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Research Article

Soluble proteins of chemical communication in the social wasp *Polistes dominulus*

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Abstract. Members of the odorant-binding protein (OBP) and chemosensory protein (CSP) families were identified and characterised in the sensory tissues of the social wasp *Polistes dominulus* (Hymenoptera: Vespidae). Unlike most insects so far investigated, OBPs were detected in antennae, legs and wings, while CSPs appeared to be preferentially expressed in the antennae. The OBP is very different from the homologous proteins of other Hymenopteran species, with around 20% of identical residues, while the CSP appears to be much better conserved. Both OBP and CSP, not showing other post-translational modifications apart from disulphide bridges, were expressed with high yields in a bacterial system. Cysteine pairing in the recombinant and native proteins follows the classical arrangements described for other members of these classes of proteins. OBPs isolated from the wings were found to be associated with a number of long-chain aliphatic amides and other small organic molecules. Binding of these ligands and other related compounds was measured for both recombinant OBP and CSP.

Key words. Odorant-binding protein; chemosensory protein; chemical communication; fluorescence binding assay; N-phenyl-1-naphthylamine; oleoamide; *Polistes dominulus*.

Social insects interact with their conspecifics in more complex ways than other insects, and therefore rely on a richer and more detailed chemical language. In addition to conveying information about sex and species, social insects need to recognise social status and colony of their conspecific, and to exchange information with other members of the colony about food source and danger. Among other chemical compounds utilised in such communication, long-chain hydrocarbons play a major role. The chemical diversity of such molecules that are released by subepidermal cells and their different relative concentrations can modulate the chemical signals in a great number of ways [1–3].

Soluble proteins, belonging to the two major classes so far described, odorant-binding proteins (OBPs) and chemosensory proteins (CSPs), are believed to play a role in such communication by modulating both the released signals and their perception. OBPs and CSPs are highly concentrated in the lymph of chemosensilla and, despite their different structure, apparently play similar roles in insect chemical communication. Both types of polypeptide have small masses (around 15 kDa for OBPs and 12 kDa for CSPs), are very soluble and are extremely resistant to thermal denaturation. OBPs present six conserved cysteines all paired in an interlocked fashion [4, 5], while the four cysteines of CSPs

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are linked by two disulphide bridges, connecting adjacent residues [6].

A great number of OBPs and CSPs have been characterised in several insect species and orders. The following references and those provided in other parts of the text represent only selected examples from the great number of publications available in the literature [7–19]. OBPs show wider diversity than CSPs both between and within species. Both classes of protein are represented in each species by a variable number of polypeptides, which have often been grouped in subclasses on the basis of their sequence similarities.

The three-dimensional structure of only one member of OBPs, the pheromone-binding protein (PBP) of *Bombyx mori* [20–22] and one member of the CSPs, CSPA6 from *Mamestra brassicae* [23, 24] have been resolved so far. Both proteins mainly contain α-helical domains and present hydrophobic binding pockets, which can accommodate ligands of medium size. However, their three-dimensional foldings are clearly different from one another and bear no similarity to other known structures.

The number of OBPs and CSPs expressed is very variable, according to the insect species. However, in most cases, the proteins reported in the literature represent only a small fraction of those actually expressed. In *Drosophila*, thanks to complete sequencing of the entire genome, we know that 51 genes exist, encoding and possibly expressing proteins of the OBP family [25].

In some cases, CSPs have been reported to be expressed in parts of the body other than sensory organs, where they could be involved in the release (and possibly in the modulation) of chemical signals. In *Drosophila,* the ejaculatory bulb simultaneously secretes the specific sex pheromone, vaccenyl acetate, and a protein of the CSP family [26]. In the lepidopteran *M. brassicae,* CSPs are also expressed in pheromonal glands [27]. In *Locusta migratoria*, one of the CSPs is highly expressed in the wings, where it is strongly associated with oleoamide [28]. In fire ants, a protein of the OBP family, which has been related to social behaviour, has been isolated from the thorax [29].

Binding assays have been described for proteins of both classes, using fluorescent probes, such as 1-aminoantharcene (1-AMA), N-phenyl-1-naphthylamine (1-NPN) and 8-anilino-1-naphthalenesulphonic acid (ANS). Generally, broad specificity has been observed, with dissociation constants in the micromolar range and a certain selectivity for some proteins towards groups of ligands [19, 23, 28, 30–33].

As a first contribution to the biochemistry of chemical communication in social wasps, we report here the isolation and characterisation of members of both families of binding proteins, as well as the identification of ligands associated in vivo with such polypeptides.

Materials and methods

Preparation of extracts

Crude extracts of antennae, legs, head, thorax, abdomen and wings of *Polistes dominulus* (Hymenoptera: Vespidae) collected in the field were prepared by homogenisation of the tissues in 50 mM Tris-HCl buffer (Tris buffer), pH 7.4 and centrifugation at 16,000 g for 20 min, at 4 °C. The crude supernatants were analysed by electrophoresis in denaturing or native conditions on 15% polyacrylamide gels, or used in chromatographic separations.

Purification of OBPs and CSPs

Low-molecular-weight proteins (14–15 kDa) were purified from crude extracts of antennae, legs and wings by a combination of gel filtration chromatography on a 1 × 30 cm Superose-12 column with 50 mM ammonium bicarbonate as the eluent, and anion-exchange chromatography on a Mono-Q column (Pharmacia, Uppsala, Sweden) with a 0–0.5 M gradient of NaCl in Tris buffer, using an FPLC system (Pharmacia).

N-terminal sequences

Low-molecular-weight fractions isolated from antennae or legs were separated by polyacrylamide gel electrophoresis under native conditions. Bands were blotted on PVDF membranes and subjected to sequential Edman degradation using a Procise 491 protein sequencer (Applied Biosystems, Foster City Calif.) equipped with a 140C microgradient apparatus and a 785A UV detector (Applied Biosystems) for the automated identification of phenylthiohydantoin-amino acids.

RNA extraction and cDNA synthesis

Total RNA was extracted from the tissues of *P. dominulus*, using the Trizol reagent kit (GIBCO BRL, Invitrogen Italia, San Giuliano Milanese, Italy), according to the manufacturer's protocol. cDNA was prepared from total RNA by reverse transcription, using an oligo-dT_{20} or the tailed oligo-dT ATTCTAGAGCGGCCGCGACATGT_{20} VN and the Thermoscript RT-PCR System (Invitrogen), following the manufacturer's protocol, in a 20-µl total volume.

Polymerase chain reaction

Aliquots of 1 µl of crude cDNA were amplified in a Bio-Rad Gene Cycler thermocycler, using 1 unit of *Thermus aquaticus* DNA polymerase (Amersham Pharmacia Biotech), 200 µM of each dNTP (Pharmacia Biotech), 1 µM of each PCR primer, 50 mM KCl, 2.5 mM $MgCl_2$ and 0.1 mg/ml bovine serum albumin in 10 mM Tris-HCl, pH 8.3, containing 0.1% Triton X-100. At the 5′ end we used a 1 : 1 mixture of degenerate primers designed on the N-terminal amino acid sequences of the putative OBP and CSP of *P. dominulus* (for OBP: GAT TCI GAT ATI

GCI GTI AAG AAA TA and GAC ACI GAC ATI GCI GTI AAA AAG TA, encoding amino acids 1–9 DSDIAVKKY; for CSP: AAA TAC GAT TAC ATI GAT CCI ATG GA and AAG TAT GAC TAT ATI GAC CCI ATG GA, encoding amino acids 9–17 KYDYIDPME.

At the 3′ end we used an oligodT_{20}VNN for OBP and the specific primer ATT CTA GAG CGG CCG CGA CAT GT for CSP. After a denaturing step at 95 °C for 5 min, the reaction was performed for 35 cycles [95 °C for 1 min, 45 °C (OBP) or 50 °C (CSP) for 1 min, 68 °C for 1 min], followed by a final step of 7 min at 72 °C. Major bands of about 800 and 500 bp were obtained for OBP and CSP, respectively.

Cloning and sequencing

The crude PCR products were ligated into a pGEM vector without further purification, using a 1 : 5 (plasmid : insert) molar ratio and by incubating the mixture overnight at 4 °C. After transformation of *Escherichia coli* XL-1 Blue competent cells with the ligation product, positive colonies were selected by PCR, using the plasmid primers SP6 and T7 and grown in LB/ampicillin medium. DNA was extracted and custom sequenced at MWG Biotech, Ebersberg Germany.

Cloning of OBP and CSP in the expression vector

The DNA encoding the OBP or the CSP of interest was obtained by PCR from the pGEM plasmid containing the appropriate sequence, using the primers reported below. Sequences were designed to include an *Nde*I restriction site with the ATG codon in the 5′ primer and an *Eco*RI restriction site in the 3′ primer. For OBP, the primers at the 5′ and 3′ ends were CGCATATGGATTCGGATATTGCGGTG and AATTGAATTCTTATTAAAGACCCATTTCTTT, respectively. For CSP the corresponding primers were: TACATATGGARGARGARGARGTNTAYTCNGA and TTGGATCCGAATTCTTATGAGT TTTGTTTCTT. Amplified DNA was again inserted into a pGEM vector that was used to transform *E. coli* XL-1 Blue competent cells. Colonies were tested for the presence of the insert by PCR, using the plasmid primers SP6 and T7, DNA was extracted, purified and digested with *Nde*I and *Eco*RI. The excised 350- to 400-bp fragments were purified from the agarose electrophoretic gel and cloned into pET5b (Novagen, EMD Biosciences, Darmstadt, Germany), previously digested with the same enzymes. The resulting plasmids pET-OBPpd-I and pET-CSPpd-I were sequenced and shown to encode the mature proteins.

Production of recombinant OBP and CSP

For protein expression, *E. coli* BL21(DE3)pLysS cells were transformed with the pET-OBPpd-I and pET-CSPpd-I plasmid. Single colonies were grown overnight in 10 ml Luria-Bertani/Miller broth containing 100 mg/l ampicillin. The culture was diluted 1 : 100 with fresh medium and grown at 37 °C until the absorbance at 600 nm reached 0.6 AU. At this stage, 0.4 mM isopropyl thio-β-D-galactoside was added to the culture to induce expression. After 2 h at 37 °C, the cells were harvested by centrifugation, resuspended in 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM phenylmethylsulphonyl fluoride and lysed by sonication. The recombinant OBP was present at this stage entirely in the supernatant and was expressed with yields of about 20 mg per litre of culture. The recombinant CSP, instead, was expressed with lower yields, about 10 mg per litre of culture, of which only half was present in soluble form.

After dialysis, the proteins were purified by two chromatographic steps on anion-exchange resin DE-52, using a gradient of 0–0.5 M NaCl in Tris buffer, followed by gel filtration on Superose-12. Fractions were analysed by SDS-PAGE for the presence of protein contaminants and by UV spectroscopy to evaluate the amount of DNA coeluted with the protein. At the end of the purification procedure, the proteins were more than 95% pure, as judged by SDS-PAGE, and virtually free from DNA.

Molecular-mass characterisation

Accurate molecular mass for native and recombinant protein samples was measured by MALDI-TOF mass spectrometry using a Voyager DE MALDI-TOF instrument (Applied Biosystems, Framingham, Conn.) under denaturing non-reducing conditions. Protein extracts were subjected to a desalting/concentration step on ZipTipC4 devices (Millipore, Bedford, Mass.) before analysis. Samples were loaded on the instrument target, using the dried droplet techniques and α-cyano-4-hydroxycinnamic acid as matrix. Mass calibration was performed using the molecular ions from different internal standards added to each sample. Data are reported as average masses.

The apparent molecular masses for native and recombinant protein samples were determined by gel filtration chromatography using the FPLC system (Pharmacia), with a Superose-12 1 × 30 cm column, and eluted with 50 mM Tris-HCl, pH 7.4, at a flow rate of 0.2 ml/min. The following proteins were used as molecular-mass standards: cytochrome c (12,300 Da), bovine carbonic anhydrase (29,000 Da) and bovine serum albumin (66,000 Da).

Enzymatic hydrolysis and peptide characterization

Proteins samples were digested with trypsin (Sigma, St. Louis, Ill.) in 25 mM ammonium bicarbonate, pH 6.5 at 37 °C for 18 h, using an enzyme : substrate ratio of 1 : 50 (w/w). Peptide mixtures were desalted/concentrated on µZipTipC18 devices (Millipore) and analysed by MALDI-TOF mass spectrometry as reported above. Data are reported as average masses.

Fluorescence measurements
Emission fluorescence spectra were recorded on a Jasco FP-750 instrument at 25 °C in a right-angle configuration with a 1-cm light path quartz cuvette and 5-nm slits for both excitation and emission. Each protein was dissolved in 50 mM Tris buffer, pH 7.4, while ligands were added as 1 mM methanol solutions.

Intrinsic fluorescence
Tryptophan intrinsic fluorescence was measured on a 1 µM solution of the protein, using an excitation wavelenght of 295 nm and recording the emission spectrum between 300 and 360 nm. Quenching of intrinsic fluorescence was measured under the same conditions and in the presence of ligands at concentrations of 1–20 µM.

Binding assays
To measure the affinity of the fluorescent ligand 1-NPN to recombinant CSP, a 2 µM solution of each protein in 50 mM Tris-HCl, pH 7.4 was titrated with aliquots of 1 mM methanol solutions of the ligand to final concentrations of 1–20 µM. The affinity of other ligands was measured in competitive binding assays, using 1-NPN as the fluorescent reporter at 2 µM concentration and concentrations of 2–20 µM for each competitor.

Data analysis
For determining binding constants, the intensity values corresponding to the maximum of fluorescence emission were plotted against free ligand concentrations. Bound ligand was evaluated from the values of fluorescence intensity assuming the protein to be 100% active, with a stoichiometry of 1:1 protein:ligand at saturation. The curves were linearised using Scatchard plots. Dissociation constants of the competitors were calculated from the corresponding IC_{50} values, using the equation: $K_D = [IC_{50}]/(1+[1\text{-NPN}]/K_{1\text{-NPN}})$, [1-NPN] being the free concentration of 1-NPN and $K_{1\text{-NPN}}$ being the dissociation constant of the complex protein/1-NPN.

Extraction and identification of endogenous ligands
Crude OBP samples, purified from about 1000 wings of male or female *P. dominulus* by gel filtration on Superose 12, were concentrated to about 0.5 ml and extracted with two aliquots of 1 ml dichloromethane. The organic phases were combined, concentrated to about 0.2 ml and analysed by GC-MS using a HP5890A/HP5971A instrument (Agilent, Palo Alto, Calif.). Gas chromatographic separations were performed on a Restek Rtx-5MS column using the following conditions: isotherm at 70 °C for 30 min, first gradient of 30 °C/min up to 150 °C, 5 min isotherm, then a second gradient of 5 °C/min to 310 °C and final isotherm of 11 min. The injector was kept at a temperature of 280 °C. Mass spectra were acquired between m/z 42 and 550.

Synthesis of fatty acid amides
Samples (0.2–1.0 g) of methyl esters of pelargonic, lauric, myristic, palmitic, oleic, elaidinic and stearic acids were dissolved in methanol (100–300 ml) and the solutions were saturated with ammonia gas at 0 °C and kept for 3 days at room temperature in sealed flasks. Evaporation of methanol afforded the pure amides in quantitative yields.

Results

Identification and purification of OBPs and CSPs
A first comparison of the crude extract from different parts of the *P. dominulus* body, as analysed by polyacrylammide gel electrophoresis in denaturing conditions, revealed prominent bands with apparent molecular masses of about 15 kDa, mainly in antennae and legs (fig. 1A). Electrophoretic analysis in native conditions also revealed fast-migrating bands in the same organs. Microsequencing of such bands, as blotted from a native electrophoretic gel, yielded two main sequences showing low but significant similarities with proteins of the OBP and CSP families (fig. 1C). In particular, a putative OBP sequence was detected in sensory parts, such as antennae and legs, as well as in wings. In contrast, a band specifically present in the antennal extract of both sexes yielded a sequence similar to CSPs.
The most prominent bands with apparent molecular weights around 15 kDa in SDS-PAGE, indicated as putative OBPs on the basis of their partial amino acid sequence, were purified for further characterisation from the antennae and tarsi of both sexes, using gel filtration chromatography, as reported in the experimental section. As an example, the electrophoretic profile of fractions obtained from a male tarsus extract is reported in figure 1B. Similar experiments were performed on putative CSPs (data not shown).

Cloning of OBP and CSP from the tarsi of *P. dominulus*
On the basis of the N-terminal sequence obtained from gel bands of putative OBPs and CSPs, degenerate primers were designed and used, together with an oligodTVNN or with a tailed oligodT to amplify cDNA, obtained from total RNA of female antennae of *P. dominulus* in PCR experiments. Bands of about 800 and 500 bp were obtained for OBP and CSP, respectively. Cloning in pGEM vector and sequencing afforded two sequences, one for OBP and one for CSP. The OBP partial cDNA contained 767 bp, with an open reading frame of 375 bp, encoding a polypeptide of 125 amino acids, corresponding to the entire mature protein. The CSP partial cDNA contained 480 bp, with an open reading frame of 276 bp, encoding a polypeptide of 92 amino acids, correspond-

ing to residues 9–100 of the mature protein. The sequences of OBP and CSP of *P. dominulus* can be accessed from GenBank under the numbers AY297026 and AY297027, respectively.

The deduced amino acid sequences are aligned with homologous proteins from other insect species in figure 2. OBP bears poor similarity to homologous proteins, with percent identities below 20%, while CSP shares generally 30–40% of its residues with proteins from other species, reaching almost 60% in one case.

Bacterial expression of OBP and CSP

Both OBP and CSP from *P. dominulus* were expressed in a bacterial system, using the protocol employed for other proteins of the same families [34, 35]. The construct included the nucleotide sequence coding for the mature protein, an ATG codon at the 5′ end and a stop codon at the 3′ end. Thus, the expressed proteins only differed from the native ones for an additional methionine residue in position 1. Both proteins were expressed in good yields (around 20 mg of OBP and 10 mg of CSP per litre of bacterial culture) (fig. 1D).

While all the OBP was produced in soluble form, only about half of the CSP was obtained in the supernatant after sonication of the cells. Both proteins were purified, using a combination of anion-exchange chromatography and gel filtration, as described in Materials and methods. The sequences of the recombinant proteins and the correct pairing of the disulphide bridges were confirmed by MALDI mass spectrometry analysis of the tryptic digests (fig. 3).

Protein expression in different tissues

To verify the specific expression of the cloned OBP and CSP in different sensory parts, low-molecular-weight protein components partially purified from antennae, legs and wings were subjected to a desalting/concentration step on C4 devices and analysed by MALDI-MS. Figure 4 shows the results obtained for tissues from female and male individuals. While a molecular species matching the exact mass of OBP, as calculated from its amino acid sequence (theoretical value 13,723.1 Da), was found in all the samples examined, a component presenting the predicted molecular weight of CSP (theoretical value 12,059.6 Da) was present only in the antennal extracts of both sexes. Additional species corresponding to

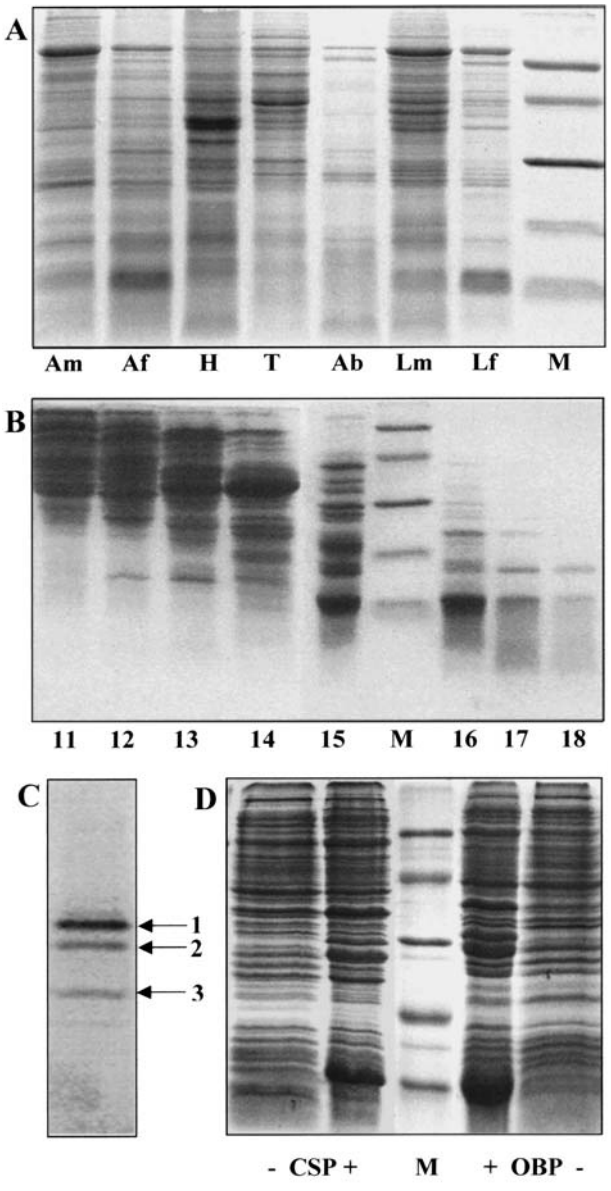
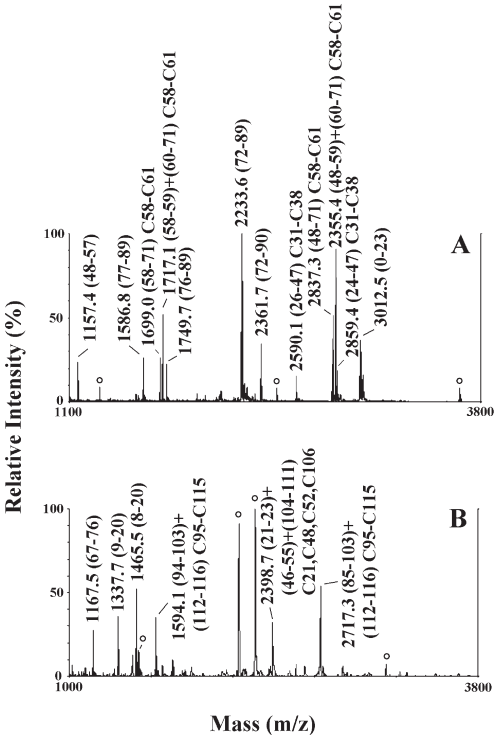


Figure 1. Electrophoretic analysis of soluble proteins of *P. dominulus* involved in chemical communication. (*A*) Electrophoretic analysis in denaturing conditions (15% SDS-PAGE) of crude extracts from different parts of the body. Am, male antennae; Af, female antennae; H, female head; T, female thorax; Ab, female abdomen; Lm, male legs; Lf, female legs; M, molecular-weight markers (from the top: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; trypsin inhibitor, 20 kDa; α-lactalbumin, 14 kDa). (*B*). Electrophoretic analysis in denaturing conditions (15% SDS-PAGE) of fractions obtained by gel filtration chromatography of a crude extract from female tarsi. M, as above. (*C*). Electrophoretic analysis in native conditions (15% SDS-PAGE) of a low-molecular-weight fraction obtained from female antennae by gel filtration chromatography. Proteins were blotted onto PVDF membrane and subjected to Edman degradation. Bands 1 and 2 gave the same N-terminal sequence DSDIAVKKYLHAVPEPVLAKKLKEE(S)GLEADKD- (limited to residues 1–18 for band 2), similar to OBPs of other species, while band 3 and other minor bands gave the sequence EEEELYSDKYDYIDPMEIVNNDRLRDQYYNXFMN- (or limited to shorter fragments), indicative of a protein of the CSP family. In these latter sequences, the concomitant presence of glutamic acid and aspartic acid in the first four residues was sometimes detected during Edman degradation, suggesting the presence of different isoforms. (*D*). Electrophoretic analysis in denaturing conditions (15% SDS-PAGE) of crude bacterial cultures expressing CSP and OBP, sampled before (–) and after (+) induction with isopropyl-beta-D-thiogalactopyranoside.

Protein	Amino acid sequence
P.dom OBP-1	DSDIAVKKYLHAVPEPVLAKCLKESGLEADKDKLLSDEST---------VDQGKFSCLIACTLKDNGAL
A.mel ASP2	IDQDTVVAKYMEYLMPDIMP-CADELHISEDIATNIQAAKNG--------ADMSQLGCLKACVMKRIEML
L.mig OBP-1	DVNMKLTGRIMDAAKEVDHTCRSSTGVPRDMLHRYAEGQT---------VDDDDFKCYLKCIMVEFNSL
D.mel PBPRP-5	RAFDEKEALAKLMESAES-CMPEVGATDADLQEMVKKQP---------ASTYAGKCLRACVMKNIGIL
S.gem OBP	SRDSAKKIGSQYDNFET-CITEHGLTEDDIFSIGEVSTGNHKTNHEDTELHKNGCVIQCMLEKDGLM
P.dom OBP-1	-VNGELKYDVLSELLSKLLTNKEDKLQERLELLKACIPEGANAKNDCEYIGKIMQCKLSKAKEMGL
A.mel ASP2	-KGTELYVEPVYKMIEVVHAGNADDIQLVKGIANECIENAKGETDECNIGNKYTDCYIEKLFS
L.mig OBP-1	SDDGVFVLEEELENVPPEIK------EEGHRVVHSCKHINH--DEACETAYQIHQCYKQSDPELYS
D.mel PBPRP-5	DANGKLDTEAGHEKAKQYTGNDPAKLKIALDIGETCAAITV-PDDHCEAAEAYGTCFRGEAKKHGL
S.gem OBP	-SGADYDEEKMREDYIKETGAQPG--DQRIEALNACMHETKDMEDKCDKSLILVACVLAAEAVLAD

Protein	Amino acid sequence
P.dom CSP-1	EEEELYSDKYDYIDPMEIVNNDRLRDQYYNCFMNTGP--CVTPDAIYFKEHFPEAVVTK
A.mel ASP3	DESYTSKFDNINVDEILHSDRLLNNYFKCLMDEGR--C-TAEGNELKRVLPDALATD
L.mig CSP-II-8	AAAYTTKYDNIDLDEILNNERLLKKYYECLMSDSDASC-TPDGKELKVSIPDALVTD
D.mel OS-D	RMVEQAYDDKFDNVDLDEILNQERLLINYIKCLEGTGP--C-TPDAKMLKEILPDAIQTD
L. hum CSP	EDLYADTYDHIEPMEILNNDELRNQYYNCVMNTGP--CMSDEQRFLKEHVAEAMATR
P.dom CSP-1	CKKCTEIQKTNFEKLAIWYNENRPDEWTALIKKFMEDAKKQNS
A.mel ASP3	CKKCTDKQREVIKKVIKFLVENKPELWDSLANKYDPDKKYRVKFEEEAKKLGINV
L.mig CSP-II-8	CSKCNEKQKEGSNKVIRFLIQKKEDLWKSLQAKYDPEGTYLKKHPELLSA
D.mel OS-D	CTKCTEKQRYGAEKVTRHLIDNRPTDWERLEKIYDPEGTYRIKYQEMKSKANEEP
L. hum CSP	CRRCTERQKDGLEKVVVWYTENRPEEWSALVVHLIEEAKKQNITPVSGGFI

Figure 2. Deduced amino acid sequences of OBP and CSP of *P. dominulus* aligned with homologous proteins from other isnsect species. Percent of identical amino acids are in the range of 17–22% for OBP and 36–58% for CSP. As previously observed, CSPs present, besides the four-cysteine motif, a number of conserved residues, while OBPs are more divergent. *P. dom*, *Polistes dominulus* (acc. no. OBP AY297026, CSP AY297027); *A. mel*, *Apis mellifera* (acc. no. OBP AF393493, CSP AF481963); *L. mig, Locusta migratoria* (acc. no. OBP AF542076, CSP AY149656); *D. mel, Drosophila melanogaster* (acc. no. OBP U05985, CSP U02546); *L. hum, Linepithema humile* (acc. no. CSP AY129672); *S. gem, Solenopsis geminata* (acc. no. OBP AF427905).



unknown components were also detected. These findings demonstrated the ubiquitous expression of OBP in all sensory tissues analysed and the specific expression of CSP in the antennae.

Moreover, to determine the protein aggregation state, these mass spectrometry measurements were compared to gel filtration chromatography experiments under native conditions. The chromatographic analysis performed with different molecular-mass standards indicated for OBP and CSP an apparent molecular mass of 25 kDa and 20 kDa, respectively. On the basis of the calculated mass of these species (see above), these experiments indicated that both OBP and CSP are apparently present in solution as dimers.

Figure 3. MALDI-MS analysis of recombinant CSP (*A*) and OBP (*B*) from *P. dominulus* following digestion with trypsin. Signals corresponding to the disulphide-bridged peptides are reported; each signal is assigned to the corresponding peptide pair and the cysteine residues involved in the S-S bond are indicated. Circles indicate ions generated from trypsin autoproteolysis or molecular markers added for internal calibration. Residue 0 corresponds to the exogenous methionine residue at the N terminus.

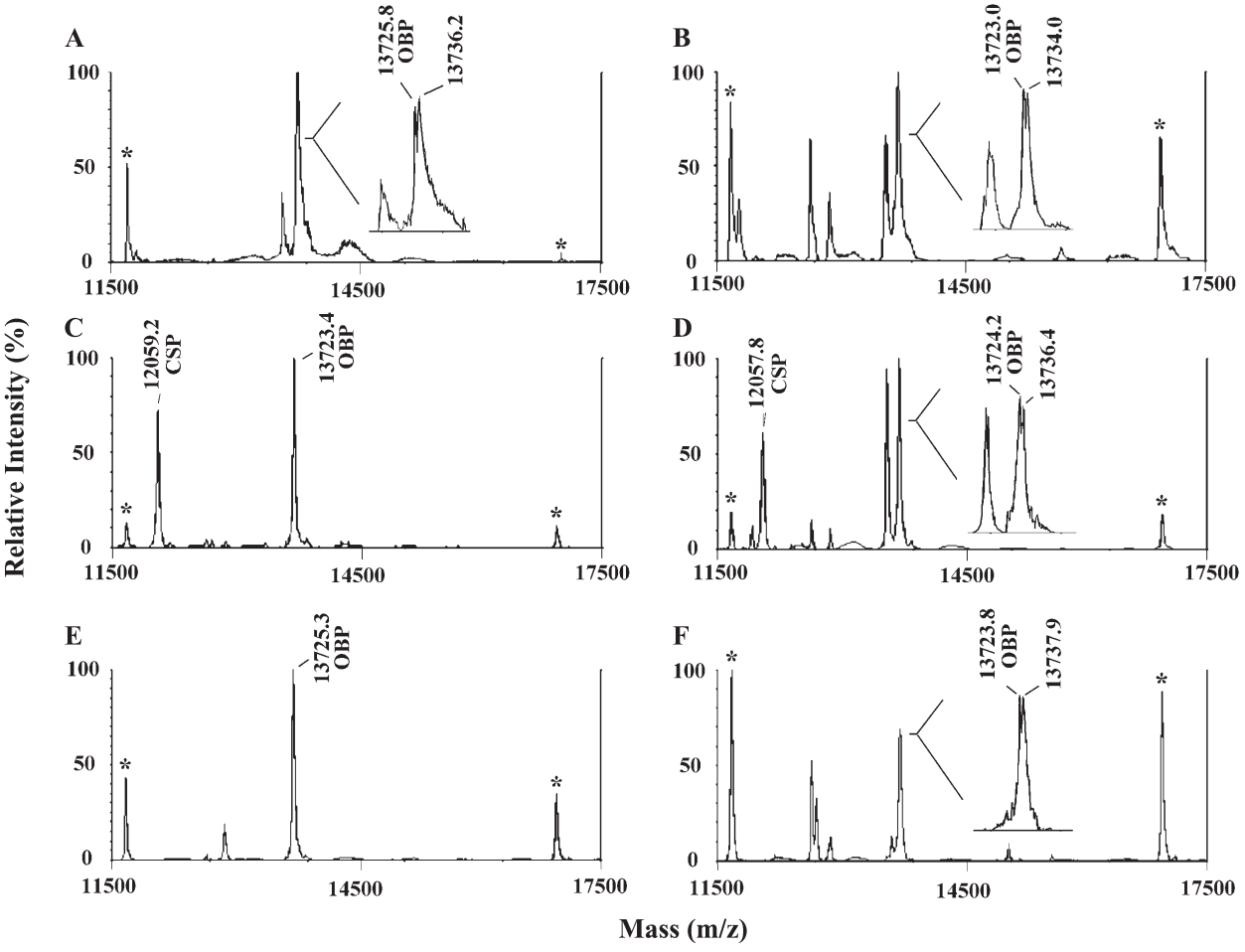


Figure 4. Specific expression of the OBP and CSP in different sensory parts of *P. dominulus*. Low-molecular-weight protein components partially purified from antennae, legs and wings were subjected to a desalting/concentration step on C4 devices and analysed by MALDI-MS. Female legs (*A*), male legs (*B*), female antennae (*C*), male antennae (*D*), female wings (*E*), male wings (*F*). Asterisks indicate molecular marker ions used for internal calibration.

Endogenous ligands of *P. dominulus* OBP

On the basis of previous observations in other insect species, we looked for the presence of soluble binding proteins in the wings of *P. dominulus* and small organic molecules possibly associated with them. Crude extracts of wings from both males and females presented a band with an apparent molecular weight of about 14 kDa in SDS-PAGE, identified as OBP on the basis of MALDI-MS analysis (see fig. 4). These proteins were further purified by gel filtration chromatography and separately extracted in aqueous solutions with dichloromethane. These organic extracts were then subjected to GC-MS experiments. This analysis revealed the presence of several organic components in both extracts, with clear differences between sexes (fig. 5). In the female extracts, together with nonanal, the only compound also present in the male sample, a series of homologous amides were identified, including those of pelargonic, myristic and oleic acids, the latter being the most abundant. In contrast, in males, such amides were absent, while the most prominent peaks could not be assigned with confidence to any structure present in the MS database.

Binding experiments

Both recombinant *P. dominulus* OBP and CSP were used to measure affinities to a number of potential ligands, using the fluorescent probe 1-NPN. Binding assays were performed according to the previously described protocol [30]. The fluorescent probe 1-NPN binds both OBP and CSP with dissociation constants of 2.1 and 2.2 µM, respectively (fig. 6A, B). In both cases, the Scatchard plot gave a linear profile, indicating the absence of cooperativity effects. The affinities of other compounds were measured in competitive binding assays, using a concentration of protein of 2 µM and of the fluorescent ligand of 4 µM, while the concentration of each competitor was increased up to 20–30 µM.

In the absence of information relative to pheromones or other molecules of some relevance for this species, we based our choice of the ligands on those found associated

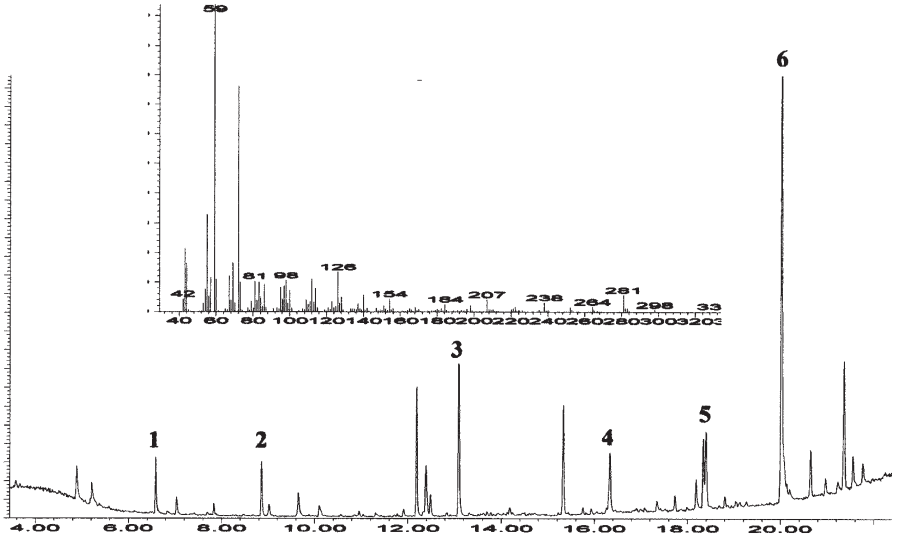


Figure 5. Gas-chromatographic profile of a dichloromethane extract from a sample of OBP purified from the wings of female *P. dominulus*. The inset reports the mass spectrum of the main peak (6), identifying this compound as oleoamide. Other peaks in the gas chromatogram were assigned to nonanal (1), thymol (2), tert-butylbenzoic acid (3), pelargonic amide (4) and myristic amide (5). A parallel analysis performed on the protein extracted from male individuals yielded a different profile, with peaks that could not be clearly assigned.

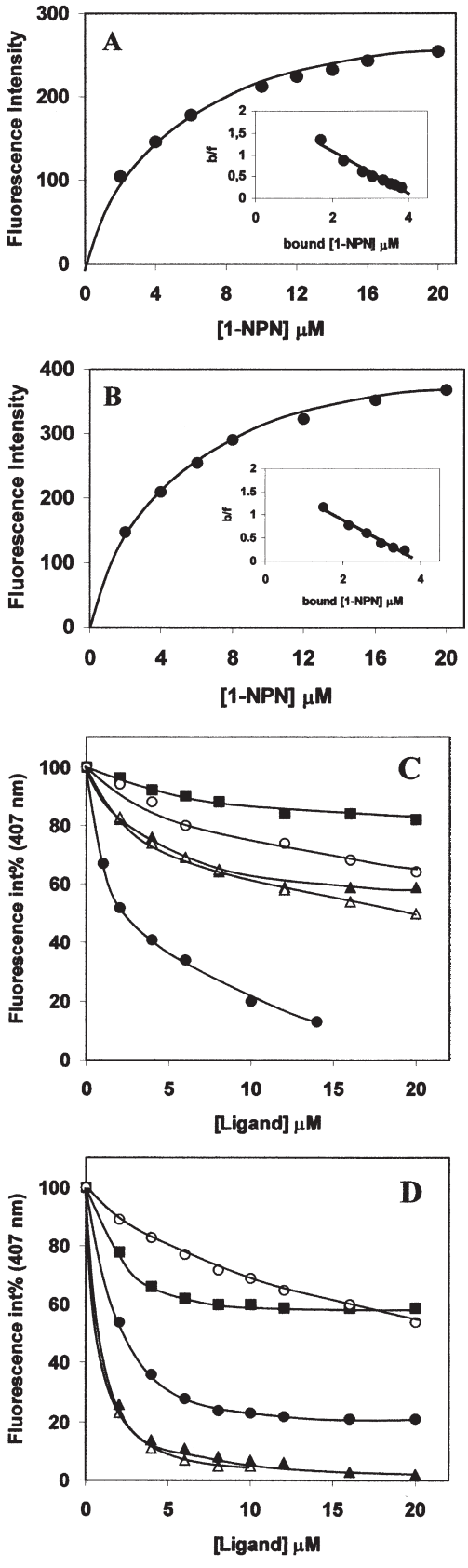
with OBPs in the wings. In particular, we used series of linear saturated amides, alcohols and carboxylic acids of 12–18 carbon atoms. A few unsaturated amides were also included, as were other compounds, such as aldehydes and esters. The results are summarised in table 1, while figure 6C, D reports the binding curves obtained with 1-NPN and the data relative to a few representative competition experiments.

OBP and CSP showed clearly different binding specificities to the amides examined. While OBP seemed to bind oleamide in a selective way, CSP exhibited a broader spectrum of activity, binding equally well saturated amides of 12–16 carbon atoms. Its affinity dropped drastically with pelargonic and stearic amides, but, surprisingly, the insertion of a double bond into stearic amide restored good binding activity. Another remarkable observation concerned the behaviour of the two geometrical isomers elaidic and oleic amide. OBP seemed to discriminate them quite clearly, whereas they bound equally well to CSP. Differences between OBP and CSP were also observed with saturated straight-chain alcohols of 12–18 carbons, OBP once more showing more selectivity. Good affinity was also measured for both proteins with long-chain compounds bearing other functional groups, such as acid, ester and amine.

Discussion

We identified and cloned members of the OBP and CSP families from the wasp *P. dominulus*. We followed a biochemical approach, starting with the identification of the proteins and the determination of N-terminal sequences, followed by the application of molecular biology tools to determine the full sequences of the mature proteins. Such an approach yielded a single sequence for both OBP and CSP. However, we cannot exclude the presence of other members of both classes of proteins. In fact, other observations have suggested a microdiversity at the level of these proteins. N-terminal Edman degradation gave multiple sequences and MALDI mass spectrometry also afforded several peaks in the same mass range. Furthermore, several members of OBPs and CSPs have been described in all other insect species so far studied. Direct identification of the expressed proteins or cloning of the relative genes often yields only a fraction of the total number present in the genome, as in the case of *Drosophila melanogaster*, where only a few of the 51 OBPs were identified before screening the genome.

An interesting and unusual characteristic of the wasp proteins is their distribution in different parts of the body. Unlike most cases so far described, OBP seems to be rather ubiquitous, being equally expressed in sensory organs, such as antennae and legs, as well as in other parts, such as wings. In contrast, CSP is preferentially expressed in the antennae. Only one case has recently been reported in the literature of a similar ‘anomalous’ distribution of these two classes of proteins, that of the argentine ant *Linepithema humile* [36]. Such findings strengthen the idea that CSPs could be as important as OBPs in pheromone and odour perception, playing similar roles.



Certain species seem likely to preferentially use OBPs and others CSPs for the same purpose.

The results of binding experiments also present some interesting aspects. Generally, OBP showed a higher specificity than CSP towards the ligands assayed. In particular, among the series of aliphatic amides, OBP appeared to be selective only for oleamide. Even closely related structures, differing from oleamide in configuration (elaidic) or the presence (stearic) of the double bound, exhibited much weaker affinity. Such specificity appears remarkable when compared with the broad spectrum of binding of other insect OBPs reported in the literature. For example, both the PBP of *Antheraea polyphemus* and that of *M. brassicae* also bind, in addition to the specific pheromones, structurally related compounds with similar activities [32]. However, when the interaction of such ligands was monitored probing the intrinsic fluorescence of a tryptophan residue located inside the binding pocket, only one of the three pheromone components of *A. polyphemus* quenched the tryptophan fluorescence of PBP1, while the others were ineffective [37]. Such specificity was further supported by the observation that only one of the three pheromone components induced a conformational change in the PBP1 of the same species [38]. These results indicate that OBPs exhibit a certain degree of specificity towards their ligands, and such specificity can be observed in the affinity of binding (as in the case described here) or only in the microscopic interaction with the protein. Oleamide, the best ligand for *P. dominulus* OBP, was also found to be associated with OBP in the wings of female individuals. This is not the first case in which long-chain amides have been identified as potential chemical messengers in insects. In the locust, we also found oleamide associated with proteins of the wings, in that case, CSPs rather than OBPs [28].

Our binding protocol did not allow evaluation of the stoichiometry of binding. In fact, we assumed that at saturation, one molecule of fluorescent probe bound to one molecule of protein; on this basis, we evaluated the spectroscopy parameters necessary for calculating the binding constant. However, for some ligands, such an assumption

Figure 6. Binding assays performed with purified samples of recombinant OBP (*A, C*) and CSP (*B, D*). In both cases, the binding curves (*A, B*) indicated a single type of binding site without any cooperativity effects. The fluorescent probe 1-NPN bound to OBP and CSP with similar affinities (K_D = 2.1 and 2.2 μM for OBP and CSP, respectively). Displacement curves were obtained using the protein at a concentration of 2 μM and the probe at 4 μM. For evaluation of the binding constants we assumed a 1 : 1 stoichiometry and a 100% activity of the protein. The following fatty acid amides were used as competing ligands: pelargonic (open circles), myristic (open triangles), stearic (filled squares), elaidic (filled triangles), oleic (filled circles).

Table 1. Binding of aliphatic amides and related compounds to OBP and CSP of *P. dominulus*.

Ligand	Number of carbons	OBP		CSP	
		$[I]_{50}$	K_D (μM)	$[I]_{50}$	K_D (μM)
Amides					
Pelargonic	9	30	11	20	7.1
Lauric	12	>20		5	1.8
Myristic	14	20	7.1	1	0.36
Palmitic	16	≫20		1	0.36
Stearic	18	≫20		>20	
Elaidic	18	>20		1.5	0.53
Oleic	18	2	0.71	2	0.71
Alcohols					
Dodecanol	12	0.8	0.29	2.5	0.89
Tetradecanol	14	8	2.8	0.8	0.29
Hexadecanol	16	>30		1	0.36
Octadecanol	18	30	11	9	3.2
Others					
Lauric acid	12	22	7.9	≫10	
Stearic acid	16	8	2.8	1.8	0.64
Methyl laurate	13	2.5	0.89	0.6	0.21

Competitive binding assays were performed using 2 μM protein solutions containing the fluorescent probe (1-NPN) at a concentration of 4 μM and the other ligands at concentrations of 1–30 μM.

may not be correct, as indicated by the recent observation that the *M. brassicae* CSP can take up into its binding site three molecules of the ligand 12-bromo-dodecanol in a cooperative manner. This process, as observed in the X-ray structure, is accompanied by a drastic conformational change in the protein, whose binding pocket becomes much larger [39].

Despite the extensive structural information available for both classes of these soluble proteins, their functional role in olfaction is not yet clear. However, the increasing evidence for a significant specificity of these proteins towards potential ligands suggests that they could be involved to some extent in the recognition of chemical messengers.

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