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Major venom proteins of the fire ant *Solenopsis invicta*: insights into possible pheromone-binding function from mass spectrometric analysis

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Abstract

Proteins in the venom of the fire ant, *Solenopsis invicta*, have been suggested to function in pheromone-binding. Venom from queens and workers contain different isoforms of these proteins, consistent with the differing pheromones they secrete, but questions remain about the venom protein composition and glandular source. We found that the queen venom contains a previously uncharacterized pheromone-binding protein paralog known as Sol i 2X1. Using imaging mass spectrometry, we located the main venom proteins in the poison sac, implying that pheromones might have to compete with venom alkaloids for binding. Using the known structure of the worker venom protein Sol i 2w, we generated three dimensional homology models of the worker venom protein Sol i 4.02, and of the two main venom proteins in queens and female alates, Sol i 2q and Sol i 2X1. Surprisingly, the models show that the proteins have relatively small internal hydrophobic binding pockets that are blocked by about ten amino acids of the C-terminal region. For these proteins to function as carriers of hydrophobic ligands, a conformational change would be required to displace the C-terminal region, somewhat like the mechanism known to occur in the silk moth pheromone-binding protein.

Introduction

Venom from the fire ant *Solenopsis invicta* Buren contains proteins resembling insect pheromone-binding proteins (Borer et al., 2012). Odorant- and pheromone-binding proteins (OBPs) are used by insects to carry hydrophobic odorants and pheromones from the external surroundings through the aqueous lymph to chemosensory receptors in the neuronal membranes inside chemosensory sensilla of the antennae (Leal, 2005), mouthparts, and tarsi (Sparks et al., 2014). The OBPs in the fire ant venom obviously are not performing this type of function, but their actual role is unknown. Typical insect genomes have large paralogous groups of OBP genes, but only a relatively small subset are expressed in chemosensory organs. For example, 18 OBP genes were identified in the *S. invicta* genome (Gotzek et al., 2011), but only 3 OBPs were detected in the antennal proteome (González et al., 2009). Some OBPs are known to be involved in non-chemoreception hydrophobic ligand transport

functions, including pheromone secretion (Pelosi et al., 2018). If the fire ant venom OBPs function as pheromone carriers in secretion, then further characterization of these proteins could be a valuable aid in the identification of *S. invicta* pheromones. Although there are well-developed methods for identifying volatile pheromones, such as electroantennographic recording (Jones & Oldham, 1999), it is more difficult to identify non-volatile pheromones that act over a short range. At least five different fire ant pheromones are released from the queen's venom (Vander Meer et al., 1980; Rocca et al., 1983; Fletcher and Blum, 1981; Vargo and Fletcher, 1986; Vander Meer et al., 1992; Vargo, 1992; Vander Meer and Alonso, 2002), and several of these have been shown to be non-volatile. One approach to identifying non-volatile pheromones would be the method known as "ligand fishing" (Zhuo et al., 2016). In this method, OBPs are used *in vitro* to capture pheromones for analysis (Oldham et al., 2000; Oldham et al., 2001; Leal et al., 2005). Pheromones captured by OBPs are thought to be held tightly until the protein encounters a change of conditions that causes the pheromone to be released, due to conformational changes between an open conformation, which is favored under conditions for pheromone binding (i.e. at the air/lymph interface where the pheromone is picked up from the air) and a closed conformation, which is favored under conditions for pheromone release (i.e. at the surface of the neuronal membrane where the pheromone is transferred to the pheromone receptor) (Horst et al., 2001; Laughlin et al., 2008). This property might be exploited to collect pheromones from a mixture and then release them for chemical analysis.

The OBP-like fire ant venom proteins have different sequence isoforms in workers and queens (Hoffman et al., 1988; Hoffman, 1993; Lockwood et al., 2012). The most abundant OBP-like protein in fire ant worker venom is called Sol i 2w, and the most abundant OBP-like protein in fire ant queen venom is called Sol i 2q. Sol i 2w and 2q have 75.6% sequence identity. Each has a molecular weight of about 13,200, and each has 119 amino acids, including seven cysteines. Six of the cysteines form intramolecular disulfide bonds, and one joins two protein molecules in a covalent homodimer through an intermolecular disulfide bond. Several minor isoforms (Sol i 4, Sol i 4.01, Sol i 4.02, Sol i 4q) were identified in worker venom by direct sequencing (Hoffman, 1993), in worker venom by immunoblots (Lockwood et al., 2012), and by sequencing cDNA derived from RNA extracts of whole worker or queen abdomens (Lockwood et al., 2012). Additional isoforms (Sol i 2X1, Sol i 2X2) are listed in the National Center for Biotechnology Information (NCBI) sequence database, derived from the *S. invicta* genome sequence (XP_011156049 and XP_011156057), but apparently these isoforms have not been observed in protein extracts. Previous protein analyses of fire ant venom used venom "milked" from ants (Hoffman et al., 1988; Hoffman, 1993; dos Santos Pinto et al., 2012; Lockwood et al., 2012), and mRNA sequences were derived from whole abdomens (Lockwood et al., 2012). This leaves open the question of the precise glandular origin of the venom proteins, because the poison sac and the Dufour's gland both deliver their contents into the sting through separate ducts (Billen, 1987). Therefore, we have re-examined the *S. invicta* venom proteins, using imaging mass spectrometry to locate the proteins in workers and queens. We have also conducted a proteomic analysis of proteins extracted from dissected poison sacs and used three-dimensional homology models to assess the potential of the venom proteins as pheromone carriers for future ligand fishing experiments.

Results

Venom proteins

Poison sac proteins in the 12–15-kDa monomer range on a 1-D SDS PAGE gel were subjected to in-gel trypsin digestion, and the extracted peptides were analyzed by tandem mass spectrometry. The main protein components (Figures 1 and 2) were identified with approximately 90% sequence coverage. As previously reported (Hoffman et al., 1988; Hoffman, 1993; dos Santos Pinto et al., 2012), the main low molecular weight worker venom proteins identified were Sol i 2w and Sol i 4. In our specimens, these proteins were in an approximately 6:1 ratio.

In egg-laying queens and in female alates (unmated queens), the main low molecular weight venom proteins identified were Sol i 2q and Sol i 2X1. In alates, the ratio of 2q to 2X1 was about 2:1, whereas in egg-laying queens the ratio was two to four times higher. Trace amounts of Sol i 4 and Sol i 2w were observed in queens and alates, and small, variable amounts of Sol i 2q, 2X1, 2X2, and 4 were detected in workers.

Imaging mass spectrometry

Dissected queen venom sacs and worker venom sacs were cryosectioned and examined by imaging mass spectrometry. Both venom sacs showed one major peak near m/z 13000, the monomer mass of Sol i 2q, Sol i 2w, Sol i 2X1, and Sol i 4.02. In queens (Figure 3), the m/z 13000 proteins were abundant in the convoluted gland and in the wall of the poison sac. In workers, the m/z 13000 proteins were abundant primarily in the convoluted gland. Sections that had been washed with chloroform/methanol to remove lipids gave sharp protein peaks at m/z 13130 ± 6 (queens), or m/z 13128 ± 4 (workers) (Figure 4). These peaks are about 100 mass units lower than expected from the amino acid sequences of Sol i 2q (13241 Da) and Sol i 2w (13217 Da), possibly resulting from free radical reactions initiated by the matrix used to volatilize protein ions, 2,5-dihydroxybenzoic acid (DHB) (Asakawa, 2016). For non-solvent-washed sections, there was a broad peak centered at about m/z 13600 that was absent or much less intense in solvent-washed sections (Figure 4). The broad higher mass peak could be due to lipid or venom alkaloid adducts in the non-solvent-washed sections. Sol i 2w is known to be a disulfide-linked dimer (Hoffman, 1993; Borer et al., 2012). The amino acid sequences of Sol i 2q and 2X1 contain the same pattern of cysteines as Sol i 2w (Figure 1), implying that these proteins also form disulfide-linked dimers. This conjecture is supported by the three dimensional homology modeling discussed below. However, our MALDI-TOF spectra showed mostly monomers. The DHB matrix is known to reduce disulfides (Asakawa, 2016), so it is likely that the intermolecular disulfide bonds are broken by photoactivation of the matrix. Non-solvent-washed sections of queen venom sacs occasionally showed a broad peak at m/z 27000 (Figure 4), indicating incomplete reduction in these samples.

Pheromone binding capacity

Three-dimensional homology models were constructed for Sol i 2q, Sol i 2X1, and Sol i 4.02, using SwissModel. For all three sequences, SwissModel selected the crystal structure of Sol i 2w (Protein Data Bank ID 2YGU) as the best template. The models for 2q and 2X1

were dimers, like the 2w template, and the model for 4.02 was a monomer, which is consistent with the fact that it lacks the cysteine that forms an intermolecular disulfide in the other three proteins. The quality parameters indicated that the models were very good. For 2q, 2X1 and 4.02, respectively, GQME (global quality model estimate) was 0.90, 0.85, and 0.73; and QMEAN (qualitative model energy analysis) Z-score was 0.16, 0.21, and -0.28. GQME values can range from 0 to 1, with 1 high; and a model having a QMEAN Z-score with an absolute value less than 1 is comparable in quality to experimentally determined Protein Data Bank structures of similar size (Benkert et al., 2011). We used the three-dimensional structures with CASTp software to determine the size of the largest interior binding pocket of each structure. Sol i 2w and 4.02 have modest-sized binding pockets, in the range of 0.4 nm³. This is approximately the molecular volume of E,E-alpha farnesene (Chem Spider 4444849), a minor component of the fire ant trail pheromone (Vander Meer et al., 1988). However, the comparable pockets in 2q and X1 are much smaller (Table 1). All four venom allergens have approximately ten C-terminal amino acids blocking access to the interior of the protein (Figure 5). In Sol i 2w, 2q, and X1 there is no access between the interior pocket and the exterior aqueous solvent. This implies that, if these are pheromone-binding proteins, there would have to be a conformational change to allow the pheromones into the interior binding sites. We also computationally tested the binding of the venom alkaloid solenopsin A (the isomer having an 11-carbon chain) to the 3D models, using Autodock software. The results (given as dissociation constants in Table 1) predict that solenopsin A binds to the interior pockets of the venom proteins with sub-millimolar dissociation constants, but only if the C-terminal ten amino acids are removed (Figure 5). With the intact proteins, solenopsin A binds only to surface sites on the proteins, and the binding is much weaker.

Discussion

The major venom allergens of worker fire ants (*Solenopsis invicta*) were identified by Hoffman and coworkers (Hoffman et al., 1988; Hoffman, 1993). Subsequently, Deslippe and coworkers (Lockwood et al., 2012) found proteins with similar but not identical sequences in queen venom. We have re-analyzed the major venom proteins of workers, alates and queens (Figures 1 and 2). We find that queen and alate venom contains significant amounts of a protein called Sol i 2 X1, that, as far as we know, has not been previously detected in fire ants but was predicted from the *S. invicta* genome. Deslippe and coworkers described a protein called Sol i 4q, using direct N-terminal sequence analysis of queen venom proteins and PCR amplification of poison sac mRNA sequences. However, we did not detect this protein in our analysis. The N-terminal sequences of the isoforms of Sol i 4 are identical, so it is possible their PCR primer was derived from Sol i 4.02, which was expressed in the queens we analyzed. They may have amplified an mRNA sequence of a Sol i 4 isoform that is expressed at very low levels in the poison sac and was not detected by our mass spectrometric analysis. Alternatively, Sol i 4 gene isoforms could be variably expressed, depending on environmental or other factors.

An analysis of Sol i 2w by X-ray crystallography led Borer et al. (Borer et al., 2012) to conclude that it has a similar structure to insect pheromone-binding proteins. Because all four of the major venom proteins (Sol i 2w, 2q, 4.02 and X1) share substantial amino acid

sequence similarity, this entire set of four proteins thus should be considered as potential pheromone transporters. Borer et al. suggested the possibility that Sol i 2w may carry the worker trail pheromone. The source of the trail pheromone is the Dufour's gland (Wilson, 1959; Vander Meer et al., 1988), which releases its contents through the sting (Billen, 1987). We do not know of any previous experiments that have rigorously demonstrated the presence of the venom proteins in the venom sac, rather than in the Dufour's gland. However, our imaging mass spectrometry results reveal that both the worker and queen venom proteins are in the convoluted glands of the poison sacs (Figure 3). Thus, the putative pheromone-binding protein, coming from the poison sac, must combine with its pheromone ligand downstream from where the poison sac duct and Dufour's gland duct join in the sting.

If the venom proteins are pheromone-binding proteins, their poison sac location poses a difficult biochemical problem. The proteins are in the aqueous phase of the venom (Hoffman et al., 1988), which presumably is in equilibrium with the piperidine alkaloid organic phase. We do not know the aqueous solubility of the venom alkaloids, but their chemical structures resemble a series of well-known detergents—the alkyl trimethylammonium halides (e.g. DTAB, CTAB). Thus, the venom proteins could be exposed to millimolar concentrations of the piperidine alkaloids, assuming the aqueous solubilities and micelle formation properties of the alkaloids are similar to CTAB (Linke, 2009). Our computational results (Table 1) suggest that the venom proteins have relatively small internal binding pockets. The structural models indicate that the C-terminal tails of the proteins block access to the proteins' interior pockets (Figure 5). The protein interiors are inaccessible to solenopsin A, but *in silico* removal of the C-terminal tails permits binding of this venom alkaloid. Although the calculated binding constants have only moderate strength (Table 1), we estimate that, at the very high alkaloid concentrations to which these proteins are exposed, the venom alkaloid could compete with other ligands for these binding sites. The C-terminal tails may have a hinge at Gly 108. This position is a conserved glycine in all four major venom proteins (Figure 1). If access to the ligand-binding site is regulated by the conformation of the C-terminal tail, it would be similar to the conformational changes observed in pheromone-binding proteins like the silk-moth (*Bombyx mori*) pheromone-binding protein (Horst et al., 2001). At acid pH, the C-terminal tail of this pheromone-binding protein displaces the pheromone bombykol from its binding pocket in the interior of the protein. Borer et al. (Borer et al., 2012) reported evidence that Sol i 2w binds to added hydrophobic ligands, but the experimental details presented are insufficient to determine whether the binding sites were internal or external, or whether binding was accompanied by a protein conformational change. In any case, the presence of high concentrations of venom alkaloid creates a problem for the venom proteins if their function is to bind pheromones. It seems unlikely that the binding proteins could pick up pheromones in the convoluted gland or poison sac, considering the competing high concentrations of venom alkaloids. And if the venom proteins function as venom alkaloid transporters, then the structure reported by Borer et al. (Borer et al., 2012) is not likely to be the biologically relevant conformation for binding. Thus, our future studies of the fire ant venom proteins will focus on whether the C-terminal tails are displaced by environmental conditions, allowing the proteins to function as a carriers of pheromones or venom alkaloids.

Experimental Procedures

Ants

Polygyne colonies of *Solenopsis invicta* were collected in Bexar County, Texas by floatation (Jouvenaz et al., 1977). Colonies were maintained in the lab in plastic trays containing petri dish nests, and they were fed water, honey water, and beef liver (Gavilanez-Slone and Porter, 2013).

Sample preparation for imaging mass spectrometry

The ants were cold-anesthetized and the poison sacs were dissected in 10 mM HEPES buffer, pH 7.0, containing 100 mM NaCl. It was necessary to keep the stinger attached to the poison sac to prevent the loss of the sac contents. Porcine gelatin (Sigma-Aldrich G1890) was prepared at a concentration of 10% by stirring on a hot plate until clear. After cooling to about 45°C, thin gelatin layers were pipetted into wells of a silicone mold (Ted Pella 10505) and allowed to gel. The dissected poison sacs were placed on the gelatin layers, one per well, and they were covered with another layer of warm liquid gelatin. After the samples were gelled at 4°C for 30 min, the mold containing the gelatin-embedded poison sacs was placed inside a small zipper seal plastic bag and flash-frozen by immersion in a dry ice/acetone mixture. Frozen blocks were stored at -20°C. Cryosectioning was conducted at -25°C on a Leica CM1850 cryostat. The frozen gelatin blocks were trimmed and then glued to specimen discs using O.C.T. (Tissue-Tek 4583). Sections (14 µm) were cut and transferred to indium tin oxide (ITO) coated glass slides that had been cleaned with trisodium carbonate (Yang and Caprioli, 2013). About 20–30 sections were placed onto each slide. Slides were stored in a vacuum desiccator. Some slides were solvent-washed, using the six-rinse protocol described by Yang and Caprioli (Yang and Caprioli, 2011). In experiments where solvent-washed and non-washed sections were compared, consecutive sections were placed alternately on two different ITO slides, one of which was subsequently solvent-washed. The slides were coated with 2,5-dihydroxybenzoic acid (DHB) by sublimation (Hankin et al., 2007), rehydrated with acetic acid for 45 sec at 80°C (Yang and Caprioli, 2011), and then dried at 80°C for 1–2 min. The sublimation and rehydration steps were completed immediately before the mass spectra were acquired.

Protein extraction and digestion

For each extract, two dissected poison sacs were transferred to a 200 µL plastic PCR tube to which was added 10 µL of 2% sodium dodecyl sulfate. The poison sacs were broken open with a pipet tip to release the contents. The supernatant was applied to a NuPAGE 4–12% BisTris gel (Thermo Fisher) for SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gels were stained with Coomassie blue. The protein band with apparent molecular weight from 12 – 15 kDa was excised, destained, and proteins reduced and alkylated prior to in-gel trypsin digestion.

Mass spectrometry

Imaging mass spectrometry was carried out using a Bruker ultrafleXtreme MALDI-TOF mass spectrometer and Fleximaging software. Calibration was done using Bruker Protein Calibration Standard I.

Tryptic peptides obtained from in-gel trypsin digestion of venom protein extracts (see previous section) were analyzed on a Thermo Fisher LTQ Orbitrap Velos Pro mass spectrometer. Protein sequences were identified with Mascot (Matrix Science), Scaffold (Proteome Software), and MaxQuant software (Cox & Mann, 2008). Label-free quantification was obtained by calculating the exponentially multiplied protein abundance index (emPAI), by the method described by Ishihama et al. (Ishihama et al., 2005). Only peptides with Mascot ions scores ≥ 95% confidence (> 46 or 47 for all data sets) were used in calculating emPAI. The emPAI values were adjusted by apportioning peptides shared among multiple proteins.

Modeling

Homology models of Sol i 2q, Sol i 2X1, and Sol i 4.02 were constructed using SwissModel (<https://swissmodel.expasy.org/>). Interior cavities were calculated from the homology models using CASTp software (<http://sts.bioe.uic.edu/castp/>). The binding of solenopsin A to the three models, with and without the C-terminal helices, was predicted using Autodock 4.2 (<http://mgltools.scripps.edu>). The atomic coordinates for solenopsin A were obtained from the Cambridge Crystallography Data Center (<https://www.ccdc.cam.ac.uk/>), structure number 951312.

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Figure 1.
Sequence alignment of venom proteins.

Figure 2.

Estimated relative amounts of venom proteins in queens (Q), alates (A) and workers (W), for two biological replicates, each containing extracts from two poison sacs. Quantities estimated using the exponentially modified protein abundance index (emPAI) for the indicated proteins, given as a percent of total emPAI.

Figure 3. Mass imaging of queen poison sac cryosection. Left panel: mass image. Relative intensity of cryosection protein peak between m/z 13209 and 13143 (color code for intensity indicated on 0–100% bar). Right panel: dark field light microscope image of same section at same scale as mass image (scale bar below intensity bar). Central oval is convoluted gland; outer ring is wall of poison sac.

Figure 4. MALDI-TOF mass spectra of single points in mass images of queen poison sac cryosections. Red: sections washed in CHCl_3 /methanol prior to coating with DHB matrix. Blue: sections not solvent-washed. Peak marked 2^+ is doubly-charged m/z 13130 ion. Inset: expanded plot of m/z 13130 peak.

Figure 5.

Three-dimensional homology model of Sol i 2q. Left: entire sequence, modeled using the X-ray structure of Sol i 2w (PDB 2YGU). The C-terminal tail is colored cyan. Atoms lining the binding pocket are shown in yellow. Middle: structure with the C-terminal tail removed. Right: same as middle image, except with solenopsin A (magenta) bound to the pocket.

Table 1

Computed properties of Sol i 2w and 3D homology models

Protein	Calculated internal pocket ^a volume (nm ³)		Calculated solenopsin A dissociation ^b , K _d (μM)	
	Intact C-tail	Minus C-tail	Intact C-tail	Minus C-tail
Sol i 2w	0.42	0.33	1140	32
Sol i 2q	0.27	0.23	130	58
Sol i 2X1	0.34	0.14	810	410
Sol i 4.02	0.46	0.46	580	130

notes:

^a largest internal pocket;^b calculated at 25°C from computed free energy of binding