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RESEARCH ARTICLE

Unique function of a chemosensory protein in the proboscis of two *Helicoverpa* species

Yu-Ling Liu*, Hao Guo*, Ling-Qiao Huang, Paolo Pelosi‡ and Chen-Zhu Wang‡

ABSTRACT

Chemosensory proteins (CSPs) are soluble proteins found only in arthropods. Some of them fill the lumen of chemosensilla and are believed to play a role similar to that of odorant-binding proteins in the detection of semiochemicals. Other members of the CSP family have been reported to perform different functions, from delivery of pheromones to development. This report is focused on a member (CSP4) of the family that is highly and almost exclusively present in the proboscis of two sibling noctuid species, *Helicoverpa armigera* and *H. assulta*. We expressed the protein in bacteria and measured binding to terpenoids and related compounds. Using specific antibodies, we found that when the moths suck on a sugar solution, CSP4 is partly extruded from the proboscis. A solution of protein can also fill a hydrophobic tube of same length and diameter as the proboscis by capillary action. On this basis, we suggest that CSP4 acts as a wetting agent to reduce the surface tension of aqueous solutions and consequently the pressure involved in sucking.

KEY WORDS: Chemosensory protein, *Helicoverpa armigera*, *Helicoverpa assulta*, Proboscis, Drink-blot, Wetting agent

INTRODUCTION

Chemosensory proteins (CSPs) are a large family of soluble polypeptides that are similar in structure but different in function. Known as OS-D when first identified in *Drosophila* (McKenna et al., 1994; Pikielny et al., 1994), they were later renamed as CSPs when ligand-binding properties and expression in the lymph of chemosensilla supported a role in olfaction and taste (Angeli et al., 1999). CSPs are single polypeptides of 100–120 amino acids without post-translational modifications, except for two disulphide bridges connecting adjacent cysteines. The motif of four cysteines at conserved positions is the signature of this class of protein (Picimbon, 2003; Wanner et al., 2004; Vogt, 2005; Pelosi et al., 2006). The three-dimensional structure of CSPs, known for only three members of the family [*Mamestra brassicae* (Lartigue et al., 2002), *Bombyx mori* (Jansen et al., 2007) and *Schistocerca gregaria* (Tomaselli et al., 2006)], is made of six α-helical domains arranged in a highly compact and stable structure that contains a binding cavity lined with hydrophobic residues. Some members of the family are present at high concentration in the lymph of chemosensilla, located on the sensory organs of insects (Angeli et al., 1999; Jin et al., 2005). This fact and the ability of CSPs to bind several organic compounds, including pheromones and other semiochemicals, has suggested, at least for some of them, a role similar to that of odorant-binding proteins (OBPs) in insect chemodetection (Ishida et al., 2002; Calvello et al., 2003; Ozaki et al., 2005; González et al., 2009).

Apart from those expressed in chemosensilla, the family of CSPs includes members present in pheromone glands or reproductive organs, where they could act as carriers for semiochemicals, assisting their release into the environment (Dyanov and Dzitoeva, 1995; Jacquin-Joly et al., 2001; Dani et al., 2011; Iovinella et al., 2011; Iovinella et al., 2013; Sun et al., 2012; Zhou et al., 2013). Most interestingly, other functions, completely unrelated to chemical communication, have been reported to be associated with some CSPs. In the cockroach *Periplaneta americana*, a member of this family named p10 seems to be involved in limb regeneration (Kitabayashi et al., 1998), while in the honeybee, CSP5, which has been only detected in the eggs, is required for embryo development, as shown by RNA interference experiments (Maleszka et al., 2007). More recently, a member of this class of protein (previously reported as CSP-I) (Ban et al., 2003) has been shown to be responsible for the transition from solitary to gregarious phase in the locust (Guo et al., 2011). The small size of CSPs, their compact structure and soluble nature are responsible for their extreme stability and have probably determined their use for several tasks. Moreover, a remarkable flexibility of the polypeptide folding, while not affecting its stability, allows the protein to bind a variety of ligands of very different sizes (Lartigue et al., 2002), an additional property that might account for the easy adaptation of these proteins to perform different tasks. The presence of proteins of the same family with different unrelated tasks is not limited to CSPs. Lipocalins probably provide the best documented examples, comprising members involved in chemical communication, such as the OBPs of vertebrates (Pelosi, 1994), carriers for other hydrophobic molecules and even enzymes (Flower, 1996).

The two sibling lepidopteran species, *Helicoverpa armigera* (Hübner 1809) and *H. assulta* (Guenée 1852) are major pests for a number of agricultural plants, including cotton, wheat, corn, tobacco tomato and pepper (Zong and Wang, 2007). Because of the high economical impact, their chemoreception system is currently the object of wide and intensive research.

Here, we report on a CSP expressed in both *H. armigera* and *H. assulta* that is uniquely and abundantly present in the proboscis. We suggest that this protein acts as a surfactant in the inner cavity of the proboscis to lower the surface tension of aqueous nutrients, thus reducing the pressure involved in sucking.

RESULTS AND DISCUSSION

The Swiss-Prot database reports 14 genes encoding CSPs in the moth *H. armigera*. Some differ by only one or two amino acid residues and could be the consequence of individual variability within a population.

Electrophoretic analyses on crude extracts from different parts of the body revealed the presence of a strong band in the proboscis

State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China.
*These authors contributed equally to this work

‡Author for correspondence (ppelosi@agr.unipi.it; czwang@ioz.ac.cn)

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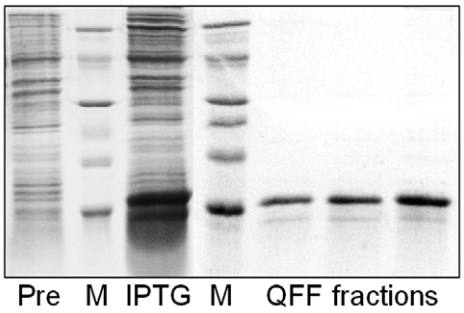

Fig. 1. Bacterial expression and purification of *Harm*CSP4. *Helicoverpa armigera* chemosensory protein 4 (*Harm*CSP4) was obtained in high yields (about 30 mg l^{-1} of culture) in its soluble form and purified by two chromatographic steps on anion-exchange resins, the first on DE-52 and the second on QFF. The figure reports the SDS-PAGE analysis relative to crude bacterial extracts before (Pre) and after induction with IPTG, as well as three fractions from the last purification step. Molecular mass markers (M) are, from the top: 66 kDa (BSA), 45 kDa (ovalbumin), 29 kDa (carbonic anhydrase), 20 kDa (trypsin inhibitor) and 14 kDa (α-lactalbumin).

of *H. armigera* as well as its sibling species *H. assulta*. On the basis of PCR analysis, performed with specific primers, followed by cloning and sequencing, we could assign this band to CSP4 (accession no. AEX07269). Given the unusual abundance of this protein in the proboscis, we decided to investigate its properties and function. CSP4 of *H. armigera* is a single polypeptide of 128 amino acids, including a signal peptide of 18 residues, with a calculated molecular weight for the mature protein of 12,805.5 and a predicted isoelectric point of 6.36. In the same species, another very similar protein has been reported, CSP9, differing from CSP4 by only two residues in the mature sequence. In the sibling species *H. assulta*, we cloned an orthologous gene encoding a protein identical to CSP4 of *H. armigera*, except for a single amino acid substitution at position 51 (K/R) of the mature protein. Among all the other CSPs of *H. armigera*, the only one showing substantial similarity to CSP4 (apart from the isoform CSP9) is CSP11 (accession no. AFR92095), which is 71% identical at the amino acid level.

We expressed the *H. armigera* CSP4 in bacteria using a construct containing the sequence encoding the mature protein preceded only by a methionine. As observed with other CSPs, our protein was produced in high yields and in its soluble form. Purification was easily accomplished by two chromatographic steps on anion-exchange resins, the first on DE-52, the second on QFF. Fig. 1 reports the electrophoretic analysis of the crude expression product and relevant fractions of the last purification step. The purified sample of the protein was extracted with dichloromethane to remove hydrophobic ligands that might be present, before further experiments were carried out.

To define the size and shape of the binding pocket and gain insight into the physiological function of CSP4, we performed competitive ligand-binding assays with a series of organic compounds, using *N*-phenyl-1-naphthylamine (1-NPN) as a fluorescent reporter. 1-NPN binds CSP4 with a dissociation constant of 9.2±0.7 μmol l^{-1} (mean ± s.d.), a value in the same range as those measured with other CSPs. Fig. 2A reports the binding curve for 1-NPN, while displacement curves for some ligands are plotted in Fig. 2B. Medium-size compounds, such as *p-tert*-butylbenzophenone and benzoates of 6–10 carbon alcohols are the best ligands. Also, floral-smelling compounds, such as geraniol and citralva, bind with good affinity. A model of the protein (Fig. 2C) shows the presence in the binding pocket of an aromatic residue (Tyr109) and some branched chains, accounting for the observed binding affinities to medium-sized terpenoids. Although these results might be in agreement with a putative involvement of this protein in chemosensing, other data could not support this hypothesis, suggesting alternative functions.

In fact, an electrophoretic analysis of crude extracts from parts of the body of *H. armigera* (Fig. 3A) and western blot experiments, using a polyclonal antiserum raised against the purified protein (Fig. 3C), revealed that CSP4 is only present in the proboscis, where it is exceptionally abundant. Weakly cross-reacting bands detectable in the tarsi of *H. armigera* are probably due to the presence of the above-mentioned CSP11, which shares 71% of its residues with CSP4. We can confidently exclude the presence of CSP4 in tarsi, based on PCR experiments (not shown), which provided a strong amplification band when we used primers for CSP11, but negative

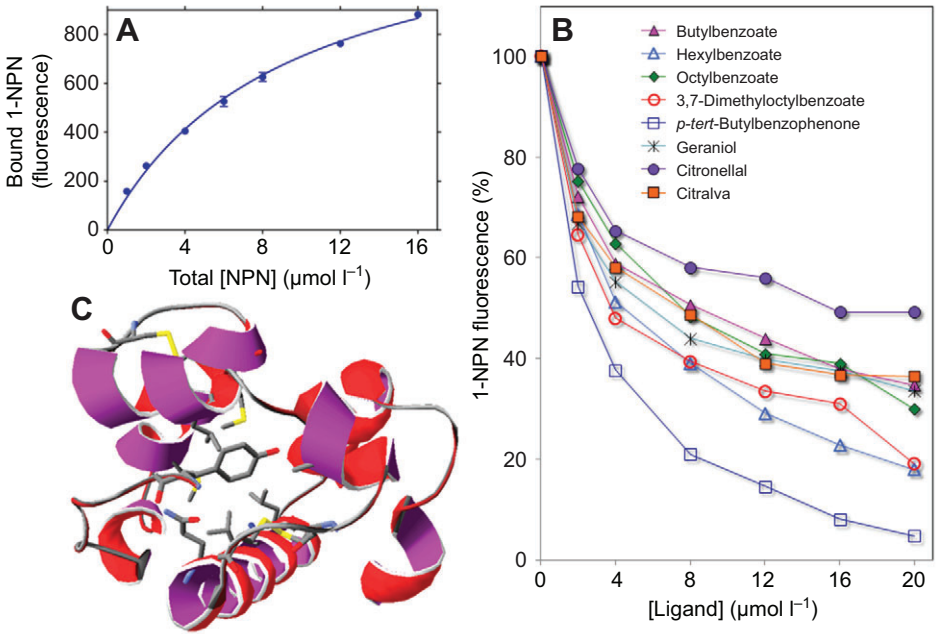

Fig. 2. Binding of 1-NPN and selected ligands to *Harm*CSP4. (A) Affinity of *N*-phenyl-1-naphthylamine (1-NPN) to the recombinant protein. A 2 μmol l^{-1} solution of the protein in Tris was titrated with 1 mmol l^{-1} solution of 1-NPN in methanol to a final concentration of 2–16 μmol l^{-1}. The data, means of three replicates, were analysed using Prism software and indicated the presence of a single binding site with a dissociation constant (k_d) of 9.2±0.7 μmol l^{-1} (mean ± s.d.). (B) Competitive binding assays. In each experiment a mixture of the protein and 1-NPN in Tris, both at a concentration of 2 μmol l^{-1}, was titrated with the competing ligand to a final concentration of 2–20 μmol l^{-1}. Fluorescence intensities are reported as a percentage of the values in the absence of competitor. (C) Three-dimensional model of *Harm*CSP4. The binding site contains a tyrosine and few branched-chain amino acids, plausibly accounting for binding to terpenoids and related structures.

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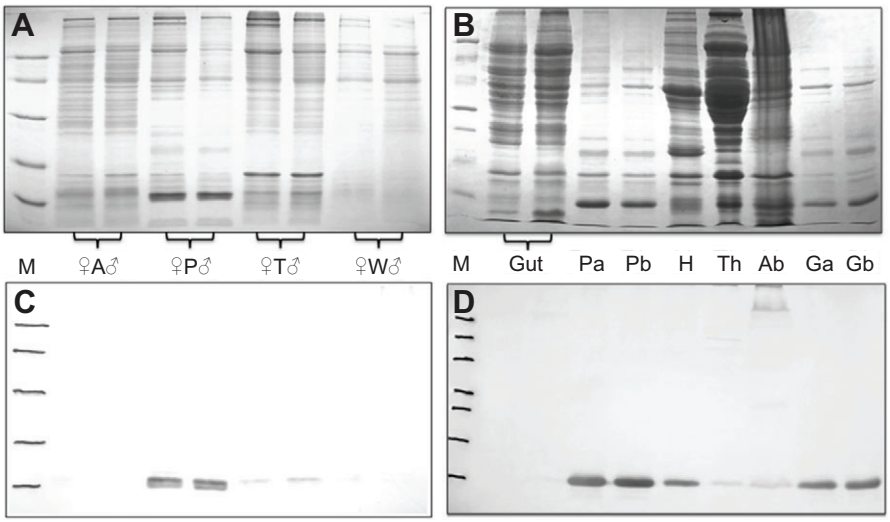

Fig. 3. Expression of CSP4 in parts of the body of *H. armigera*. (A,B) SDS-PAGE and (C,D) western blot analysis of crude extracts from parts of the body of adult *H. armigera*. A, antennae; P, proboscis; T, tarsi; W, wings; Gut, foregut and midgut; Pa, proboscis after 24 h fasting; Pb, proboscis after drinking; H, head without antennae and mouth parts; Th, thorax; Ab, abdomen; Ga, single galea dissected after feeding; Gb, single galea washed in buffer. Molecular mass markers (M) are as in Fig. 1.

results for CSP4. A very similar pattern of expression was obtained in parallel experiments performed on the sibling species *H. assulta* (data not shown).

The proboscis of noctuids exhibits sensilla on its surface, but all are concentrated in the terminal region (Zenker et al., 2011). When we analysed the presence of CSP4 in three sections of the proboscis, using western blot experiments, we observed that the protein is not concentrated in the distal region, where sensilla are present, but is evenly distributed along the length of the proboscis. Fig. 4A,B reports the results from one of several experiments performed with the proboscis of female and male *H. armigera*. Similar results were obtained with *H. assulta* (data not shown). We always detected CSP4 all along the proboscis, rather than specifically in the distal region, where sensilla are visible. Occasionally, as can be visualised with the female sample (Fig. 4A), the distal part did not contain appreciable amounts of the protein, but variability was quite large and we could not detect any significant difference between sexes. Sensilla have also been observed inside the proboscis of the butterfly *Papilio xuthus*, where they are probably involved in the detection of sugars (Inoue et al., 2009), but their number is relatively small (about 80 sensilla per proboscis) and cannot account for the large amount of CSP4 (several micrograms) that, on the basis of electrophoretic analyses, we judged to be contained in a single proboscis. These results seem to exclude the possibility that CSP4 could be mainly associated with sensilla of any type and consequently with chemodetection.

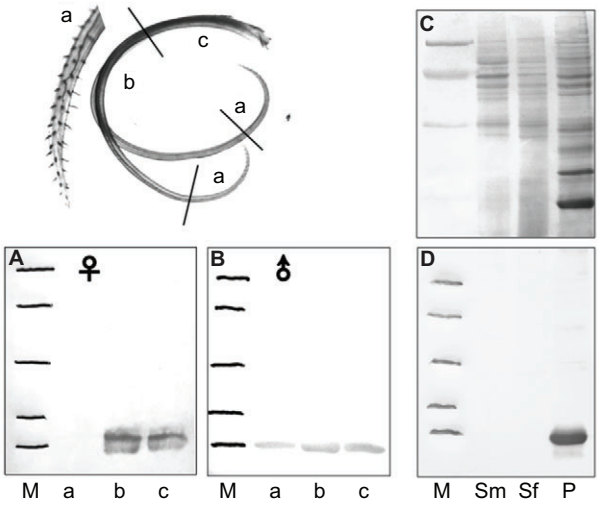

Fig. 4. Expression of CSP4 along the proboscis. To understand whether CSP4 was only associated with sensilla, which are present in the distal region of the proboscis, crude extracts prepared from three sections of the entire proboscis (a, b and c) were analysed by SDS-PAGE (not shown) and western blot (A,B). The figure reports the findings of a representative of several experiments performed with different moths. CSP4 was always found in the middle (b) and proximal (c) region, and most of the time in the terminal part (a). These results exclude a role for CSP4 in a sensory function. To investigate the source of production of CSP4, extract from salivary glands of males (Sm) and females (Sf) were analysed by SDS-PAGE (C) and western blot (D). The extract of a single proboscis (P) was also loaded on the gel as a positive control. On the basis of the negative result, we can exclude the salivary glands as site of production of CSP4. Molecular mass markers (M) are as in Fig. 1.

Therefore, we looked for an alternative function. The proboscis is a relatively long channel (about 10 mm), but extremely thin, with an average inner diameter of about 600–700 μm. Considering that its wall is made of hydrophobic cuticle, the surface tension of water would greatly increase the pressure needed for sucking an aqueous liquid, such as a sugar solution, through the full length of the proboscis. The presence of a protein, whose concentration we can estimate to be above 100 μmol l^{-1}, could greatly reduce the surface tension of water, making sucking a feasible process.

If CSP4 was actually present in the food channel of the proboscis and used as a surfactant, during the process of sucking the liquid filling the proboscis and containing such a protein could be excreted from the tip of the proboscis and detected. To this end, we set up a simple method that we call ‘drink-blot’, using a sheet of nitrocellulose membrane soaked in a solution of sucrose. We then let a moth land on the membrane surface and suck the sugar solution with its proboscis. In this way, microscopic dots of CSP4 were transferred to the membrane and stained with antiserum against CSP4, using a classic dot-blot protocol. The results of this experiment are reported in Fig. 5 where microscopic dots are clearly visible, indicating that CSP4 was indeed released from the proboscis. Incidentally, we envisage that this simple method could have a variety of applications in the study of feeding behaviour in insects, and could be also applied to investigate the release of salivary proteins in blood-sucking insects.

Subsequently, we performed experiments to measure the effect of the protein on the surface tension of water. First, we observed the shape of drops of CSP4 solution when deposited on the hydrophobic

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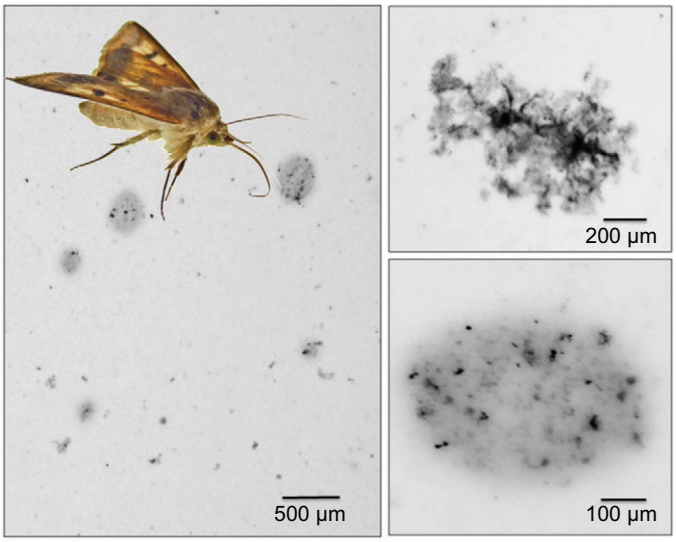

Fig. 5. Drink-blot experiment. To visualise small amounts of CSP4 that are probably extruded from the proboscis during the process of feeding, a moth was allowed to walk on a nitrocellulose sheet soaked in a sugar solution and suck in different spots. The membrane was then developed as in a classic western blot experiment, using a polyclonal antiserum raised against recombinant *Harm*CSP4. The image of the insect has been added to the figure to show how the proboscis is extended while feeding.

surface of Parafilm, and compared this with the drop shape of other CSPs of the same species (CSP11, accession no. AFR92095 and CSP13, accession no. AFR92097), as well as pure buffer (Fig. 6). Clearly, the protein solution drops appear flatter and more spread out on the surface than the drop of buffer. We then used plastic capillary tubes of about the same inner diameter (about 0.7 mm) as the moth's proboscis and observed the height of a water solution with and without protein (100 µmol l^{-1} CSP4, CSP11 or CSP13) that was allowed to enter the tubes by capillarity. To allow clearer visualisation, the solutions were spiked with a trace of Bromophenol Blue. Fig. 6 shows that, while pure Tris buffer could not wet the inner surface of the capillary and its level inside was equal to that on the outside, in the presence of any of the three proteins the level of the inner solution was about 2 cm higher than that outside. We can conclude that CSP4 certainly has a surfactant effect, although this property is not unique to this CSP but can be found to different extents in many other proteins. This conclusion is not surprising as any other protein with the amphipathic character could have caused this effect. To the best of our knowledge this is the first report of a protein otherwise involved in chemical communication that is used as a surfactant. In fact, there are not many reports on surfactant proteins being excreted; one of the most recent describes the action of latherin, a protein abundant in horse sweat, whose function is to help the sweat wet the hydrophobic hair and increase water evaporation (McDonald et al., 2009).

We could also hypothesise that CSP4, thanks to its good affinity for hydrophobic compounds, could help with solubilising terpenoids present in flower nectar. Although the moths survive and reproduce without problems when reared in the lab on a diet of sucrose, we cannot exclude the possibility that some additional compounds might be required for other non-vital functions. The affinity of CSP4 for terpenes and terpenoids could suggest such a role. This idea is also suggested by a recent report (Ishida et al., 2013) of a protein in the OBP family that is present in the oral disc of the blowfly *Phormia regina*, which helps to solubilise fatty acids present in the diet.

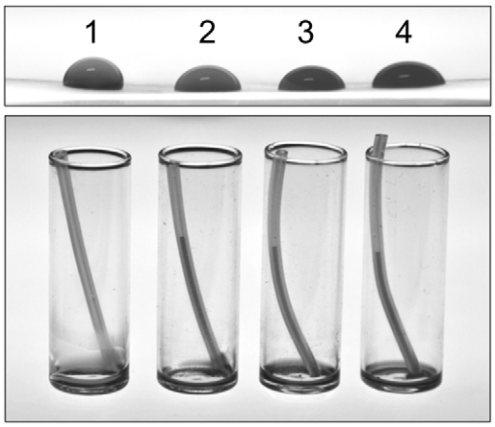

Fig. 6. Surfactant properties of *Harm*CSP4. Upper panel: 20 µl drops of water (1) and 100 µmol l^{-1} solutions of CSP4 (2, accession no. AEX07269), CSP13 (3, accession no. AFR92097) and CSP11 (4, accession no. AFR92095), all from *H. armigera*. The shape of the drops is flatter when any of the protein is present. Lower panel: the surfactant property of 100 µmol l^{-1} solutions of the three CSPs (2–4) produces a rise of about 2 cm in a hydrophobic capillary tube of about the same diameter as the proboscis. No effect was observed with pure buffer (1).

However, it is also reasonable to conceive that this protein, originally involved in chemosensing, may have been adapted to a surfactant function without losing its binding characteristics.

To trace the origin of the liquid filling the lumen of the proboscis, we first looked at the salivary glands as the most likely source of the protein. However, western blot experiments performed with crude extract of the whole glands from male and female moths failed to detect even traces of the protein, while the extract from a single proboscis yielded a very intense band, when loaded on the same gel (Fig. 4C,D). Extracts of the gut, another possible source of the protein, also did not react with the antiserum (Fig. 3B,D). In contrast, an extract from the head, devoid of antennae and mouth parts, still presented a band cross-reacting with the antiserum against CSP4, indicating that this protein is synthesised in other parts of the head. These data are not enough to identify the exact location where the protein is synthesised, but we can conclude that some glands in the head, other than salivary glands, or else in the proboscis are the most likely candidates.

Taken together, our results strongly support a role of surfactant for CSP4, although we cannot exclude the possibility that the protein might be involved, at the same time, in other additional tasks.

The fact that CSP4 might perform a function totally unrelated to chemical communication is not surprising, as other members of the same family have been reported to be involved in different tasks, such as development in cockroaches and honey bees (Kitabayashi et al., 1998; Maleszka et al., 2007), while an OBP helps solubilising nutrients in the blowfly (Ishida et al., 2013). Nevertheless, this is the first case of a CSP acting as a surfactant, a rather simple function for a complex molecule like a protein. This fact might appear as a waste of energy for a job that could be performed by cheaper surfactants, such as organic molecules. However, using this protein might well be the easiest solution for the moth, and not too expensive, considering that there is no waste of material, as most of the protein is recycled. However, other examples of apparent waste are known in biology, such as the fact that mice excrete proteins at concentrations of few milligrams per millilitre in order to solubilise semiochemicals and deliver them into the environment (Cavaggioni and Mucignat-Caretta, 2000; Beynon and Hurst, 2003).

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In conclusion, we present evidence for a novel task of a CSP, confirming that this class of protein, despite their high conservation across evolution, includes members of diverse unrelated functions, a fact related to the highly successful architecture of such polypeptides (Iovinella et al., 2013). More generally, our results support the idea that proteins that have developed a stable structure and are easy and inexpensive to synthesise have been adopted for several, perhaps unrelated, tasks. Moreover, they represent the first use of a CSP as a lubricant, apparently an expensive way to solve the problem of water surface tension, but once again an additional example that nature sometimes follows a different logic. Our results also provide a new original target for insect population control, interfering with their feeding behaviour rather than with their chemoreception system. Finally, the drink-blot assay we propose could find different applications in monitoring the excretion of proteins and studying related behaviours.

MATERIALS AND METHODS

Insects

Helicoverpa armigera and *H. assulta* were collected as larvae from Zhengzhou, Henan province of China and maintained in the laboratory on artificial diet, as previously described (Sun et al., 2012).

Reagents

All enzymes were from New England Biolabs. Oligonucleotides were custom synthesised at Augct Biotechnology, Beijing, China. All other chemicals were purchased from Sigma-Aldrich and were of reagent grade, except selected compounds used in binding assays that were prepared via conventional synthetic routes.

RNA extraction and cDNA synthesis

Total RNA was extracted from TRI Reagent (Invitrogen), following the manufacturer's protocol. cDNA was prepared from total RNA by reverse transcription, using 200 units of M-MLV reverse transcriptase (Promega), according to the protocol provided.

Cloning and sequencing

Using specific primers designed to both ends of the genes encoding CSPs in *H. virescens*, DNA sequences were amplified in PCR experiments, cloned into pGEM vector and custom sequenced at Augct Biotechnology, Beijing, China.

For expression of *H. armigera* CSP4 (*Harm*CSP4; accession no. AEX07269), at the 5′ end we used a specific primer encoding the first six amino acids of the mature protein, preceded by an *Nde*I restriction site (5′-CATATGCGTCCTGACGGCGCCAC-3′). At the 3′ end, the primer contained the sequence encoding the last six amino acids, followed by a stop codon and an *Eco*RI restriction site (5′-GAATTCTTAAGCCTTGACTT-CTTT-3′).

Subcloning into expression vectors

pGEM plasmid containing the sequence encoding the mature *Harm*CSP4, flanked by the two restriction sites, was digested with *Nde*I and *Eco*RI restriction enzymes for 2 h at 37°C and the digestion product was separated on agarose gel. The obtained fragment was purified from gel using TaKaRa MiniBest Plasmid Purification Kit and ligated into the expression vector pET30b (Novagen, Darmstadt, Germany), previously linearised with the same enzymes. The resulting plasmid was sequenced and shown to encode the mature protein.

Preparation of the protein

For expression of the recombinant *Harm*CSP4, pET-30b vector containing the sequence encoding the mature protein was used to transform BL21 *E. coli* cells. Protein expression was induced by addition of IPTG to a final concentration of 0.4 mmol l^{-1} when the culture had reached OD_{600}=0.8. Cells were grown for a further 2 h at 37°C, then harvested by centrifugation and sonicated. After centrifugation, *Harm*CSP4 was present in the supernatant. Purification of the protein was accomplished by two chromatographic steps on anion exchange resin DE-52 (Whatman). The purified protein was delipidated by extraction with dichloromethane before being used for the experiments.

Preparation of the antiserum

An antiserum against *Harm*CSP4 was obtained by injecting a rabbit subcutaneously and intramuscularly with 300 µg of recombinant protein, followed by three additional injections of 150 µg after 10 days each time. The protein was emulsified with an equal volume of Freund's complete adjuvant for the first injection and incomplete adjuvant for further injections. The rabbit was bled 1 week after the last injection and the serum was used without further purification. The rabbit was housed in a large cage, at constant temperature, and all operations were performed according to ethical guidelines to minimise pain and discomfort to the animal.

Western blot analysis

After electrophoretic separation under denaturing conditions (14% SDS-PAGE), duplicate gels were stained with Coomassie Blue R250 or electroblotted on Trans-Blot nitrocellulose membrane (Bio-Rad Laboratories) following a previously described procedure (Kyhse-Andersen, 1984). After treatment with 2% powdered skimmed milk/Tris overnight, the membrane was incubated with the crude antiserum against the protein at a dilution of 1:500 (2 h), then with goat anti-rabbit IgG horseradish peroxidase conjugate (dilution 1:1000; 1 h). Immunoreacting bands were detected by treatment with 4-chloro-1-naphthol and hydrogen peroxide.

Drink-blot assay

A nitrocellulose membrane was soaked in a solution of about 5% sucrose in water, drained and gently pressed between filter paper to absorb the excess liquid. While the membrane was still humid, a moth was allowed to land on it and walk around, probing and sucking the sucrose solution. After a few minutes, the moth was removed and the membrane was processed following the classic western blot protocol reported above.

Fluorescence binding assays

The affinity of the fluorescent ligand 1-NPN to *Harm*CSP4 was measured by titrating a 2 µmol l^{-1} solution of the protein with aliquots of 1 mmol l^{-1} ligand in methanol to a final concentration of 2–16 µmol l^{-1}. The probe was excited at 337 nm and emission spectra were recorded between 380 and 450 nm, following an established protocol (Ban et al., 2002). The affinity of other ligands was measured in competitive binding assays, where a solution of the protein and 1-NPN, both at a concentration of 2 µmol l^{-1}, was titrated with 1 mmol l^{-1} methanol solutions of each competitor to a final concentration of 2–16 µmol l^{-1}. The dissociation constant (k_d) for 1-NPN and the stoichiometry of binding was obtained by processing the data with Prism software. Dissociation constants of the competitors were calculated from the corresponding IC_{50} values (the concentration of ligand halving the initial fluorescence value of 1-NPN), using the equation: $k_d=IC_{50}/1+[1\text{-NPN}]/k_{1\text{-NPN}}$, where [1-NPN] is the free concentration of 1-NPN and $k_{1\text{-NPN}}$ is the dissociation constant of the complex protein/1-NPN.

Molecular modelling

A three-dimensional model of *Harm*CSP4 was generated using the on-line program SWISS MODEL (Guex et al., 1997; Schwede et al., 2003; Arnold et al., 2006). The structure of the CSP of *M. brassicae* (Lartigue et al., 2002) was used as a template (identity between the two proteins: 60%). Models were displayed using the SwissPdb Viewer program 'Deep-View' (Guex and Peitsch, 1997) (http://www.expasy.org/spdbv/).

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Competing interests

The authors declare no competing financial interests.

Author contributions

Conceiving and designing the experiments: C.-Z.W. and P.P. Performing the experiments: Y.-L.L., H.G., P.P., L.-Q.H. and C.-Z.W. Analyzing the data: P.P.,

Y.-L.L., H.G. and C.-Z.W. Contributing reagents/materials/analysis tools: C.-Z.W. and P.P. Drafting and revising the article: P.P. and C.-Z.W.

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