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Research Article

Expression and immunolocalisation of odorant-binding and chemosensory proteins in locusts

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Abstract. We have identified, cloned and expressed a new chemosensory protein (CSP) in the desert locust *Schistocerca gregaria* belonging to a third sub-class of these polypeptides. Polyclonal antibodies stained a band of 14 kDa, as expected, in the extracts of antennae and palps of the adults, but not in the 4th and 5th instars. In the related species *Locusta migratoria*, instead, the same antibodies cross-reacted only with a band of apparent molecular mass of 35 kDa in the extract of 1st–5th instars, but not in the adults. The recombinant protein binds the fluorescent probe N-phenyl-1-naphthylamine, but none of the compounds so far reported as pheromones for *S. gregaria*. The expression of the odorant-binding protein (OBP) and of CSPs of sub-classes I and II was also monitored in antennae, tarsi, palpi, wings and other organs of solitary and gregarious locusts in their nymphal and adult stages. OBP was found to be antenna specific, where it is expressed at least from the 3rd instar in both solitary and gregarious locusts. CSPs, instead, appear to be more ubiquitous, with different expression patterns, according to the sub-class. Immunocytochemistry experiments revealed that OBP is present in the sensillum lymph of sensilla trichodea and basiconica, while CSP-I and CSP-III were found in the outer sensillum lymph of sensilla chaetica and in the sub-cuticular space between epidermis and cuticle of the antenna. Sensilla chaetica on other parts of the body showed the same expression of CSP-I as those on the antenna.

Key words: Odorant-binding protein; chemosensory protein; Western blot; immunocytochemistry; sensillum; *Schistocerca gregaria*; *Locusta migratoria*.

Two major families of soluble proteins are expressed in chemosensilla of insects and seem to be involved in the perception of volatile and contact stimuli: odorant-binding proteins (OBPs) and chemosensory proteins (CSPs). Both are small polypeptides (120–140 residues for OBPs and 100–120 for CSPs) mainly folded in α-helical domains [1, 2]. However, amino acid sequences and three-dimensional structures are completely different between the two classes of protein. The typical signature of OBPs is a set of six cysteine residues, whose relative positions and interlocked pairing is conserved. Apart from this motif, OBPs of different insect orders may share only a few residues, with a percentage of identical amino acids often lower than 15%. Even within the same species, OBPs are extremely divergent, as in the case of *Drosophila melanogaster*, where 39 genes encode polypeptides with the OBP signature, with similarities between members

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ranging from well below 10% to 40% of identical residues, the only exception being OSE and OSF with 68% [3]. CSPs present a motif of only four cysteines paired in a non-interlocked way, thus defining two small loops of 8–10 and 4 residues, including the two cysteines. While the three disulphide bridges of OBPs clearly contribute to the rigidity of their structures, in CSPs, the presence of the two small loops does not seem to improve the stability of the protein. CSPs are also more conserved than OBPs across evolution, with often around 50% of identical residues even between members of phylogenetically distant species [for reviews on OBPs and CSPs see refs 4–9].

Immunocytochemistry experiments have shown that both OBPs and CSPs are highly concentrated in the lymph of chemosensilla [10–15]. The OBPs of moths have been further sub-divided into pheromone-binding proteins (PBPs), localised predominantly in sensilla trichodea, responding to pheromones, and general odorant-binding proteins (GOBPs), localised predominantly in sensilla basiconica, generally involved in detecting plant odours [12, 16]. However, with more antibodies becoming available, a more complex picture of the distribution pattern of different sub-classes of OBPs and CSPs among the various types of sensilla has arisen, particularly when looking at insects of other orders [14, 17].

In the locust (*Locusta migratoria*), cloning has revealed the presence of two closely related genes encoding OBPs, whose expression products seem to be by far the major, if not the only, components detected by MALDI mass spectrometry [18]. By contrast, at least 20 genes have been described for CSPs in the same species and a great diversity has been observed at the protein level. Based on their similarities at the amino acid level, these sequences can be segregated into two sub-classes. Members of the same sub-class can differ by only a few or up to 50% of their amino acid residues [10, 19, 20]. Nevertheless, OBPs and CSPs of hemimetabolous insects are still much less thoroughly investigated than in holometabolous orders, in particular, Lepidoptera and Diptera.

Here we report the identification of the first member of a third sub-class of CSPs in the desert locust *Schistocerca gregaria*. Using polyclonal antibodies, we have also monitored the expression of OBP and the three CSP sub-classes in nymphal and adult stages of solitary and gregarious locusts, and have localised their presence in the sensillum lymph of different types of chemosensilla.

Materials and methods

Identification of the gene encoding CSP-III

Total RNA was extracted from the antennae of instars and adult *S. gregaria*, using the Trizol reagent kit (GIBCO BRL), according to the manufacter's protocol.

The subtractive analysis was performed by a Representational Difference Analysis of cDNA according to the published procedure [21]. Briefly: for each sample (adult = Tester and third instar = Driver), an amplified cDNA was synthesised and digested with DpnI (Invitrogen). Only the Tester was then ligated with specific linkers and mixed at a ratio of 1:100 with the Driver. The obtained solution, after denaturation and renaturation, was digested with the Exo Mung nuclease (Invitrogen) and reamplified with the same primers previously used as linkers. The obtained product was reintroduced into the same procedure twice and the final amplified DNA fragments were cloned into a pGem T vector system (Promega) and custom sequenced at MWG, Ebersberg, Germany. Complete cDNA sequences of the clones of interest were then obtained adopting a 5′- and 3′-RACE approach, using the Invitrogen Kit RACE System for Rapid Amplification of cDNA Ends.

Cloning of locust CSP-III in the expression vector

The DNA encoding *Sgre*CSP-III was obtained by PCR from the pGEM plasmid containing the appropriate sequence, using the forward 5′-TCTCCATATGGAGGCTGCACCGCAGGA-3′ and the reverse 5′-CTGGGAATTCTCAGAAGGTGATGCCGC-3′ primers. Sequences were designed to include an *Nde*I restriction site with the ATG codon in the forward primer and an *EcoR*I restriction site in the reverse primer. Amplified DNA was again inserted into a pGEM vector that was used to transform *Escherichia coli* XL-1 Blue competent cells. Colonies were tested for the presence of the insert by PCR, using the plasmid primers SP6 and T7, and DNA was extracted, purified and digested with *Nde*I and *EcoR*I. The excised 350-bp fragment was purified from an agarose electrophoretic gel and cloned into pET5b (Novagen), previously linearised with the same enzymes. The resulting plasmid, pET-*Sg*-CSPIII was sequenced and shown to encode the mature protein *Sgre*CSP-III

Expression and purification of CSP-III

For protein expression, *E. coli* BL21(DE3) cells were transformed with the pET-*Sg*-CSP-III 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 3 h at 37 °C, the cells were harvested by centrifugation, resuspended in 50 mM Tris/HCl pH 7.4, 300 mM NaCl, 1 mM PMSF and lysed by sonication. The recombinant CSP was present at this stage entirely in the supernatant and was expressed with yields of more than 30 mg per litre of culture.

After dialysis, the protein was purified by anion-exchange chromatography on DE-52, followed by gel filtration on Superose 12 and cation-exchange chromatography on CM-52. At the end of the purification procedure, the protein was more than 95% pure, as judged by SDS-PAGE and virtually free from DNA, as shown by its UV spectrum.

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. The protein was dissolved in 50 mM Tris buffer, pH 7.4, while ligands were added as 1 mM methanol solutions. The tryptophan intrinsic fluorescence was measured on a 1 μM solution of the protein, using an excitation wavelength of 295 nm and recording the emission spectrum between 300 and 360 nm. Quenching of intrinsic fluorescence was measured in the same condition and in the presence of N-phenyl-1-naphthylamine (1-NPN) at concentrations of 1–20 μM.

Binding assays

To measure the affinity of the fluorescent ligand 1-NPN to recombinant *Sgre*CSP-III, a 2 μM solution of protein in 50 mM Tris, pH 7.4, was titrated with aliquots of 1 mM methanol solutions of the ligand to final concentrations of 2–20 μM. The affinity of other ligands was evaluated in competitive binding assays, using 1-NPN as the fluorescent reporter at 2 μM concentration and concentrations of 2–300 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 was 100% active, with a stoichiometry of 1:1 protein:ligand. The curves were linearised using Scatchard plots.

Preparation of antisera

Antisera were obtained by injecting an adult rabbit subcutaneously and intramuscularly with 500 μg of recombinant protein, followed by two additional injections of 300 μg after 15 and 30 days. The protein was emulsified with an equal volume of Freund's complete adjuvant for the first injection and incomplete adjuvant for further injections. Animals were bled 10 days after the last injection and the serum was used without further purification. Rabbits were individually housed in large cages, at constant temperature, and all operations were performed according to ethical guidelines in order to minimise pain and discomfort to animals. The following antisera were used in this study: anti-*Sgre*CSP-I and anti-*Sgre*CSP-III, produced against the recombinant CSP-I [10] (Acc. No. AF070964) and CSP-III (this work; Acc. No. AY583763), respectively, of *S. gregaria*, and anti-*Lmig*OBP and anti-*Lmig*CSP-II, produced against the recombinant OBP [18] (Acc. No. AF542076) and CSP-II [19] (Acc. No. AY149658), respectively, of *Locusta migratoria*.

Western blot analysis

After electrophoretic separation under denaturing conditions (14% SDS-PAGE), proteins were electroblotted on a nitrocellulose membrane, by the procedure of Khyse-Andersen [22]. After treatment with 0.2% dried milk/0.05% Tween 20 in PBS overnight, the membrane was incubated with the crude antiserum against the protein at a dilution of 1:500 and then with goat anti-rabbit IgG-horseradish peroxidase conjugate (dilution 1:1000). Immunoreacting bands were detected by treatment with 4-chloro-1-naphthol.

Scanning electron microscopy

For scanning electron microscopy (SEM), the antennae of adult males and females were cut into several parts and fixed in 70% ethanol for 2 h, and then cleaned in an ultrasonic bath (250 W) for 1 min in the same solution. After treatment with 100% ethanol for 30 min, the samples were dried in air. The preparations were mounted on holders and examined by SEM (FEI Quanta200) after gold coating.

Immunocytochemical localisation

Antennae, palps and legs were chemically fixed in a mixture of paraformaldehyde (4%) and glutaraldehyde (2%) in 0.1 M PBS (pH 7.4), dehydrated in an ethanol series and embedded in LR White resin (Taab) with polymerisation at 60 °C. Ultrathin sections (60–80 nm) were cut with a glass knife on a RMC MT-XL or with a diamond knive on a Reichert Ultracut ultramicrotome.

For immunocytochemistry, the grids were subsequently floated on droplets of the following solutions: PBS containing 50 mM glycine, PBGT (PBS containing 0.2% gelatine, 1% bovine serum albumin and 0.02% Tween-20), primary antiserum diluted with PBGT, six changes of PBGT for washing, secondary antibody diluted with PBGT, twice each on PBGT, PBS glycine, PBS and water. Optional silver intensification [23] increased the size of the gold granules from 10 to about 40 nm, and 2% uranyl acetate increased the tissue contrast for observation in the transmission electron microscope (HITACHI H-7500 or Zeiss EM 10A).

The primary antisera were used at dilutions of 1:1000–1:5000 for OBP and 1:5000–1:30,000 for CSPs and incubated at 4 °C overnight. As a control, the primary antiserum was replaced by serum from a healthy rabbit at the same dilution. None of the controls showed any labelling exceeding some scattered background grains. The sec-

ondary antibody was anti-rabbit IgG, coupled to 10-nm colloidal gold (AuroProbe EM, GAR G10; Amersham) diluted 1:20 and incubated at room temperature for 60–90 min. Immunocytochemical labelling was done on sections of three adults and three nymphs (3rd, 4th and 5th instar) of each sex in *L. migratoria* and of two male and one female adults in *S. gregaria*.

Results

Cloning of CSP-III in S. gregaria

Using a subtractive approach, we isolated clones mainly or exclusively expressed in the adult stage of the desert locust. One of these clones encoded a protein with significant similarity to CSPs. A RACE approach afforded a full-length sequence of 617 bp, with an open reading frame of 387 bp (GenBank Acc. No. AY583763). The first 18 residues of the protein show the characteristics of a signal peptide, indicating that the mature protein starts at amino acid 19 and is constituted by 111 residues, with a calculated molecular mass of 12,832.60 and an isoelectric point of 7.89. The protein presents the typical four-cysteine signature of CSPs, but appears to be markedly different from the two sub-classes of these polypeptides, as previously described in locusts [10, 19, 20]. Figure 1 reports the deduced amino acid sequence of *Sgre*CSP-III, aligned with other representative CSPs. The similarity of this protein with other CSPs locusts as well as other insect species is particularly low and supports its assignment to a third sub-group of CSPs.

The mature protein was expressed in *E. coli* BL-21 cells, using the Novagen pET5b plasmid as a vector. The construct bears the starting triplet ATG in addition to the nucleotide sequence coding for the mature protein. Therefore, the amino acid sequence of recombinant CSPs only differ from the native ones for the addition of an initial methionine. The protein was obtained at high yield (30 mg/l of culture) and was completely soluble. It was purified by a combination of gel filtration and ion-exchange chromatography, as described in the Materials and methods. Due to the relatively high isoelectric point of this polypeptide (calculated pI: 7.64), anion-exchange chromatography yielded the protein in the first eluate, while retaining most of the impurities. The purified recombinant protein was used for ligand-binding assays and for the production of polyclonal antibodies.

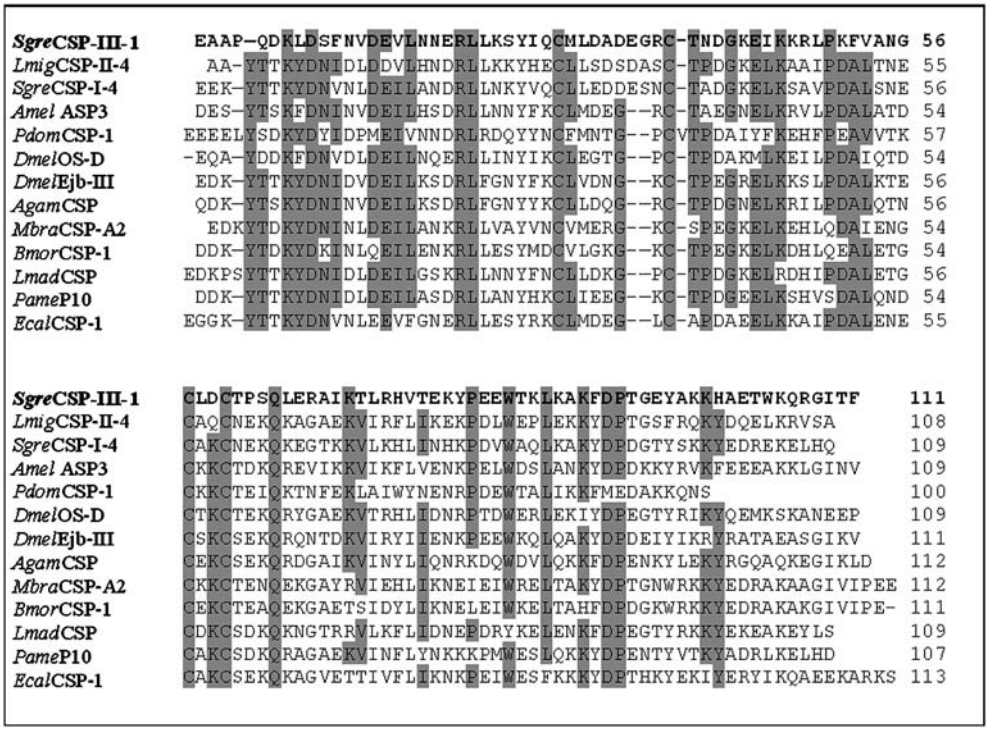

Figure 1. Deduced amino acid sequence of CSP-III of *S. gregaria* aligned with representative CSPs. The first 16 residues of mature *D. melanogaster* OS-D have been omitted. Percentage of conserved amino acids are 37 and 41 with CSP-II and CSP-I, respectively, and 28–38 % with the other proteins. CSP-III appears to be more divergent than other CSPs that on average share around 50% of their residues. Accession numbers are *S. gregaria Sgre*CSP-III-1: AY583763; *L. migratoria Lmig*CSP-II-4: CAB65180; *S. gregaria Sgre*CSP-I-4: AY149651; *Apis mellifera Amel*ASP3c: AF481963; *Polistes dominulus Pdom*CSP-1: AAP55719; *D. melanogaster Dmel*OS-D: AAA21358; *D. melanogaster* ejaculatory bulb Ejb: NP_524966; *Anopheles gambiae Agam*CSP: AAL84186; *Mamestra brassicae Mbra*CSP-A2: AAF19648; *Bombyx mori Bmor*CSP-1: AF509239; *Leucophaea maderae Lmad*CSP: AY116616; *Periplaneta americana Pame*P10 AAB84283; *Eurycantha calcarata Ecal*CSP-1: AAD30550

Ligand-binding experiments

Ligand binding was measured by adopting a fluorescent assay already used for several OBPs and CSPs. The fluorescent probe 1-NPN binds recombinant CSP-III with a dissociation constant of about 8 µM (not shown). The same compound quenches the intrinsic tryptophan fluorescence of the protein in a dose-dependent fashion (not shown). Aromatic compounds, such as phenyl acetonitrile and different phenols, as well as other compounds, reported as putative pheromones and semiochemicals for the desert locust [24–26], were ineffective in displacing the fluorescent probe from the complex.

Tissue and temporal expression

We used polyclonal antibodies raised against recombinant OBP and CSP-II of *L. migratoria*, as well as CSP-I and CSP-III of *S. gregaria*, to monitor, in Western blot experiments, the expression of the four proteins in different parts of the body and during development in both species of insects. We used individuals of *L. migratoria* in both the solitary and gregarious phases, whereas for *S. gregaria,* all experiments were performed with insects in the latter phase. Some representative Western blots are shown in figure 2, while all the data obtained can be summarised as follows.

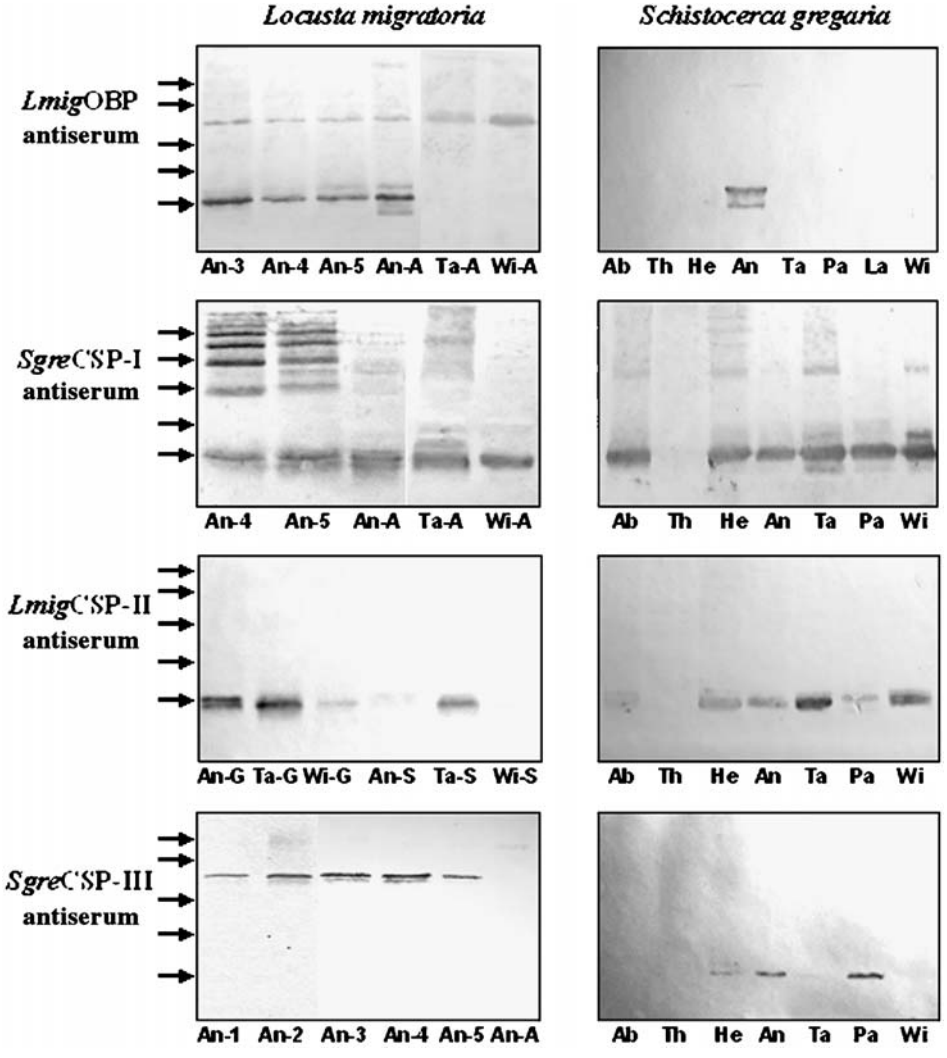

Figure 2. Typical Western blots obtained with antisera against OBP and the three CSPs on crude extracts from *L. migratoria* and *S. gregaria*. OBP was expressed only in the antennae of adults as well as of nymphal stages. CSP-I, instead, was present in most parts of the body. The antiserum recognised several bands in the antennae of the nymph, migrating with molecular weights multiple of the protein mass and indicating the presence of oligomers. CSP-II was also expressed in several organs, but nearly absent in wings of *L. migratoria*. The anti-CSP-III antiserum, which recognised an expected 14-kDa band in the antennae and palpi of *S. gregaria*, failed to stain any protein in the adult sample of *L. migratoria*, while it cross-reacted with a band of apparent mass of 35 kDa in the antennal extracts of the nymph. Arrows indicate the positions of the molecular-weight markers: from the top 66, 45, 29, 20 and 14 kDa. Numbers refer to the different instars, -A stands for adults, -S and -G for solitary and gregarious adults, respectively. Ab, abdomen; Th, thorax; He, heads (without antennae); An, antennae, Ta, tarsi; Pa, palpi; La, labrum; Wi, wings.

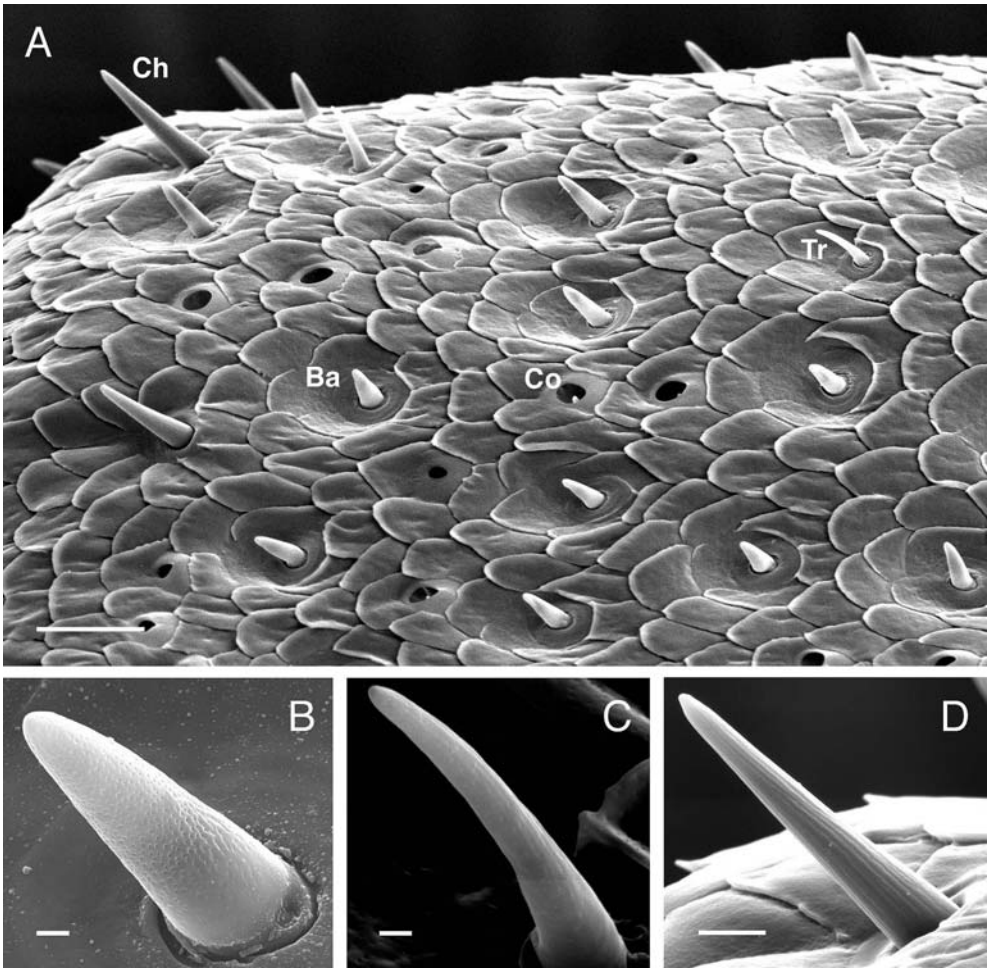

Figure 3. The different types of chemosensitive sensilla on an antenna of adult *L. migratoria* as seen in the scanning electron microscope. (*A*) Sensilla chaetica (Ch) are mainly localised close to the segmental borders. S. basiconica (Ba) and s. coeloconica (Co) are the most common types, whereas s. trichodea (Tr) are fairly rare. The surface of the antenna is sculptured by scale plates. Bar, 20 µm. (*B*) At high magnification numerous wall pores are observed in a s. basiconicum Bar, 1 µm. (*C*) The few pores in a s. trichodeum are hardly visible at high magnifcation. Bar, 1 µm. (*D*) A s. chaeticum at high magnitication displays shallow grooves on the cuticular wall. The tip pore is not visible in this micrograph. Bar, 5 µm.

Generally, a good cross-reactivity was observed between antisera raised against protein of one species and orthologous proteins of the other species. Such results were expected based on the high similarity between orthologous sequences in the two species, at least for CSPs of class I and class II [19, 27].

No differences in the tissue distribution or in the temporal expression of all four proteins were observed between sexes or between individuals of *L. migratoria* belonging to the two phases, with the single exception of CSP-II, present at lower levels, particularly in the antennal extract, in the tissues of solitary locusts (fig. 2), although comparable amounts of protein were loaded, as judged after Coomassie staining of the electrophoretic gels (not shown).

No differences were also observed in the tissue distribution and temporal expression between the two insect species for OBP, CSP-I and CSP-II, whereas CSP-III showed unique expression patterns that will be discussed below.

Looking in detail at each specific protein, we observed that OBP was strictly antenna-specific, while CSP-I was present in all parts of the body examined, with the exception of the thorax, and CSP-II was also widely distributed, but mainly in the tarsi. CSP-III was recognised by the specific antiserum only in antennae and palpi (a weak cross-reactivity in the deantennated head extract was likely due to protein present in the palpi) of *S. gregaria*, while the same antiserum failed to stain any band in the samples of adult *L. migratoria*.

For the expression of OBP and CSPs in the antennae of nymphal stages, we generally observed (again with the exception of CSP-III) synthesis of the proteins at least from the 3rd instar (due to the small size of 1st and 2nd instars, we used such samples in only one experiment). A strange phenomenon was observed with the antennae of

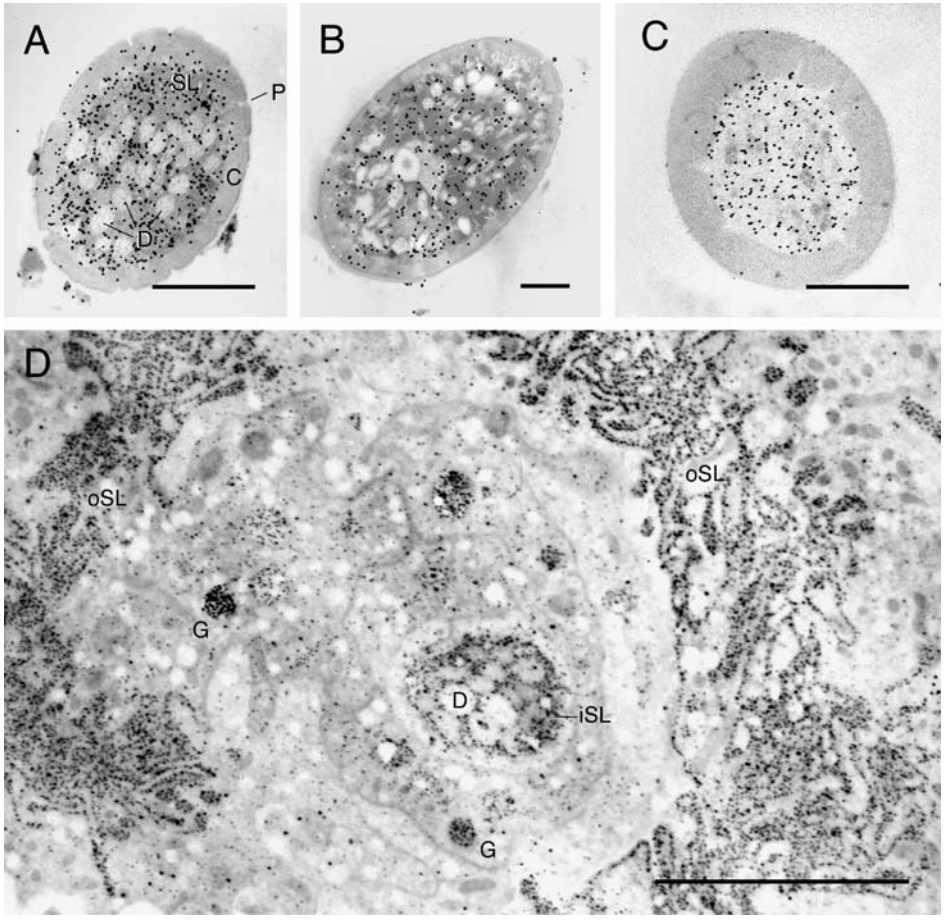

Figure 4. Immunocytochemical localisation of OBP in olfactory sensilla of adult *L. migratoria* (*A*, *C*, *D*) and *S. gregaria* (*B*). In both species, only s. basiconica (*A*, *B*) and s. trichodea (*C*) express OBP. The sensillum lymph (SL) around the dendrites (D), but not the dendrites themselves are heavily labelled. A section through a s. basiconicum below the hair base is shown (*D*). Strong labelling is evident in the inner (iSL) and outer (oSL) sensillum lymph cavity, the latter being bordered by extensive microvilli and microlamellae. OBP is also expressed in some intracellular granules (G) in the auxiliary sensillar cells. C, cuticle; P, pore. Bars, 1 µm in *A*, *B*, *C*; 5 µm in *D*.

the 4th and 5th instars, where the antiserum against CSP-I stained, in addition to the expected 14-kDa band, additional bands migrating with higher masses, possibly corresponding to multiples of the protein molecular weight. Such a phenomenon, observed in both species, was limited to nymphal stages, being absent in all samples from adults.

Finally, CSP-III showed a completely different expression pattern in the two species. In *S. gregaria* the antiserum recognised a 14-kDa band in antennae and palpi of adults, but did not show any cross-reactivity with nymphal samples. In *L. migratoria*, instead, no cross reactivity was observed with samples from adults (solitary and gregarious phase), while the same antiserum specifically stained a band of about 35 kDa in extracts of the antennae, tarsi and wings of all nymphal stages, but not in the adults.

Immunocytochemical localisation

Four types of chemosensilla, sensilla basiconica, s. trichodea, s. chaetica and s. coeloconica, are present on the antenna of nymphs and adults of *L. migratoria* [28, 29] and *S. gregaria* [30]. They respond to olfactory or gustatory stimuli. Olfactory sensilla either belong to the single-walled sensilla trichodea and basiconica or to the double-walled sensilla coeloconica; sensilla chaetica are gustatory and mechanosensitive (fig. 3). S. trichodea appear slender and are characterised by a thick cuticular wall penetrated by relatively few wall pores, the outer dendritic segments in the lumen being unbranched (figs. 3C, 4C, 5C, D). Sensilla basiconica have a thin wall, perforated by numerous and fairly large pores; the lumen contains a large number of dendritic branches surrounded by sensillum lymph (figs. 3B, 4A, B, 5A–D). The sensilla chaetica have a terminal pore and no wall pores; they are characterized by two lumina, one containing the unbranched outer dendritic segments surrounded by the inner sensillum lymph, the other containing outer sensillum lymph only (figs. 3D, 5A, B, 6A–F).

Anti-*Lmig*OBP labelled all single-walled sensilla basiconica and s. trichodea in *L. migratoria*, whereas sensilla chaetica and sensilla coeloconica were not labelled

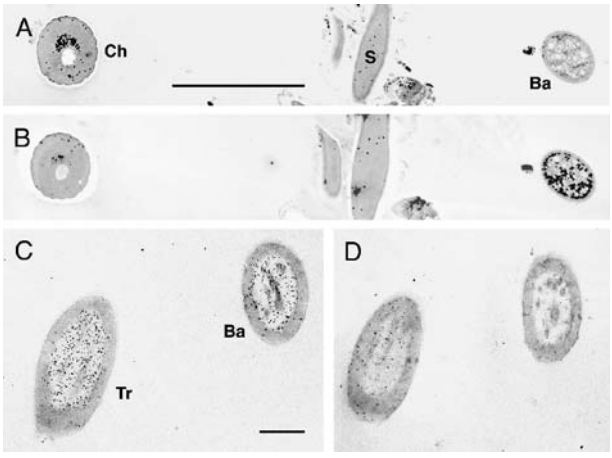

Figure 5. Complementary labelling for OBP and CSP on the antennae of adult *S. gregaria* (*A*, *B*) and *L. migratoria* (*C*, *D*). The consecutive sections in *A* and *B* display an identical pair of s. chaeticum (Ch) and s. basiconicum (Ba). With anti-CSP-I, only the s. chaeticum was labelled (*A*), while with anti-OBP, only the s. basiconicum was labelled (*B*). The few grains on the non-labelling sensilla, as well as on the antennal surface scales (S) are background labelling. Consecutive sections through a s. basiconicum (Ba) and a s. trichodeum (Tr) were also labelled with anti-OBP (*C*) and anti-CSP-I (*D*). These sensilla express only OBP but not CSP-I. Bars, 10 μm in *A*, *B*; 1 μm in *C*, *D*.

(figs 4, 5). Similar results were obtained with *S. gregaria* (fig. 4B and data not shown). Labelling intensity was variable, even when comparing different sensilla on the same section, some being heavily labelled, while others were barely above background. Sections of *S. gregaria* in general were less intensely labelled than those of *L. migratoria*, when using the same antibody concentration, possibly owing to a lower cross-reactivity of the antiserum with the OBP of the latter species.

Within the sensory hairs, the sensillum lymph, but not the dendrites was labelled. The sensillum lymph filling the large sensillum lymph cavity below the hair base was also strongly labelled (fig. 4D). Labelled intracellular secretory granules were found in the trichogen and tormogen cells, but not in the thecogen cell (fig. 4D).

When using anti-*Sgre*CSP-I as primary antiserum, a complementary pattern of labelling was observed in both *L. migratoria* and *S. gregaria*. Only s. chaetica, suggested to be gustatory sensilla, were labelled, but never the olfactory s. trichodea or s. basiconica (fig. 5). S. chaetica on antennae, palps and tarsi displayed the same labelling properties. Label was observed on the outer sensillum lymph in the non-innervated lumen, but not on the inner sensillum lymph around the dendrites (fig. 6A, B, D, E). Similar results were obtained when using anti-*Lmig*CSP-II as primary antiserum (data not shown). Anti-*Sgre*CSP-III also labelled s. chaetica in *L. migratoria*, but only in nymphs (fig. 6E, F).

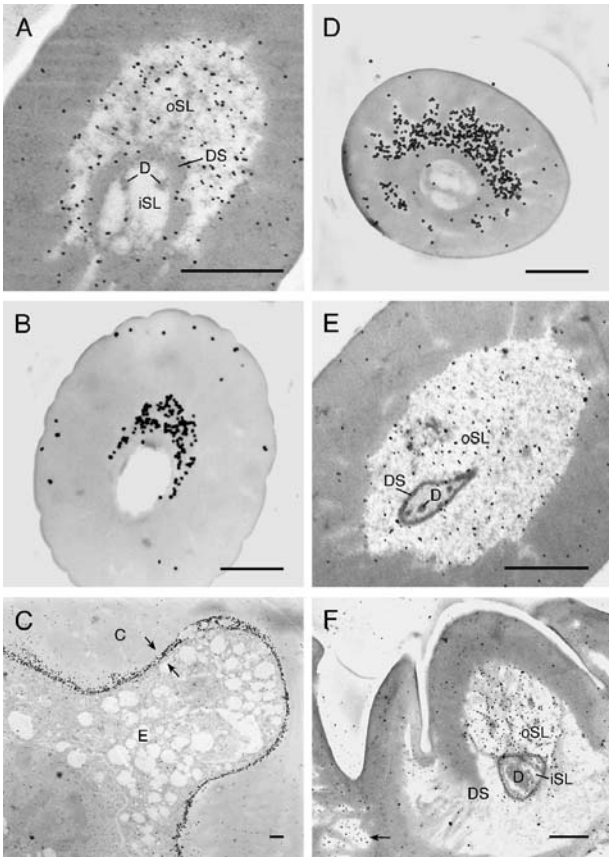

Figure 6. CSP expression in *L. migratoria* (*A*, *E*, *F*) and *S. gregaria* (*B*, *C*, *D*). CSP-I was expressed in all s. chaetica, not only on the antennae (*A*, *B*), but also on the tarsi (*D*), palps and wings (not shown). However, label was found only in the crescent-shaped lumen containing the outer sensillum lymph (oSL), which is separated by a massive dendrite sheath (DS) from the lumen containing the inner sensillum lymph (iSL) with the dendrites (D). CSP-I was also expressed in the subcuticular space (arrows) between epidermal cells (E) and antennal cuticle (C) (*C*). CSP-III was expressed in antennal s. chaetica of 4th-instar nymphs of *L. migratoria*, but again only the outer sensillum lymph (oSL) was labelled (*E*, *F*). The sub-cuticular space around the sensillar base also showed more than background labelling (arrow). Bars, 1 μm.

With anti-*Sgre*CSP-I and anti-*Sgre*CSP-III, the subcuticular space between normal epidermal cells and the antennal cuticle was also labelled (fig. 6C, F).

Discussion

We have identified a new soluble protein, expressed in the antennae and wings of adult *S. gregaria*, but not in its nymphal stages. On the basis of some conserved residues, including the four-cysteine motif, it belongs to the CSP family, but its low similarity (32–42% of identical residues) with other proteins of the same class suggests classifying this protein in a third sub-class of chemosensory proteins, CSP-III.

Like other CSPs, this new member reversibly binds the fluorescent probe 1-NPN; however, such an observation alone does not necessarily indicate a role in chemoreception. In fact, similarly to vertebrate lipocalins, which are known to perform several functions, among which the carrying of odorants and pheromones, CSPs could also represent a large superfamily, including members involved in completely different functions. At least one example supporting this view is p10 of the cockroach *Periplaneta americana*, whose synthesis is related to leg regeneration [31].

Another anomalous aspect is the absence of CSP-III in adults of the closely related species *L. migratoria*, whereas CSPs of class I and class II have otherwise extremely similar orthologues in *S. gregaria* and *L. migratoria* [19, 27]. In fact, in adult *L. migratoria,* the anti-*Sgre*CSP-III antiserum fails to detect any electrophoretic band, while in the nymphal stages it strongly cross-reacted with a protein of about 35 kDa. High-molecular-weight bands in the nymph but not in the adult of *L. migratoria* are also stained by the antiserum against *Sgre*CSP-I. In both cases, the proteins recognised by the antisera could be oligomeric forms of CSPs. This phenomenon could be related to the occurrence in the genomes of *D. melanogaster* and other Diptera of sequences encoding two or three OBPs linked in tandem, the physiological significance of which however, has not been investigated [32].

Immunocytochemical localization of CSP-III has so far only been studied in nymphal antennae of *L. migratoria*, where this protein is expressed in the subcuticular space and in the outer sensillum lymph of sensilla chaetica, like CSP-I and CSP-II. With the expression of OBP and the CSPs of class I and II, we observed that in both locust species, OBP is strictly antenna-specific, while CSP-I is ubiquitous and CSP-II presents an intermediate behaviour. This is underlined by the results of immuno-EM, as olfactory sensilla trichodea and basiconica, which were shown to express OBP, are only found on the antennae. Gustatory sensilla chaetica expressing CSP, on the other hand, are found on most parts of the body including wings, legs and ovipositor.

In most insect species, OBPs are specifically expressed in the antennae and therefore have been associated with olfaction, whereas CSPs are expressed also in other body parts [7, 33]. However, this is not always the case, as in some Hymenoptera, such as the argentine ant *Linepithema humile* and in the paper wasp *Polistes dominulus*, the situation is reversed: CSP is antenna-specific, while OBP is expressed in different parts of the body [34–36]; immunocytochemical data for these species are so far not available. Noteworthy in this context is that in the stick insect *Carausius morosus,* another CSP is expressed in olfactory sensilla [15].

The observation that CSP-I was found in contact chemosensilla suggests a role in contact chemoreception. A role as carrier for stimulus molecules as hypothesised for the OBPs, nevertheless, is unlikely, because CSP is expressed in the outer sensillum lymph only, which is not in contact with the sensory dendrites. The same protein is also expressed by cells underlying non-sensillar cuticle, where it could act as a carrier for the so-called 'cuticular hydrocarbons', important semiochemicals mediating individual recognition [37, 38]. A role of CSPs in carrying and releasing semiochemicals to the environment is also supported by the association of these proteins with endogenous ligands, when they were purified from the wings of locusts [19]. Interestingly, in the paper wasp, where the expression pattern of OBPs and CSPs is reversed, CSPs being antenna specific, it is the OBP of the wings that is loaded with endogenous ligands [35].

No differences have been detected between sexes and only minor quantitative differences (only with regard to CSP-II) have been observed between individuals in solitary or gregarious phases. Neither OBP nor any of the three types of CSPs seem, therefore, to be involved in the transition from the solitary to the gregarious phase, a phenomenon related to the devastating effects of locusts to crops.

In the desert locust *S. gregaria*, both nymphs and adults produce aggregation pheromones, which activate receptor neurones housed in s. basiconica; in additon, a possible sex pheromone activates receptor neurones in s. trichodea. Receptor neurones present in s. coeloconica respond to green-leaf odours and organic acids [39]. The olfactory reception system of *L. migratoria* is presumed to be very similar to that of *S. gregaria*. Given this diversity of function of the different sensillum types, it is somewhat surprising that anti-*Lmig*OBP labels both types of single-walled olfactory sensilla in both locust species, whilst in moths as well as in *Drosophila,* s. trichodea and s. basiconica show pronounced differences in the type of OBP they express [12, 14, 17]. At present we cannot say whether the antibody is less specific or whether the OBPs in locusts are less diverse than in moths. As orthopterans are a fairly old evolutionary group, the great diversification of OBPs in Lepidoptera and Diptera might not yet have occurred.

In holometabolous insects, such as moths, PBP expression starts about 3 days before emergence of the adult [40]. Lepidopteran larvae express only GOBP but no PBP [41]. In the hemimetabolous locusts, Western blots of OBP were similar in adults and 3rd, 4th and 5th instar nymphs (fig. 2). Nymphs of migratory locusts are known to show aggregation, migration and feeding behaviour. As a result, nymphs must have a relatively complex olfactory reception system. However, whether there are several, closely related sub-classes of OBPs or whether a single OBP can serve all these different functions remains to be shown.

Not only adults but also 2nd, 3rd, 4th and 5th-instar nymphs have been reported to respond to their own

volatiles with no significant differences [42]. These behavioural characteristics are in agreement with the result that labelling for OBP was of almost equal intensity in adult locusts and nymphs. Likewise, other than e.g. in moths, no sexual dimorphism is observed in the sensillar outfit [39] nor are there differences between the sexes in the response to sex pheromones [43]. In the immunocytochemical experiments also, we found no significant difference between sexes but, again, there may be subtle differences which cannot be discriminated by the antiserum used.

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