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The major antennal chemosensory protein of red imported fire ant workers

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Abstract

Some chemosensory proteins (CSPs) are expressed in insect sensory appendages and are thought to be involved in chemical signaling by ants. We identified fourteen unique CSP sequences in EST libraries of the red imported fire ant, *Solenopsis invicta*. One member of this group (Si-CSP1) is highly expressed in worker antennae, suggesting an olfactory function. A shotgun proteomic analysis of antennal proteins confirms the high level of Si-CSP1 expression, and also shows expression of another CSP and two odorant-binding proteins (OBPs). We cloned and expressed the coding sequence for Si-CSP1. We used cyclodextrins as solubilizers to investigate ligand binding. Fire ant cuticular lipids strongly inhibit Si-CSP1 binding to the fluorescent dye N-phenyl-naphthylamine, suggesting cuticular substances are ligands for Si-CSP1. Analysis of the cuticular lipids shows that the endogenous ligands of Si-CSP1 are not cuticular hydrocarbons.

Keywords

chemosensory proteins; *Solenopsis invicta*; proteomics; cuticular lipids; cyclodextrin

Introduction

Social insects restrict the benefits of altruism to their kin by using nestmate recognition signals. The recognition system involves production, detection, and interpretation of chemical signals on the surface of the cuticle (Vander Meer & Morel, 1998). Often the nestmate recognition signals consist of cuticular hydrocarbons (Howard & Blomquist, 2005). In the carpenter ant, *Camponotus japonicus*, specialized antennal sensilla capture cuticular hydrocarbons by means of a hydrophobic ligand-binding protein known as a chemosensory protein (CSP), which transports them to the olfactory receptor neurons (Ozaki et al., 2005). Insects express CSP paralogs in a variety of tissues (Wanner et al., 2004; Pelosi et al., 2006; Zhou et al., 2006; Forêt et al., 2007). The hydrocarbon-binding CSP of *C. japonicus* is expressed at high levels in the antenna, and it is the only CSP detected there. Antennal CSPs with similar sequences have been reported in several ant, wasp and bee species (Ishida et al., 2002; Briand et al., 2002; Cavello et al., 2003; Cavello et al., 2005; Forêt et al., 2007; Leal & Ishida, 2008). Nestmate recognition signals consisting of cuticular hydrocarbons have been found in many species of ant (Thomas et al., 1999; Lahav et al., 1999; Wagner et al., 2000). However, non-hydrocarbon cuticular lipids (e.g. fatty alcohols, fatty acids, wax esters) are often present in the extracts used for behavioral studies, but their roles in recognition are rarely studied (Vander Meer & Morel,

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1998; Howard & Blomquist, 2005; Hefetz, 2007). It is not known how widespread is the use of cuticular hydrocarbons as nestmate recognition signals by social insects, nor is it known whether the major antennal CSP is the exclusive carrier of nestmate recognition signals to the olfactory receptor neurons. We have now identified Si-CSP1 as the major antennal CSP of the red imported fire ant, *Solenopsis invicta*. We report here on the ligand-binding properties of recombinantly expressed Si-CSP1, using fluorescence spectroscopy as well as the ligand capture method we previously developed (Renthal, 2003).

Results and discussion

Fire ant chemosensory proteins

We used a pattern search of a preliminary version of the Lausanne Fire Ant EST library, which revealed 11 chemosensory protein (CSP) sequences. Subsequently, we identified three additional sequences using BLAST. The 14 CSP sequences are shown in Fig. 1, and they are also listed in Table 1 with the Genbank accession numbers. We also found additional sequences that differ at a small number of positions from the sequences in Fig. 1, but it is not yet clear whether the variation is due to sequencing errors, polymorphism or to functionally different CSPs. We constructed three dimensional models of the *S. invicta* CSPs, using the Swiss-Model Server (Guex and Peitsch, 1997; Schwede *et al.*, 2002) with amino acid sequence homology to the liganded and unliganded forms of CSPMbraA6 from *Mamestra brassicae* (Campanacci *et al.*, 2003; Protein Data Bank files 1n8u and 1kx8). The liganded models are all hollow cylinders, with the interior cavities in most models open at both ends and spanning the entire length of the molecule. The volume and shape of the cavities differ for each CSP, suggesting that some of the CSPs are selective for specific ligand molecules.

The fourteen chemosensory protein (CSP) sequences we identified in *S. invicta* EST libraries (Table 1 and Fig. 1) compare with 20 reported in *Locusta migratoria* and *Tribolium castaneum*, 10 in *Mamestra brassicae* and *Bombyx mori*, 9 in *Nasonia vitripennis*, 7 in *Anopheles gambiae*, 6 in *Apis mellifera*, and 4 in *Drosophila melanogaster* (Pelosi *et al.*, 2005; Forêt, 2007). Thus, *S. invicta* has more CSPs than are yet known for any other hymenopteran.

A neighbor-joining tree of hymenopteran CSPs (Fig. 2) shows three sequence groups supported by high bootstrap values. Group I is similar to a clade identified by Forêt *et al.* (2007) and characterized as “Divergent CSPs”, since these CSPs lack some of the sequences conserved across many orders. Group I CSPs, including Si-CSP9, may have a role in development, as this group includes Am-CSP5, which is expressed only in the egg (Forêt *et al.*, 2007), and appears to function in development of the embryonic integument (Maleszka *et al.*, 2007). Group II contains the fire ant CSPs that we identified in the antenna, Si-CSP1 and Si-CSP3 (Table 2) (discussed below), along with Si-CSP2, Si-CSP5 and Si-CSP10. Group II lacks known CSPs from any other species. A BLAST search of these sequences against all insect DNA and EST sequences also showed no other closely related CSPs. The complete *A. mellifera* genome and several large wasp EST libraries were included in this search. Therefore, it appears likely that group II contains CSPs which are unique to ants, and perhaps unique to Myrmicine ants. Similar paralogous expansions of CSPs were previously observed in other large analyses of CSPs (Wanner *et al.*, 2004; Pelosi *et al.*, 2006; Forêt *et al.*, 2007). Group III is similar to Hymenoptera Class 1 identified by Wanner *et al.* (2004). It includes the *A. mellifera* protein Am-CSP1, which is strongly expressed in the antenna, as well as in other tissues (Forêt *et al.*, 2007), and also *L. humile*, *C. japonicus* and *P. dominulus* CSPs which are known to be expressed in the antenna (Ishida *et al.*, 2002; Cavello *et al.*, 2003; Ozaki *et al.*, 2005). Four fire ant CSPs are in this clade: Si-CSP4, 8, 13 and 14. However, these proteins were not detected in the proteomic analysis of fire ant worker antennae (Table 2) (discussed below).

Expression of Si-CSP1 in *S. invicta* worker antenna

Proteins extracted from fire ant worker antennae were analyzed by two-dimensional gel electrophoresis (Fig. 3). The major low molecular weight protein was subjected to N-terminal Edman degradation, giving the sequence GDLGLYPSEL... This corresponds to the sequence beginning 22 amino acids from the presumed initiation methionine in the protein sequence translated from Genbank cDNA sequence AY713302 (Leal and Ishida, 2008), and also to the protein sequence translated from near the 5' end of EE134548 (SiJWH08ACX) in the Lausanne Fire Ant EST Library. We refer to this antennal CSP as Si-CSP1. To test whether other CSPs are expressed at high levels in worker antennae, we used a shotgun proteomics approach. Fifty *S. invicta* worker antenna pairs were extracted and cleaved with trypsin. The tryptic peptides were directly analyzed by capillary LC/MS/MS to view the worker antennal proteome. The results (Table 2) show that Si-CSP1 is detected in the antenna at a high level. We also detected peptides from Si-CSP3. The expression of Si-CSP1 in the antenna was further localized by Leal and Ishida (2008) to the antennal club (the two distal antennal segments). They found no Si-CSP1 in the funiculus, pedicel or scape. We previously showed that all of the porous sensilla are in the club of the *S. invicta* antenna (Renthal et al., 2003). Since the hemolymph and cuticle are continuous throughout the antenna, Si-CSP1 must be expressed in a compartment that is unique to the antennal club, most likely olfactory sensilla.

Of the 42 proteins identified in Table 2, many have known or possible functions in olfaction, including two members of the odorant-binding protein (OBP) family, glutathione- and peroxide-related enzymes (Rogers et al., 1999; Novoselov et al., 1999), and hydrophobic ligand-binding proteins, including a lipocalin and apolipoprotein III, which we previously identified as a major antennal protein in the fire ant (Guntur et al., 2004). The two antennal OBPs are Si-OBP9 (Gp-9) and Si-OBP1. Previous studies suggested that Gp-9 is present in the hemolymph (Krieger and Ross, 2002), so it is not clear whether it is expressed in the antenna and whether it has a particular role in olfaction. However, Si-OBP1 is a sequence homolog of *A. mellifera* OBP1, known to be expressed only in the honeybee antenna (Forêt and Maleszka, 2006) and reported to bind to the queen pheromone (Danty et al., 1999). Our finding of Si-OBP1 and Si-OBP9 (Gp-9) is the first report of a member of the OBP family in an ant antenna. Previous analyses of ant antennal proteins found only CSPs (Ishida et al., 2002; Ozaki et al., 2005), and this generated a discussion of whether some ants might preferably use CSPs rather than OBPs for olfaction (Cavello et al., 2005). The presence of Si-OBP1 in the fire ant antenna suggests that *S. invicta* may use both OBPs and CSPs for olfaction.

Recombinant Si-CSP1

Because the most abundant CSP in *C. japonicus* worker antennae was shown to bind to nestmate recognition signals, we reasoned that Si-CSP1, the most abundant CSP in *S. invicta* worker antennae, may also be involved in nestmate recognition. Therefore, we expressed recombinant Si-CSP1 in order to determine its ligand binding properties. Transformed *E. coli* cells were induced to express Si-CSP1. Extraction from the cell pellet and purification (Fig. 4) typically yielded 20 mL of 50–100 µM Si-CSP1 per liter of medium. For some experiments, a final gel permeation step on a Superdex-75 column was included. Calibration of the column with proteins of known molecular weight showed that Si-CSP1 (expected molecular weight = 10.7 kDa) eluted at the same position as horse myoglobin (molecular weight = 17.7 kDa), indicating that Si-CSP1 is a dimer under physiological conditions.

Recombinant Si-CSP1 was cleaved with trypsin and the tryptic peptides were analyzed by capillary LC/MS/MS, confirming the expected amino acid sequence with 74% sequence coverage. Although an N-terminal methionine had been engineered into the sequence from the Nde I restriction site, the mass spectrum shows that this amino acid was post-translationally removed by *E. coli* methionine N-terminal peptidase.

Ligand binding properties of Si-CSP1

We studied the ligand-binding properties of recombinant Si-CSP1, the major fire ant worker antennal CSP. Because the major CSP of *Camponotus japonicus* was shown to be involved in nestmate recognition (Ozaki et al., 2005), we speculated that knowing the endogenous ligands of Si-CSP1 could provide information about the nestmate recognition signaling system of fire ants. Previous studies of CSPs from other insects showed that 1-N-phenylnaphthylamine (NPN) undergoes a strong fluorescence enhancement upon binding (Ban *et al.*, 2002). Therefore, we measured fluorescence excitation spectra of NPN added to Si-CSP1, monitoring the emission of NPN at 410 nm, a wavelength where it has low fluorescence in water (Fig. 5A). The upper curve of Fig. 5A indicates strong binding to Si-CSP1: the emission of the dye shifts to 410 nm, showing that it is in a non-polar environment. Also, a peak appears at 280 nm, due to fluorescence resonance energy transfer (Lakowicz, 2006) from tryptophan in Si-CSP1 to the bound NPN, indicating that the dye and the tryptophan are in close spatial proximity. The homology model for liganded Si-CSP1 shows a tryptophan near each open end of the ligand-binding cavity (Trp 24 and Trp 83). Plotted as a binding curve, the fluorescence enhancement levels off at high NPN concentrations, indicating binding to discrete sites (Fig. 5B). We have modeled the binding with a cooperative Adair-type sequential binding model (Imai, 1973), assuming two interacting binding sites. The line in Fig. 5B is fit with dissociation constants of $K_1 = 30 \mu\text{M}$ and $K_2 = 8 \mu\text{M}$. The stronger binding occurs only after the weaker site is filled.

We used NPN binding to test the relative affinities for Si-CSP1 of components of *S. invicta* cuticular lipids. Ligands for CSPs in other insects were shown to compete with NPN for the CSP binding site (Ban *et al.*, 2002; 2003), causing a decrease in fluorescence with increasing added ligand. Alkane solvent extracts of fire ant cuticle are known to contain a variety of polar lipids in addition to hydrocarbons (Lok et al., 1975). We concentrated pentane washes of fire ant worker cuticle and separated the components by thin layer chromatography (TLC) into four fractions. Based on R_f values (Kates, 1972), we tentatively identify the main components of fraction I as hydrocarbons and wax esters, fraction II as triglycerides, fraction III as fatty acids, and fraction IV as sterols. The Si-CSP1/NPN complex was titrated with purified fractions. The per cent displacement of NPN by the TLC fractions was: I, 13%; II, 7 %; III, 21%; and IV, 0%. The results for fraction III are shown in Fig. 6. However, conclusions from this experiment are limited by the low resolution of TLC separations, and also by the insolubility of the cuticular extracts in aqueous buffer.

Cyclodextrins as hydrophobic ligand transfer agents

In the competitive binding experiments (Fig. 6), the amount of cuticular lipids added were approximately in a 10-fold molar excess over protein, yet the results suggest that the ligands cannot displace all of the NPN from Si-CP1 (estimate based on the cuticular lipid analysis of Lok *et al.*, 1975). To test whether this could be an artifact of the low aqueous solubility of cuticular lipids, we sought a solubilizing agent which would not compete with the ligands for the CSP binding site. Cyclodextrins (CDs) are widely used as carriers for water-insoluble substances in the pharmaceutical industry (Szejtli, 1998). Therefore, we tested the effect of β -CD on the binding of 12-bromododecanoic acid (12-BDA) to Si-CSP1. Brominated fatty acid esters were previously shown to quench the intrinsic fluorescence of CSPs from moths (Campanacci *et al.*, 2003). We found that, as expected, 12-BDA quenches the tryptophan fluorescence of Si-CSP1 (Fig. 7), indicating that it binds to Si-CSP1. However, the quenching was greater when the same amount of 12-BDA was added along with β -CD. The results suggest that 12-BDA, which is only slightly soluble in water, is more soluble if β -CD is present. Lines fitted to the data in Fig. 7 show that cyclodextrin does not enhance the affinity of 12-BDA for Si-CSP1, but instead β -CD makes more 12-BDA available for binding.

Although CDs enhance the amount of ligand available for binding to Si-CSP1, the competition between endogenous ligands and NPN binding cannot be measured in the presence of CDs because NPN binds to β - and γ -CD, with fluorescence enhancement that renders it useless for competition studies. However, we found that ligand-transfer and NPN competition could be done in separate steps. First Si-CSP1 was electrostatically adsorbed onto a solid support (DEAE cellulose). Next, the adsorbed Si-CSP1 was equilibrated with a cuticular lipid extract dispersed in a mixture of α -, β -, and γ -CD, allowing Si-CSP1 to select the components for which it has a high affinity. Then, the lipid-CD complex was washed away, leaving Si-CSP1 adsorbed on the solid support bound to any lipids it captured. Finally, the Si-CSP1 was eluted from the solid support with a salt wash. The eluted Si-CSP1, completely free of CD, was then titrated with NPN to assess the available binding sites. We found that Si-CSP1 that had been exposed to a CD dispersion of cuticular lipids showed virtually no binding to NPN (Fig. 8). The cuticular lipids equilibrated with the sample had approximately a 6:1 molar ratio of hydrocarbons to protein and about 1:1 fatty acids and esters to protein (Lok *et al.*, 1975). A control sample of Si-CSP1 was equilibrated on DEAE cellulose with CD containing no cuticular extract, and we found that there was no inhibition of NPN binding (Fig. 8). Thus, components of the fire ant cuticular extract bind tightly to Si-CSP1 and cannot be displaced by NPN.

Previous studies of hydrophobic ligand-binding proteins have been limited by the problem of water-insolubility of the ligands. A variety of methods to overcome this problem have been reported, including transferring the ligands across a liquid/liquid or liquid/solid phase boundary (Du and Prestwich, 1995; Margaryan *et al.*, 2006), or dispersing the ligand in detergent micelles (Ozaki *et al.*, 2005). There are drawbacks to these methods. Using two-phase systems makes it difficult to measure meaningful binding free energy changes. Using detergents can be a problem because the detergent monomer may compete with ligands for the binding site on the protein. One advantage of cyclodextrins as dispersing agents for hydrophobic ligands is that, unlike detergents, cyclodextrins do not directly compete with ligands for binding sites on the protein. Furthermore, cyclodextrins may permit exact measurements of binding free energy changes by linked equilibria.

Nestmate recognition signals

In order to find out what components of cuticular lipids had been captured by Si-CSP1, we extracted the eluted Si-CSP1 with methylene chloride and analyzed the extract by GCMS and HPLC. The results show that Si-CSP1 does not bind to cuticular hydrocarbons (Fig. 9), but Si-CSP1 does bind to polar cuticular lipids (Fig. 10). The compounds captured by Si-CSP1 have elution times characteristic of fatty acids (Fig. 10A) and esters such as triglycerides (Fig. 10B). If the major *S. invicta* CSP carries nestmate recognition signals, by analogy to Cj-CSP1 (Ozaki *et al.*, 2005), then cuticular hydrocarbons are not involved in nestmate recognition in *S. invicta*. Alternatively, if the nestmate recognition signals in *S. invicta* are composed of cuticular hydrocarbons, then the major antennal CSP does not carry this signal. Tschinkel (2006) summarized many behavioral and chemical experiments reported by Vander Meer and colleagues and argued that cuticular hydrocarbons were unlikely to be the nestmate recognition signal in *S. invicta*. To clarify this issue, we plan to identify the substances captured by Si-CSP1 (Fig. 10) and test them for activity in nestmate recognition.

Experimental procedures

Ants

Polygyne colonies of *S. invicta* were collected by floatation (Jouvenaz *et al.*, 1977) from nests found in Bexar County, Texas, and maintained in the lab in plastic trays.

2D gel electrophoresis and N-terminal sequencing

Two dimensional gel electrophoresis and N-terminal sequencing were done as previously described (Guntur *et al.*, 2004).

Identification of CSP sequences in the Lausanne fire ant EST library

The Lausanne fire ant EST library (Wang *et al.*, 2007) was downloaded onto a Dell 670 workstation in the UTSA Computational Biology Core Facility. The library was expanded into a six-frame translation. Stand-alone Blast was used to run a Seedtop pattern search, with the following Prosite pattern: C-x(6)-C-x(18,19)-C-x(2)-C. Other hymenopteran CSP sequences were identified with BLAST, using the translated *S. invicta* sequences to search hymenopteran EST libraries. Sequences were aligned using CLC Sequence Viewer software (CLC bio, Arhus, Denmark) and arranged in a phylogenetic tree using Phylip software.

Recombinant CSP1

cDNA derived from fire ant antennae, containing the Si-CSP1 gene, was amplified by PCR with the following primers: forward, 5' TTAGCATATGGGAGACTTGGGACTCTATCC; and reverse, 5' TTAGGCTCGGGCATCTTCAAGTATTATTTTCCAAA. The primers introduced an Nde I restriction site in the forward primer and an Xho I restriction site in the reverse primer. The PCR product was cloned into pCR-blunt-II™ (Invitrogen, Carlsbad, CA). The gene was then moved into pET21a and used to transform BL21 (λDE3) *E. coli* cells. Expression was induced overnight at 28° in lactose-containing LB medium. The cell pellet was extracted with *t*-butanol/water and the aqueous phase was precipitated with 0.5 M ammonium sulfate. The supernatant was dialyzed and applied to a DE52 column. The 0.35 M NaCl eluent was applied to a phenyl sepharose column in 0.5 M ammonium sulfate and eluted with 20 mM Tris, pH 8.4. A final purification step was done on a Superdex-75 column (GE Healthcare, Piscataway, NJ) in 0.02 M Tris, pH 7.2, 0.1 M NaCl.

Mass spectrometry

Antennal extracts (Guntur *et al.*, 2004) or purified recombinant CSP1 fractions were unfolded in 8 M guanidinium containing 5 mM dithiothreitol at 60°C for 1 hr followed by treatment with 20 mM iodoacetamide. After addition of excess dithiothreitol and six-fold dilution with buffer, the protein was hydrolyzed with trypsin (Gold, mass spectrometry grade, Promega; 1:20 w:w) for 1 hr at 37°C. Capillary liquid chromatography-tandem mass spectrometry (LC/MS/MS) was performed with a 50 µm internal diameter capillary LC column packed with 10 cm of 5 µm C18 particles, a linear gradient from 2 to 98% acetonitrile/0.1% formic acid at 500 nL/min (Eksigent, Livermore, CA) in 60 min, and a linear ion trap (ThermoFisher, San Jose, CA) where the top 7 ions are fragmented by collision-induced dissociation. MASCOT (Matrixscience, London, UK) searches of MS/MS spectra were performed with a 10 node Linux cluster against a 6-frame translation of the NCBI *S. invicta* EST database with 1000 ppm and 0.8 Da precursor and product ion mass tolerances, respectively.

Cuticular lipid analysis

For each extraction, thirty ice-anesthetized foraging workers were extracted for 5 min at room temperature in 2 mL pentane. After evaporation under N₂, the residue was solubilized in a small volume of pentane and spotted on a glass-backed silica TLC plates (250 µm silica gel 60, Sigma). The plates were developed first with petroleum ether:toluene 9:1 v:v and then with hexane:ethyl acetate:acetic acid 6:4:0.4 v:v:v. Lipids were visualized by ashing (5% concentrated sulfuric acid/ethanol spray followed by heating to 110°C). Regions on non-ashed plates corresponding to lipid spots were scraped, eluted with 1 mL methanol, and concentrated to 200 µL by evaporation under N₂. Hydrocarbon analyses of extracts were done on a HP 5971A GC/MS using an Agilent HP-1 column. For some experiments (e.g. Fig. 10), cuticular

pentane washes were fractionated on Waters (Milford, MA) Sep-Pak Classic silica cartridges and then eluted with pentane, followed by chloroform:methanol 1:1 (v:v). HPLC analysis was done on a Grace (Deerfield, IL) Alltima HP C18 HL 5 μ column, using LDC Constametric pumps and a Varian 380-LC evaporative light scattering detector. The Sep-Pak fractions were dried under N₂ and applied to the C18 column in hexane (pentane fraction) or acetonitrile:isopropanol 1:1 (v:v) (chloroform:methanol fraction). Prior to sample injection, the column was equilibrated with acetonitrile:isopropanol 1:1. Three minutes after injection, a 30 minute linear gradient was run with chloroform, ending at 60% acetonitrile:isopropanol 1:1 and 40% chloroform. With this separation system, palmitic acid eluted at 5.1 min and tripalmitin eluted at 24.2 min.

Ligand binding

Fluorescence spectra were measured on a PTI QuantaMaster QM4 fluorometer. Ligand capture experiments were done as follows. Twenty worker ants were washed with 2 mL pentane for 5 min. and the pentane was removed and evaporated under a flow of nitrogen. The residue was dissolved in 20 μ L acetonitrile and added to 1.2 mL 20 mM Tris, pH 8.4 containing 0.17 mM each of α -, β -, and γ -cyclodextrin. In some experiments (e.g. Fig. 10) separate dried pentane and chloroform/methanol Sep-Pak fractions were solubilized in acetonitrile:isopropanol 1:1 and added to aqueous cyclodextrins. A solution of 0.8 mL of Si-CSP1 (80 nmol) in 20 mM Tris, pH 8.4 was combined with the cyclodextrin-solubilized cuticular extract and added to 250 mg buffer-washed DEAE cellulose (Whatman DE52; equilibrated with 5 mL 20 mM Tris, pH 8.4 and washed twice, by centrifugation and resuspension, with 5 mL portions of 20 mM Tris, pH 8.4). The suspension was stirred gently with a magnetic stirring bar for 20 min at room temperature and then centrifuged. After two washes with 20 mM Tris, pH 8.4, the Si-CSP1 was eluted from the DE52 by gently stirring for 10 min with 1 mL of 20 mM Tris, pH 7.4, containing 150 mM NaCl. After centrifugation, the supernatant was removed and extracted with hexane, or methylene chloride, or chloroform and methanol (Bligh and Dyer, 1959). The organic layer was removed, evaporated, and resuspended in a smaller volume of solvent for subsequent analysis.

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1      20      40      60      80      100
Si-CSP1 --GDGLYPSELDLDVVALADAAWRQSDDEFLNKG--G--SEEQKYLNDLFREAVRTDERSTDKQRGIMNTITEWYEQNEADVWKIILEDARA-----
Si-CSP2 --EELQPPSEYD-IYVPKILANDVVRQKAVDYLKKG--C--TEQEKLATDLFRDALKTNKKGGEKQKEHVKILTEWYVKNQPDTWKLI IENVDS-----
Si-CSP3 --EELWFYSGEFDMDVLSIL--EAQAEQEVDCYMKRG--C--LEQQRIADSIREAIRTNRRCTPKQKQIQLITDWYKSRMPQNWELIVANVDL-----
Si-CSP4 --EEELYSNRYDDIDRIDLENKKLLRQYNYCFMDTEP--C--RTADAKFFHEVISEAMQTQRRRTTEKQKVLNRMADWYTQNAPEQWEAFIRKLTLEDTLQKKG-----
Si-CSP5 --EEELYPREIDIDVLKILSDDAWRRAEDCYFKRVP--C--AKEQYLSDFKDLTKCEKTEKQKLVKTATEWYEQNEPDTWKLI LEDAHS-----
Si-CSP6 --GLVSGIEYFS--DNIDVDAIINSDRLLNQYVNCILDKGP--C--TADGRSLKHFLPDAIATTEKSEKQKGTARKIKYLVKKNPNIWAEFLERYPPDEEHVAFYKEFLAQGGA--
Si-CSP7 --EEKYSTKYDNIHLDITLKSRLKKNYVNCILDKGN--C--TPDGKELRETLPDALMTECKKSEKQKEGTEKVI RYLVNKKPETWEQLKKYDPNGQYTAKYLD EAHKQG IN V
Si-CSP8 --QDLYSDKFDHIDVASIVTNDKLRNEYYSICIMDTSP--C--KTADAKFLKEIFAEALNNDKKCTEKQKEHMTIQDWYTTNKPDEWQAQVAKA-EDLKK-NARK-----
Si-CSP9 --QTGRSRYSDE-QLNIALSDKRYLNRLKCALGEAP--C--DPVGRRLKSLVPLVLRGSPQESPEEIRQIKKVLSHIQRSFPKEWNRIVQYQYAS-----
Si-CSP10 --EDLHSEL-DLDIPKILANDAERGVYIDILLENAS--C--TELETKAAAIKDALTNQAGSDKRKENMKIITDWFNQNPDTWTLVVAQVNS-----
Si-CSP11 --DEKYTRKYDDVNVNDILQNNRVLTNYIRCLMDEGP--C--TAEGRRLRKTVPDALSSGCKDNDKQKAMTEKVIDHLTKRSRDWDLVAKYDPPNGEYKKRYEKS-----
Si-CSP12 --EEKYSTKYDNIIDQILQNDRLKRYVDFLEKPNVRC--PAEALAKAHIQEALDDECAKSDHQKEMSKKVI RHLITNKRDMNELKAKYDPPNGEYAKRYEAKKEGVEI
Si-CSP13 --QEDLYSDKFDGIDVKSII TNNRLRNEYYSICIMDTSP--C--VTADAKFFKDIFFDALGNKKRTEKQKEYMKIQDWYTTNPNPKWQAQVAKS-EDLKKKNARK-----
Si-CSP14 --QEDLYSDKFDGVDVASIIVNDKLRNEYYSICIMDTSP--C--ITADAKFFKGVFADALNNKCRTEKQKEHMDYIQDWYTKNKPDEWQALVKSIEDLKKKNARK-----

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Figure 1.

Alignment of CSPs from *S. invicta*. Sequences were aligned by CLC Sequence Viewer 4. Conserved cysteines are shaded. The N-terminal amino acid for Si-CSP1 was determined by Edman degradation. Most other N-termini were predicted from signal sequence cleavage sites (not shown) using SignalP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>). The EST for Si-CSP7 lacks a full signal peptide, and its N-terminus was predicted by similarity with Si-CSP12. The EST for Si-CSP11 is a fragment that lacks both the N-terminus and C-terminus of the protein; the full sequence shown was obtained by PCR.

Figure 2.

Phylogenetic tree of hymenopteran CSPs. Neighbor-joining tree compares fire ant CSP sequences with other known hymenopteran CSPs. Si, *Solenopsis invicta*; Am, *Apis mellifera*; Bi, *Bombus ignitus*; Cj, *Camponotus japonicus*; Lh, *Linepithema humile*; Ln, *Lasius niger*; Lt, *Lysiphlebus testaceipes*; Mm, *Microplitis mediator*; Nv, *Nasonia vitripennis*; Pd, *Polistes dominulus*; Sg, *Scleroderma guani*; Vc, *Vespa crabro*; Vs, *Vespula squamosa*. Background shading highlights three proposed groups of hymenopteran CSPs. Colored markers at branches indicate bootstrap values: red = 90–100%, yellow = 80–89%, green = 70–79%, blue = 60–69%.

Figure 3. Two-dimensional gel electrophoresis of *S. invicta* worker antenna proteins. Proteins were electro-blotted onto PVDF film, stained with Coomassie blue, and imaged. The circled spot was cut out and subjected to N-terminal Edman degradation. Sequence corresponds to Si-CSP1.

Figure 4. Purification of Si-CSP1 from *E. coli*. Lane A: mol. wt. markers, kDa; B: whole cell extract; C: DEAE column flowthrough; D: 0.2 M NaCl wash; E: 0.35 M NaCl wash; F: phenyl sepharose eluent.

Figure 5. Binding of 1-N-phenyl naphthylamine (NPN) to Si-CSP1. A. Fluorescence excitation spectra. Curve 1: 0.6 μM Si-CSP1 plus 10 μM NPN in 20 mM Tris buffer, pH 7.2. Curve 2: 10 μM NPN in 20 mM Tris buffer, pH 7.2. Curve 3: 0.6 μM Si-CSP1 in 20 mM Tris buffer, pH 7.2. B. Change in fluorescence as NPN is added to Si-CSP1. Excitation wavelength, 340 nm. Line fitted to data assuming two binding sites and cooperative interaction. Ordinates are in arbitrary units of fluorescence intensity.

Figure 6.

Binding of cuticular lipids to Si-CSP1. Fraction III from TLC separation of cuticular lipids added to NPN/Si-CSP1 complex. Fluorescence decrease shows components of fraction III (probably fatty acids) displace NPN from its binding site on Si-CSP1. Ordinate is fluorescence relative to no addition of fraction III.

Figure 7.

Cyclodextrin transfers 12-bromododecanoic acid (12-BDA) to Si-CSP1. A. Binding of 12-BDA to Si-CSP1 detected by quenching of tryptophan fluorescence. B. Same as A. except with 0.5 mM β -cyclodextrin. Conditions: 0.6 μ M Si-CSP1, 20 mM Tris buffer, pH 7.2. 12-BDA added as 1 μ L aliquots of 1 mM or 10 mM stock solutions in methanol. Excitation wavelength, 280 nm. Emission wavelength, 335 nm. Lines fitted to data assuming saturation binding of 12-BDA to a single site on Si-CSP1.

Figure 8.

Binding of cuticular lipids to Si-CSP1. Binding of NPN to Si-CSP1 (circles) enhances the dye fluorescence (similar to Fig. 5B). However, Si-CSP1 which first had been exposed to cuticular lipids carried by cyclodextrins (triangles) shows NPN fluorescence diminished to the same level as a control sample lacking Si-CSP1 (squares). Si-CSP1 bound to DEAE-cellulose and exposed to cyclodextrins lacking the cuticular extract (diamonds) had no effect on subsequent NPN binding.

Figure 9.

Cuticular hydrocarbon GC analysis of ligands captured by Si-CSP1. A). GC trace of pentane extract of fire ant cuticle showing 5 main components of cuticular hydrocarbons. B). Si-CSP1 bound to DEAE cellulose was equilibrated with the same extract as in A dispersed in cyclodextrins, eluted with salt, and extracted with methylene chloride. GC trace in B is scaled to the amount of extract in A, assuming each Si-CSP1 molecule can capture at least one hydrocarbon molecule. Mass spectrum of peak in trace B at 18.1 min identifies it as a polysiloxane contaminant. Total ion current $\times 10^{-5}$.

Figure 10.

Cuticular lipid HPLC analysis of ligands captured by Si-CSP1. Si-CSP1 bound to DEAE cellulose was equilibrated with cyclodextrin-dispersed CHCl_3 /methanol fraction (A), or cyclodextrin-dispersed pentane fraction (B), of silica-purified cuticular lipids, followed by salt elution and CHCl_3 /methanol extraction. HPLC analysis on C18 column, eluted with linear gradient between acetonitrile:isopropanol 1:1 and chloroform. Dashed lines: whole extract; solid lines: captured by Si-CSP1. In (A) whole extract is at 1/5 scale for clarity.

Table 1
Sequences coding for CSPs in Fire Ant EST Libraries

For many of the CSPs, the same or similar sequences appear in several ESTs. Only one example is given for each. Numbers 1–14 were assigned arbitrarily.

Name	Accession #	EST name
Si-CSP1	EE134548	SiJWH08ACX
Si-CSP2	EE145825	SiJWB07CAO2
Si-CSP3	EE145392	SiJWE02CAI2
Si-CSP4	EE137035	SiJWH09BBE
Si-CSP5	EE141402	SiJWD02BDOC
Si-CSP6	EE130243	SiJWH07ABA
Si-CSP7	EH413134	13B10
Si-CSP8	EE138976	SiJWF11BCE
Si-CSP9	EE129471	SiJWB03ACR
Si-CSP10	EE142271	SiJWG02BBO2
Si-CSP11	FJ748890	SiJWE02AAV
Si-CSP12	EE148685	SiJWG08ABE
Si-CSP13	EE132485	SiJWA03ABI
Si-CSP14	EE141365	SiJWE03BDJ

Table 2
Proteins identified in capillary LC/MS/MS analysis of *S. invicta* antennal proteins

GenInfo Identifier	EST	Protein	Mascot Score
I21964777	C317	β -Tubulin	252
I21964735	C248	Actin	201
I24240097	SiJWE08BBF	Histone 2A	161
I24222403	SiJWC04AAD	Chitin-binding	154
I21964734	SiJWH08ACX	Si-CSP1	151
I24232022	SiJWD12BBF	Tropomyosin	138
I21964680	C180	Glyceraldehyde-3-phosphate dehydrogenase	124
I24222949	SiJWB03AAO	Chitin-binding	112
I21974028	SiJWA06BBS	Si-OBP1	111
I24224024	SiJWA05ADL	Histone 3A	110
I21964340	20H2	F1 ATP synthase β	108
I24228667	SiJWB02AAB	Peroxisomal protein	106
I24232601	SiJWH11BBT	Troponin T	88
I24231738	SiJWD04BAP	Histone H1/5	84
I21964804	C402	Aldolase	79
I24222954	SiJWE02CAI2	Si-CSP3	78
I24222931	SiJWC09AAO	Aldo-keto reductase/K channel	77
I21964716	C220	Annexin	75
I24234459	SiJWG08BCG1	Glutathione S transferase	73
I21964299	SiJWB01BCJ2	Fatty acid binding protein	68
I21964667	SiJWA05BBH	Si-OBP9 (Gp-9), B allele	66
I24223427	SiJWG01AAX	DNA-binding protein	65
I24229994	SiJWC03ABM	Protease M16	65
I24229908	SiJWD12ABJ	Medium chain acyl-CoA dehydrogenase	63
I21963759	9B1	JH-inducible protein	63
I24234194	SiJWE09BBC2	Catalase	61
I24230070	SiJWA12ABU	Ubiquinol-cytochrome c reductase hinge protein	61
I24228506	SiJWE06ACA	Histone H4	61
I24230878	SiJWA07ADR	F1 ATP synthase α	58
I21964677	C188	Calreticulin	57
I24240522	SiJWG05BBT	Malate dehydrogenase	56
I21962904	IGJ2	Multiple inositol polyphosphate phosphatase	56
I24228315	SiJWB07ACG	Chitinase	53
I21964856	SiJWD06ABI	Apolipoprotein III	52
I24228358	SiJWB09AAD	Phospholipid hydroperoxide glutathione peroxidase	51
I21964514	SiJWF10BAO	Enoyl-CoA hydratase	47
I24231612	SiJWH10ABW	Sec63 domain protein	46
I24236391	SiJWC08BCW2	Mitochondrial porin	46
I21964623	C22	Elongation factor-1 α	46
I24236266	SiJWH11CADR7G01	Seryl-tRNA synthetase	42
I24230254	SiJWD12ADC	α -Tubulin	42
I21964611	C5	Paramyosin	42