass are indicated in bold characters; italics indicate those peculiar to either subclass ALP1 or ALP2; X, undetermined amino acid.

or RPLC-purified proteins revealed that honeybees possess some 19 very similar proteins with a molar mass of 13–14 kDa, which are all closely related (Figure 5). Some proteins exhibited identical N-terminal sequences, but in some cases their electrophoretic mobility was significantly different and in other cases they originated from different tissues or sexes. Consequently, in the absence of definitive data (precise molar mass), we cannot conclude whether they are identical or not. The conserved cysteine at position 17 was determined for the most abundant worker and drone leg proteins. The unidentified amino acid at this position in native unalkylated proteins was therefore assumed also to be a cysteine. Sequence alignments allow the classification of ALP into two subclasses, ALP1 and ALP2 (Figure 5). When comparing ALP with the ASP1 subclass, a significant homology can be observed, but starting at residue 17 (Figure 3C). ALP also contain the consensus sequence CXXE commonly found in moth PBP and related proteins, as illustrated by the comparison with the *Manduca sexta* PBP in Figure 3C. On the condition that these partial homologies are confirmed by complete sequencing, ALP would therefore be composed of a mosaic of antennal protein sequences.

From a quantitative point of view, ALP were found to be more abundant in legs than in antennae (Tables 1 and 2), 4-fold more abundant in workers and 16-fold more abundant in drones. In antennae, the ALP represented 33% of the total acidic soluble proteins in workers and 13% in drones, while in legs, ALP comprised all the acidic soluble proteins both in workers and drones. Even though they were related to OBP, the functional role of this large multigenic family needs further investigation. However, the partial amino-acid homology with the ASP1 supports the hypothesis that these proteins may share common properties.

Sexual dimorphism

Social insects such as honeybee can be characterized by a more complex chemical communication than that of moths (Vogt, 1995). The queen pheromone acts as a sex attractant for drones, but also controls social behavior and sexual physiology of workers (Free, 1987). Moreover, workers communicate with a large number of different pheromones, cuticular hydrocarbons or plant volatiles. This complexity is depicted at the level of a number of different olfactory sensilla types (Esslen and Kaissling, 1976) and olfactory neuron performances (Masson *et al.*, 1993). Such diversity is probably supported by variations of specific proteins involved in odorant detection. From a quantitative point of view, a large contrast differentiates drones from workers: ASP1 and ASP3b account for 93% of the drone ASP, whereas ASP2 and ASP3a are mainly present in workers, in which they reach 83% of the ASP. Nevertheless, all the ASP are found in both sexes (Table 2) except ASP3b (DA6a), which is found only in drones. The ASP2 subclass is the most abundant ASP in workers (45%) but only represents a minor fraction in drones (4%), suggesting an important functional significance of this protein for workers. The ASP3 subclass represents a major fraction of both worker and drone ASP (38 and 36% respectively), which suggests that they may play an important role in olfaction. Nevertheless, the relative abundance of ASP3a varied notably with sex (38% in workers but only 3% in drones). This discrepancy is, however, counterbalanced by the amount of the drone-specific ASP3b (33%) that might be specifically involved in addressing queen pheromonal signals to activate neurons devoted to sex attractant detection in drones. Qualitative differences observed between drones and workers are mainly restricted to this difference between ASP3a and ASP3b. It is restricted to a minute, but possibly functionally important, (de)amidation of a single residue occurring in the ASP3 subclass, since

ASP3a only differs from ASP3b in electrophoretic mobility (Figure 4). It is possible that a drone-specific post-translational modifica- tion of ASP3a into ASP3b might occur that would be involved in sex pheromone detection. Such a hypothesis is in agreement with the largely depicted pheromonal system of Lepidoptera, in which PBP have been proposed to play a role in pheromone translocation through the sensillar lymph to membrane receptors. In this system several isoforms have been identified in each species, suggesting a certain ligand-binding specificity (Prestwich *et al.*, 1995). The ASP1 subclass represents ~60% of drone ASP but only 17% in workers (Table 2), which suggests that these proteins might also be possibly involved in queen pheromone detection.

Conclusion

We isolated, for the first time in a social insect, the honeybee *A. mellifera* L., a large number (~25) of acidic soluble proteins from the antennae and legs of adults, which show the common properties of OBP-like proteins, i.e. molar mass and N-terminal sequence. According to physico-chemical criteria, the antennal specific proteins (ASP) could be classified into three subclasses. Another class of ~19 biochemically related proteins was commonly found in the legs or in the antennae and legs. This novel protein family raises an unexpected question about their function as putative binding proteins. The abundance of each OBP-like protein is largely variable from one protein to the other. Sexual variability between workers and drones was mainly found at the quantitative level, although a protein variant was observed solely in drones. The question of whether these honeybee proteins, which present common properties with OBP, are indeed able to bind odorants or pheromones remains to be studied. If they are, the peculiar multiplicity of these proteins in the honeybee could contribute to the remarkable capacity of the olfactory system to discriminate between a wide range of odor molecules. A panel of ligands corresponding to biological function in the honeybee social life, foraging or sexual behavior could be selectively tested to study the functional role of these proteins in honeybee olfaction.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and **Lipman, D.J.** (1990) *Basis local alignment search tool.* J. Mol. Biol., 215, 403–410.

Danty, E., Michard-Vanhée, C., Huet, J.-C., Genecque, E., Pernollet, J.-C. and **Masson, C.** (1997) *Biochemical characterization, molecular cloning and localization of a putative odorant-binding protein in the antennae of honeybee* Apis mellifera *L. (Hymenoptera: Apidea).* FEBS Lett., 414, 595–598.

Dickens, J., Callahan, F., Wergin, W. and **Erbe, E.** (1995) *Olfaction in a Hemimetabolus insect, antenna-specific protein in adult* Lygus lineolaris *(Heteroptera: Miridae).* J. Insect Physiol., 41, 857–867.

Du, G., Ng, C.-S. and **Prestwich, G.** (1994) *Odorant binding by a pheromone binding protein: active site mapping by photoaffinity labeling.* Biochemistry, 33, 4812–4819.

Esslen, J. and **Kaissling K.** (1976) *Zahl und Verteilung Antennaler Sensillen bei der Honigbiene (*Apis mellifera *L.).* Zoomorphology, 83, 227–251.

Feixas, J., Prestwich, G.D. and **Guerrero, A.** (1995) *Ligand binding specificity of pheromone-binding proteins of the processionary moth.* Eur. J. Biochem., 234, 521–526.

Free, J. (ed.) (1987) Pheromones of Social Bees. Chapman & Hall, London.

Györgyi, T., Robi-Shemkovitz, A. and **Lerner, M.** (1988) *Characterization and cDNA cloning of the pheromone-binding protein from the tobacco hornworm,* Manduca sexta. Proc. Natl Acad. Sci. USA, 85, 9851–9855.

Kaissling, K. and **Renner, M.** (1968) *Antennae Rezeptoren für Queen Substance und Sterzelduft bei der Honigbiene.* Z. Vgl. Physiol., 59, 357–361.

Laemmli, U. (1970) *Cleavage of structural proteins during assembly of the head of bacteriophage T4.* Nature, 227, 680–682.

Laue, M., Steinbrecht, R. and **Ziegelberger, G.** (1994) *Immuno-cytochemical localization of general odorant-binding proteins in the olfactory sensilla of the silkmoth* Antherea polyphemus. Naturwissenschaften, 81, 178–180.

Masson, C., Pham-Delègue, M.-H., Fonta, C., Gascuel, J., Arnold, G., Nicolas, G. and **Kerszberg, M.** (1993) *Recent advances in the concept of adaptation to natural odour signals in the honeybee,* Apis mellifera. Apidologie, 24, 169–194.

McKenna, M., Hekmat-Scafe, D.S., Gaines, P. and **Carlson, J.R.** (1994) *Putative* Drosophila *pheromone-binding proteins expressed in a subregion of the olfactory system.* J. Biol. Chem., 269, 16340–16347.

Moritz, R., Eddes, J., Reid, G. and **Simpson, R.** (1996) *S-Pyridylethylation of intact polyacrylamide gels and in-situ digestion of electrophoretically separated proteins: a rapid mass spectrometric method for identifying cysteine-containing peptides.* Electrophoresis, 17, 907–917.

Nagnan-Le Meillour, P., Huet, J.-C., Maibeche, M., Pernollet, J.-C. and **Descoins, C.** (1996) *Purification and characterization of multiple forms of odorant/pheromone binding proteins in the antennae of* Mamestra brassicae *(Noctuidae).* Insect Biochem. Mol. Biol., 26, 59–67.

Ozaki, M., Morisaki, K., Idei, W., Ozaki, K. and **Tokunaga, F.** (1995) *A putative lipophilic stimulant carrier protein commonly found in the taste and olfactory systems.* Eur. J. Biochem., 230, 298–308.

Pelosi, P. (1994) *Odorant-binding proteins.* Crit. Rev. Biochem. Mol. Biol., 29, 199–228.

Pelosi, P. (1996) *Perireceptor events in olfaction.* J. Neurobiol., 30, 3–19.

Pelosi, P. and **Maida, R.** (1995) *Odorant-binding proteins in insects.* Comp. Biochem. Physiol., 111B, 503–514.

Pikielny, C., Hasan, G., Rouyer, F. and **Rosbach, M.** (1994) *Members of a family of* Drosophila *putative odorant binding proteins are expressed in different subsets of olfactory hairs.* Neuron, 12, 35–49.

Prestwich, G.D., Du, G. and **Laforest, S.** (1995) *How is pheromone specificity encoded in proteins?* Chem. Senses, 20, 461–469.

Rassmussen, H., Van Damme, J., Puyde, M., Gesser, B., Celis, J.E. and **Vandekerrckhove, J.** (1991) *Microsequencing of proteins in human two dimensional gel protein databases*. Electrophoresis, 12, 873–882.

Riley, C.T., Barbeau, B.K., Keim, P.M., Kezdy, F.J., Heinrikson, R.L. and **Law, J.H.** (1984) *The covalent protein structure of insecticyanin, a blue biliprotein from the hemolymph of the tobacco hornworm,* Manduca sexta. J. Biol. Chem., 259, 13159–13165.

Tuccini, A., Maida, R., Rovero, P., Mazza, M. and **Pelosi, P.** (1996) *Putative odorant-binding proteins in antennae and legs of* Carausius morosus *(Insecta: Phasmatodea)*. Insect Biochem. Mol. Biol., 26, 19–24.

Vareschi, E. (1971) *Duftunterscheidung bei der Honigbiene-Einzelzell-Albeitungen und Verhalten reaktionen*. Z. Vgl. Physiol., 75, 143–173.

Vogt, R.G. (1995) *Molecular genetics of moth olfaction, a model for cellular identity and temporal assembly of the nervous system*. In Goldsmith, M.R. and Wilkins, A.S. (eds), Molecular Model Systems in the Lepidoptera. Cambridge University Press, Cambridge, pp. 341–367.

Vogt, R.G. and **Riddiford, L.M.** (1981) *Pheromone binding and inactivation by moth antenna*. Nature, 293, 161–163.

Vogt, R., Köhne, A., Dubnau, J. and **Prestwich, G.D.** (1989) *Expression of pheromone-binding proteins during antennal development in the gypsy moth* Lymantria dispar. J. Neurosci., 9, 3332–3346.

Vogt, R., Rybczynski, R. and **Lerner, M.** (1991) *Molecular cloning and sequencing of general odorant-binding proteins GOBP1 and GOBP2 from the tobacco hawkmoth* Manduca sexta*: comparison with other insect OBP and their signal peptides*. J. Neurosci., 11, 2972–2984.

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