# ANALYSIS OF ANTENNAL PROTEINS OF THE RED IMPORTED FIRE ANT

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## ABSTRACT

We have found protein composition differences between male, queen and worker antennae, as analyzed by polyacrylamide gel electrophoresis. Female antennal clubs contain three low molecular weight proteins, Siap1 (18 kDa), Siap2 (16 kDa) and Siap3 (14 kDa) with acidic isoelectric points. Tryptic peptide maps obtained by MALDI-TOF mass spectrometry show that Siap1 in worker segment 9 is essentially the same protein as Siap1 in segment 10, and Siap2 from worker segments 9 and 10 are nearly identical. A mass fragment of m/z 477.1 (the 2+ ion for a 952.2-Da peptide) from Siap2 in worker segment A9 was analyzed by electrospray ionization mass spectrometry and found to have the sequence (K/R)-I/L-I/L-I/L-P-V-S-I/L-A-K. This appears similar to the sequence of residues 97-107 of a putative odorant-binding protein from *A. mellifera*. The male antenna has one major acidic low molecular weight protein, Sim1. It is likely that Siap1-3 and Sim1 are odorant- and pheromone-binding proteins. The segments containing antennal glands in workers and queens show a prominent protein band near 23 kDa, Siap0, which is not seen in any other segment and may be a gland-related protein.

## INTRODUCTION

The antennae of several ant species have been studied in detail, revealing a variety of mechano- and chemosensilla, which are thought to play a major role in ant colony behavior (Jaisson 1969; Masson & Friggi 1971; Masson & Gabouriaut 1973; Hashimoto 1990; Kleineidam et al. 2000). The extracellular fluid inside insect olfactory sensilla, the antennal lymph, contains high concentrations of odorant-binding proteins (OBPs) or pheromone-binding proteins (PBPs) (Pelosi & Maida 1995). To date, no work on OBPs or PBPs from ant antennae has been reported. We are currently studying antennal proteins of the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), with the intent of exploiting the olfactory communication system for pest management.

The role of OBP/PBPs in insect olfaction has been studied in great detail. Since volatile odorants or pheromones typically are not very soluble in water, the OBPs or PBPs capture and concentrate these molecules. At least one OBP has been shown to be essential for detection of a specific odor (Kim et al. 1998). OBP/PBPs may also be involved in interaction with the olfactory receptors and in removal of odorants from the antennal lymph. Often more than one OBP and PBP occurs in a particular insect species. The different OBPs or PBPs are localized to particular subgroups of sensilla (Vogt et al. 1991; Pikelny et al. 1994). Some sensilla may

contain more than one type of OBP (Hekmat-Scafe et al. 1997). Thus, their localization resembles the distribution of olfactory receptor neurons, which appear to specialize in particular odors, but which may occur together with different types of receptor neurons in the same sensillum. However, the OBPs and PBPs are not as specific in their binding characteristics as the membrane-bound olfactory receptors. For example, two different species of moth, which use two different isomers of the same molecule as the sex pheromone, have the same PBP for both molecules (Willett & Harrison 1999). Furthermore, a PBP was found to be incapable of distinguishing between the R and S enantiomers of a beetle pheromone, in contrast to the olfactory receptors for this pheromone, which are enantiomer-specific (Wojtasek et al. 1998). The three dimensional structure of a moth PBP was determined in two different states: at neutral pH with the pheromone bound (Sandler et al. 2000), and at acidic pH with the pheromone released (Horst et al. 2001).

In order to obtain OBP/PBPs from ant antennae, we examined the anatomy of the antenna to locate olfactory sensilla (Renthal et al., to be published elsewhere). Female fire ant antennae (Fig. 1, upper and middle) have a long scape (segment A1), a narrow funicle (segments A2-A8), and a prominent club. The worker club consists of segments A9 and A10, whereas the queen club consists of segments A9-A11. Staining experiments indicate that most of the porous sensilla are on the club segments, suggesting that the receptors for pheromones and general olfaction are in these segments. The female antennae contain secretory glands of unknown function (Isidoro et al. 2000). The glands occur in segments A9 and A10 of the queen and segment A9 of the worker. The male antenna consists of 12 segments (Fig. 1, lower). The male scape (A1) is short and the antenna lacks a club. Sensilla from segments A3-A12 appear to be porous. We have now analyzed, by polyacrylamide gel electrophoresis, the protein compositions of the male, queen and worker antennae, in order to find biochemical differences that reflect the anatomical and functional differences between them.

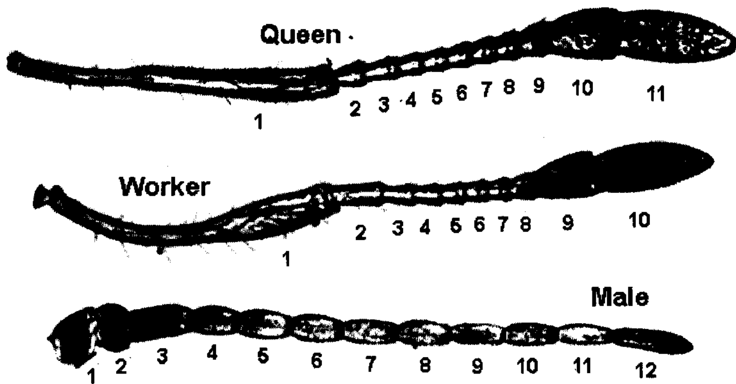


FIG. 1. Red Imported Fire Ant Antennae. Light micrographs of queen (upper), worker (middle) and male (lower) antennae, with segments numbered from scape = 1.

## EXPERIMENTAL METHODS

Polygyne colonies of *S. invicta* from Bexar County, Texas were collected by the floatation method (Jouvenaz et al. 1977) and maintained in the laboratory in plastic trays. Female specimens were selected from the colonies within 1-4 weeks of collection. Workers included a variety of sizes and task specializations. The queens described in these experiments were all alates. Males were removed from the colonies at the time of collection and either dissected immediately or frozen at -20°C. Antennae were dissected on a cold plate (initial temperature, -20°C) and, in some experiments, subdivided into individual antennal segments. Under a dissecting microscope, pooled segments from 100-300 antennae were transferred to a small (3 cm diameter) ceramic mortar in 20 µL of Laemmli (1970) SDS sample dilution buffer and ground quickly with a pestle to break the cuticle. The extract and fragments were washed into a 1.5 mL polypropylene centrifuge tube with two additional 20 µL aliquots of sample buffer. After centrifugation for 5 min at 12,000 X g, the supernatant, usually about 20 µL, was withdrawn and 1 µL of 2-mercaptoethanol was added to it. After boiling for 1 min, the supernatant was applied to a 15% polyacrylamide gel and the proteins were separated by SDS-PAGE (Laemmli 1970). Gels were stained with Coomassie blue R-250. The stain intensity was quantitated by video image analysis (Greiss & Serwer 1991), using a GBC 500 ccd camera (CCTV Corp., N.Y., NY) and a LG-3 frame grabber (Scion Corp., Frederick, MD). In some experiments, two-dimensional gels were run, with the first dimension isoelectric focusing in urea-containing tube gels (O'Farrell 1975). The pH gradient was generated with Bio-Lyte 3/10 ampholyte (Bio-Rad Laboratories, Hercules, CA). For analysis by mass spectrometry, Coomassie-stained bands from the 14 – 23 kDa range were excised and digested with trypsin, as described by Shevchenko et al. (1996). The mass distributions of the tryptic fragments were analyzed in an Applied Biosystems Voyager Elite MALDI-TOF mass spectrometer. Selected peptides were further analyzed by $MS^n$ to obtain sequence information using a Finnigan LCQ electrospray mass spectrometer. Sequences were compared with the NCBI database using BLAST (http://www.ncbi.nlm.nih.gov/).

## RESULTS

*Female Antennae.* Analysis of extracts from female antennal clubs by SDS-polyacrylamide gel electrophoresis (Fig. 2) shows three low molecular weight proteins, which we refer to as Siap1 (18 kDa), Siap2 (16 kDa) and Siap3 (14 kDa) ("Si" for *Solenopsis invicta* and "ap" for antennal protein). In-gel tryptic digestion was performed on the Siap1 and Siap2 bands from pooled worker A9 segments and A10 segments. Tryptic peptide maps obtained by MALDI-TOF mass spectrometry (Table 1) show that Siap1 in worker segment 9 has the same tryptic fragments as Siap1 in segment 10, and Siap2 from worker segments 9 and 10 also have the same tryptic fragments. Therefore, it is likely that the protein or proteins present in Siap1 in segments 9 and 10 are the same, and similarly, Siap2 is composed of the same proteins in segments 9 and 10. The tryptic fragments were further analyzed by electrospray ionization mass spectrometry. A mass fragment of *m/z* 477.1 (the +2 ion of a 952.20 Da peptide) from Siap2 in segment A9 was found to have the sequence (K/R)-I/L-I/L-I/L-P-V-S-I/L-A-K. This appears similar to the sequence of residues 97-107 of a putative odorant-binding protein from *Apis mellifera* (GenBank BE844326) (Table 2). Densitometry from video images was used to measure the relative amounts of the three bands in queens and workers. In the queen antenna, Siap1 is less abundant in segments A9 and A10 compared with A11, whereas in workers, Siap1 appears to be equally distributed in the club segments.

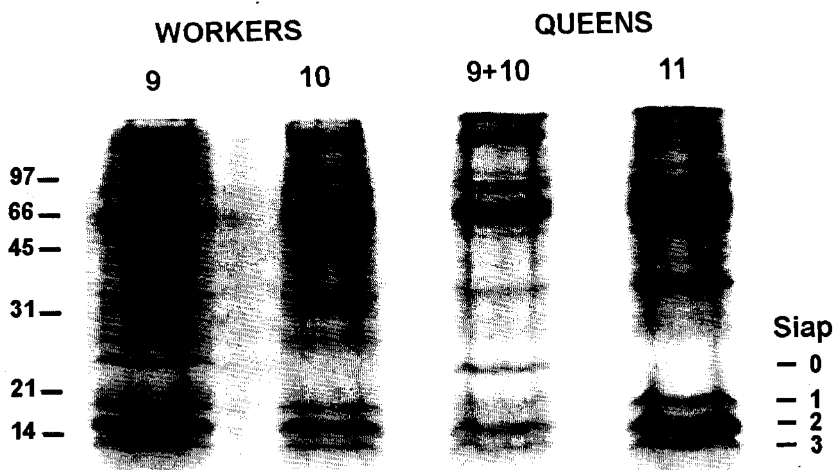


FIG 2. Polyacrylamide Gel Electrophoresis of Female Antennae. Molecular weight markers indicated at left.

The segments containing antennal glands (A9 in workers; A9 and A10 in queens) show a prominent protein band near 23 kDa, Siap0, which is not seen in any other segment. The stain intensity of Siap0 in queens and workers was measured by densitometry from video images. In queens, Siap0 has about twice the intensity as in workers.

TABLE 1. Mass Spectral Analysis of Tryptic Fragments of Antennal Proteins Siap1 and Siap2 in Worker Segments 9 and 10.

| Siap1 | | | | Siap2 | | | |
|---|---|---|---|---|---|---|---|
| Segment 9 | | Segment 10 | | Segment 9 | | Segment 10 | |
| *m/z* | rel.int. | *m/z* | rel.int. | *m/z* | rel.int. | *m/z* | rel.int. |
| 715.42 | 54 | 715.42 | 42 | 816.46 | 33 | 816.46 | 15 |
| 788.47 | 80 | 788.48 | 91 | 900.48 | 13 | 900.47 | 16 |
| 831.49 | 79 | 831.50 | 61 | 944.54 | 100 | 944.43 | 100 |
| 1032.59 | 100 | 1032.60 | 100 | 953.60 | 15 | 953.57 | 7 |
| 1349.72 | 52 | 1349.71 | 42 | 1149.57 | 13 | 1149.58 | 7 |
| 1363.71 | 22 | 1363.72 | 18 | 1729.46 | 8 | 1729.44 | 9 |
| 1667.80 | 29 | 1667.78 | 14 | 1791.68 | 12 | 1791.69 | 11 |

Although sharp bands were obtained in one-dimensional gels, it is likely that the bands are mixtures of proteins having similar molecular weights. The extraction of antennal proteins in SDS probably breaks nuclear membranes, contaminating the antennal lymph with histones. These nuclear proteins have molecular weights in the same range as OBP/PBPs, and we can detect them in the mass spectrometric analysis (data not shown). To overcome this problem, we used two-dimensional gel electrophoresis (O'Farrell 1975). This method first separates the proteins by charge, using isoelectric focusing, followed by SDS-PAGE separation by molecular

weight. The extraction buffer for the first dimension lacks SDS, which minimizes the possibility of contamination with histones. However, if any nuclei are broken during the extraction, the histones will migrate away from the OBP/PBPs during isoelectric focusing, because their isoelectric points (pIs) are known to be basic, in contrast to the acidic pIs of OBP/PBPs. A two-dimensional gel of queen antennal proteins is shown in Fig. 3. Protein spots in the molecular weight range of Siap1-3 are observed at the acidic side of the gel, as expected for OBP/PBPs.

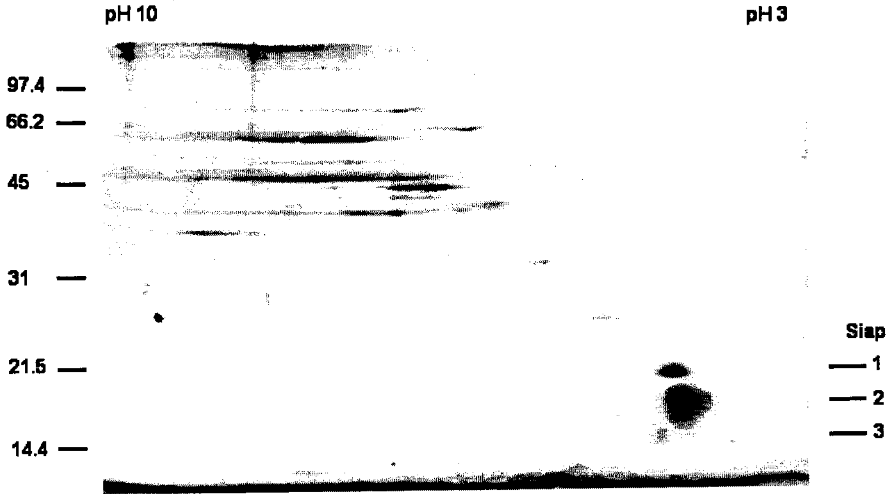


FIG. 3. Two Dimensional Electrophoresis of Queen Antennal Proteins. Extracted in 8 M urea. First dimension (horizontal axis): isoelectric focusing, pH 3-10. Second dimension (vertical axis): SDS-PAGE. Molecular weight markers indicated in kDa on left scale.

*Male Antenna.* Using two-dimensional gel electrophoresis, we found that the male antenna contains only one major low molecular weight protein, at an isoelectric point of about 3.7 and a molecular weight of about 20 kDa (data not shown).

TABLE 2. Sequence Comparison. Fragment from putative OBP of *S. invicta* worker (Siap2) compared with amino acids 97-107 of OBP from *A. mellifera*. Sequence similarities or identities are shown in bold.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Siap2, 952.2 Dalton fragment | K/**R** | - | L/**I** | **L**/I | **L**/I | P | V | S | L/**I** | A | **K** |
| *A. mellifera* GB BE844326 residues 97-107 | **R** | M | **I** | **L** | **L** | E | E | Y | **I** | P | **R** |

DISCUSSION

We have identified a group of antennal proteins in red imported fire ants which are likely to be odorant- or pheromone-binding proteins (OBPs and PBPs). The molecular weights and isoelectric points are consistent with the sizes of OBPs and PBPs reported from a variety of insects (Pelosi & Maida 1995). Three bands were identified on one-dimensional polyacrylamide

gels (Fig. 2) and three spots on two dimensional polyacrylamide gels (Fig. 3) were identified as probable OBP/PBPs, labeled Siap1-3. We find that the composition of each band does not vary between the two segments of the worker antennal club (Table 1). Considering the complexity of chemical signaling in ants (Hölldobler 1995), Siap1-3 could contain more than three OBP/PBPs. Workers and queens would be expected to require a larger repertoire of odorant and pheromone sensitivities in order to receive the many types of chemical signals they must be capable of detecting. By contrast, the male antenna has a simpler protein composition. This is compatible with what is known about *S. invicta* males: they are not observed to tend brood or to forage, and their sole function in the colony appears to be participation in nuptial flights. Therefore, we expect the male antenna to have a relatively simple repertoire of olfactory and pheromone receptors compared to the female antenna.

A 952.2 Da tryptic fragment of Siap2, sequenced by $MS^n$ (Table 2), is similar to residues 97-107 of a putative odorant-binding protein from honey bees (*A. mellifera*), although a gap must be introduced to obtain the match. Residues 97-107 of the bee OBP correspond to residues 77-87 of the pheromone-binding protein from silk moths (*B. mori*), a PBP of known three-dimensional structure (Sandler et al. 2000). This sequence forms a loop between helices D and E, which joins with the amino and carboxyl termini to cover the pheromone-binding cavity. The gap in Table 2 is consistent with previous sequence alignments of this region showing varying loop lengths in different OBPs and PBPs (Kim et al. 1998; Robertson et al. 1999). The 952.2 Da tryptic fragment also has sequence similarity to residues 98-108 of a sensory appendage protein (SAP) from *A. mellifera* (GenBank BE844261), after introducing a gap. In this case, however, sequence alignments do not indicate length variability in this region (Robertson et al. 1999). Therefore, we consider the assignment of this peptide to the OBP/PBP family more likely than to the SAP family.

The 23-kDa protein Siap0 is found only in the gland-containing segments. The queens have about twice the amount of Siap0 as workers. Since queens have about twice the number of antennal gland pores as workers (Isidoro et al. 2000), it is likely that Siap0 is a gland-related protein. The molecular weight of Siap0 is similar to a gland-associated protein previously reported in a cockroach (*L. maderae*) (Korchi et al. 1999) and to a butterfly (*A. alcinous*) tarsal protein involved in sensing oviposition sites (Tsuchihara et al. 2000).

Future studies will focus on determining the complete sequences of the proteins identified in this paper. Purified pheromone-binding proteins may be useful as a tool for isolating and identifying pheromones from ants. Ant primer pheromones have proven to be more difficult to isolate than releaser pheromones. Part of the difficulty is due to the diversity and complexity of chemical signaling by ants, and part is due to the complex glandular origins of ant pheromones: in some cases, different components of a pheromone blend come from different glands (Hölldobler 1995), and sometimes the same pheromone is found in more than one gland (Vargo & Hulsey 2000).


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