



Contents lists available at SciVerse ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Identification, expression, and immuno-reactivity of Sol i 2 & Sol i 4 venom proteins of queen red imported fire ants, *Solenopsis invicta* Buren (Hymenoptera: Formicidae)

Stephanie A. Lockwood^{a,*}, Jilla HaghiPour-Peasley^a, Donald R. Hoffman^b, Richard J. Deslippe^a

^a Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409-3131, USA

^b Department of Pathology and Laboratory Medicine, Brody School of Medicine at East Carolina University, Greenville, NC 27834, USA

ARTICLE INFO

Article history:

Received 20 April 2012

Received in revised form 10 May 2012

Accepted 13 May 2012

Available online xxxx

Keywords:

Allergen

DNA sequence

Protein sequence

Venom gland

ABSTRACT

We report on two low-molecular weight proteins that are stored in the venom of queen red imported fire ants (*Solenopsis invicta*). Translated amino acid sequences identified one protein to have 74.8% identity with the Sol i 2w worker allergen, and the other protein was found to have 96/97% identity with Sol i 4.01w/4.02w worker allergens. Both Sol i 2 and Sol i 4 queen and worker proteins were expressed using pEXP1-DEST vector in SHuffle™ T7 Express lysY *Escherichia coli*. Proteins were expressed at significant concentrations, as opposed to the µg/ml amounts by our previous expression methods, enabling further study of these proteins. Sol i 2q protein bound weakly to human IgE, sera pooled from allergic patients, whereas Sol i 2w, Sol i 4.01w, and Sol i 4q proteins bound strongly. Despite Sol i 2w and Sol i 2q proteins having 74.8% identity, the queen protein is less immuno-reactive than the worker allergen. This finding is consistent with allergic individuals being less sensitive to queen than worker venom.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

The medical significance of red imported fire ants (RIFAs) is substantial with an estimated 14 million people being stung by worker RIFAs each year (Drees, 2002). Workers sting by first clasp the skin with their mandibles and then injecting up to ~0.66 nl of venom into the skin with their modified ovipositor (stinger) (Tschinkel, 2006). Within several hours a clear pustule develops at the injection site, and though sterile due to alkaloids in the venom, can become secondarily infected (Lockey, 1974; Hoffman, 1995; Tankersley, 2008). The alkaloids comprise 99.9% of the RIFA venom composition, and are different

combinations of the same basic 2,6-dialkylpiperidines (C_{11:0}, C_{13:0}, C_{13:1}, C_{15:0}, C_{15:1}, C_{17:0}, and C_{17:1}) in either *trans*- or *cis*-forms, where the *trans* isomers are dominant (Brand et al., 1973; Blum et al., 1992; Chen et al., 2010). Strikingly different than the worker venom, the queen RIFA venom is only composed of two main alkaloid components: *cis*-C_{11:0} and *trans*-C_{11:0}, where *cis*-C_{11:0} is at least twice as abundant (Brand et al., 1973).

About 5% of people that get stung require medical treatment and 2% of fire ant sting patients have serious systemic allergic reactions (Yaeger, 1978; Stafford et al., 1989a,b). Depending on the patient, allergic reactions can manifest as coughing, wheezing, hoarseness, choking, numbness, fever, lightheadedness, dizziness, nausea, sweating, low blood pressure, headaches, and shortness of breath (Lockey, 1974; Rhoades, 1977; Drees, 2002). In extreme cases, stings from these ants have resulted in anaphylactic shock (0.6–6%) and even death (>80 cases) (Drees, 2002). Baer et al. (1979) established that the

Abbreviations: RIFAs, Red imported fire ants; Sol i 2, *Solenopsis invicta* venom allergen number 2; Sol i 4, *Solenopsis invicta* venom allergen number 4.

* Corresponding author. Tel.: +1 806 742 2715; fax: +1 806 742 2963.

E-mail address: stephanie.lockwood@ttu.edu (S.A. Lockwood).

0041-0101/\$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
<http://dx.doi.org/10.1016/j.toxicon.2012.05.011>

Please cite this article in press as: Lockwood, S.A., et al., Identification, expression, and immuno-reactivity of Sol i 2 & Sol i 4 venom proteins of queen red imported fire ants, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), *Toxicon* (2012), <http://dx.doi.org/10.1016/j.toxicon.2012.05.011>

allergenic activity of the venom is a result of the protein component. Using anti-immunoglobulin E (IgE) radio-labeled with ^{125} Iodine, Baer et al. (1979) measured differences in radioactivity, a relative measure of the quantity of IgE antibody against venom, among *Solenopsis invicta* (workers and queens), *Solenopsis geminata*, and *Solenopsis xyloni*. They determined *S. invicta* workers are most reactive followed by *S. invicta* queens, *S. xyloni*, and *S. geminata*, respectively. Baer et al. (1979) tested several patients allergic to fire ant stings and found that some patients reacted more to worker venom than queen venom. Allergic reactions are caused by IgE antibodies made specifically to recognize certain allergens; in imported fire ants called IFA IgE. Individuals never stung by a fire ant lack specific IFA IgE, where in allergic patients specific IFA IgE can be detected 100% of the time (Hoffman et al., 1988).

In 1987, Hoffman confirmed the allergenic component of the venom as proteinaceous and that the allergenic activity is conformation dependant. Hoffman (1987) further suggests that the protein is allergenic due to its tertiary structure as opposed to its primary structure. In 1988, Hoffman et al. originally named and described four worker allergens (Sol i 1, Sol i 2, Sol i 3, and Sol i 4), which only composes 0.01% of the RIFA venom composition. Herein, we added “w” or “q” designations after the allergen name to differentiate between worker and queen versions. Sol i 1w has been purified and found to have 309 amino acids, a molecular weight of 37,000 Da, and by cation exchange found to have three charged forms (Hoffman et al., 1988; Hoffman, 1993). Sol i 2w was found to be 119 amino acids long with a molecular weight of 13,217 Da (the native form is a dimer and has a molecular weight of ~28,000 Da), Sol i 3w 212 amino acids long with a molecular weight of 24,040 Da, and Sol i 4w 117 amino acids long with a molecular weight of 13,340 Da (Hoffman, 1993). Sol i 2w and Sol i 4w sequences only showed a 35% identity with each other and no significant identity to any other proteins researched (Hoffman, 1993). Sol i 2w and Sol i 3w are the major proteins in the venom, and Sol i 1w and Sol i 4w are only found in small amounts (Hoffman et al., 1988). Sol i 2w and Sol i 4w are considered among the most potent allergens due to their potency to dose ratio, where Sol i 2w exhibited the most significant reactivity in patients followed by Sol i 4w (Hoffman, 1993). The crystal structure of Sol i 2w was recently determined by Borer et al. (2012). The structure is an intramolecular disulfide-linked homodimer connecting two identical subunits, each composed of five helices that enclose a hydrophobic cavity and in stabilized by three intramolecular disulfide bridges. Borer et al. (2012) hypothesized that the natural function of Sol i 2w could be to act in capturing and/or transporting hydrophobic ligands, because Sol i 2w is structurally similar an odorant and pheromone binding protein, LUSH, from *Drosophila* fruit flies.

In this study queen venom proteins were identified, sequenced, and compared with allergens found in conspecific RIFA workers, as well as other previously identified *Solenopsis* spp. venom allergens. Once the queen venom protein sequences were determined, the best protocols for expression (using *Escherichia coli*) and purification were determined for each queen and homologous

worker venom allergen. Finally, once sufficient amounts of protein were obtained the differences in immuno-reactivity between *S. invicta* queen and worker castes were investigated. We hypothesized that the queen proteins would have a reduced immuno-reactivity than the worker allergens based on Baer et al.'s results (1979).

2. Materials and methods

2.1. Ants

Red imported fire ant colonies were collected as needed from Brazoria, Lubbock, and Travis Counties, Texas. The buckets were undisturbed for one day to allow the ants to reorganize their colony. Water was then dripped slowly into the buckets for 24 h which caused ants to form a living raft. The raft was scooped and transferred from water to plastic dish containers coated with Fluon™ (Northern Products) to prevent the ants from escaping. Ants were maintained at 29 °C, watered daily, and fed sugared cereal, cashews, and sausages.

2.2. Protein identification & sequencing

To screen the venom of alate and dealate queens for proteins, each sample was prepared in a chilled, sterile 2 ml microcentrifuge tube containing 47.5% Laemmli sample buffer, 47.5% chilled Phosphate Buffered Saline (PBS) and 5% β-mercaptoethanol. Queens were removed from colonies, immediately frozen and dissected under a dissection microscope using needles and microsheers cleaned with 90% isopropyl alcohol. For whole body rinses, the queens were rinsed in sample buffer but not homogenized. A BioRad Criterion™ PreCast Gel System (BioRad Inc.) was used to perform all Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analyses. Electrophoresis was performed for 85 min at 140 V. Upon completion, gels were soaked in chilled 3 mM CAPS buffer, pH 11, containing 10% methanol. A BioRad Criterion™ Blotter tank (BioRad Inc.) was used to electroblot the gels onto Sequi-Blot™ PVDF membrane (0.2 μm). Selected protein bands were sequenced from the N-terminus at the Texas Tech University Biotechnology Core Facility on a Porton Instruments 2020 sequencer with on-line Beckman Gold HPLC system and a Beckman 32 karat analysis system.

To obtain full-length cDNA sequences, RNA was extracted from both workers and queens. For each extraction, only a single queen abdomen or eight worker abdomens were used as starting material. Immediately prior to extracting RNA, alate queens and workers were individually flash frozen in liquid nitrogen and their abdomens were excised with microsheers. Abdomens were ground using a mortar and pestle cooled in liquid nitrogen. The samples were then placed in cooled microtubes and homogenized using a hand pestle homogenizer. Total RNA was extracted using a RNeasy® Micro Kit (Qiagen Inc.). Extracted RNA was added to 20 μl of DEPC water in microtubes and placed in a 67 °C water bath for 10 min to rid the RNA of tertiary structures. After heating, the following contents were added to each tube: 10 μl 5× MuMLV Buffer (Promega Inc.), 4 μl dNTPs (Takara, Otsu,

Shinga Inc., Japan) (2.5 μ M), 5.6 μ l oligo dT primer (Promega Inc.), 1 μ l Rnasin (Promega Inc.), and 4.4 μ l DEPC water to bring each tube to a total volume of 50 μ l. Each tube was mixed by pipetting and incubated at room temperature for 10 min. The tubes were then placed in ice. After 5 min, 2.5 μ l MuMLV-RT (Promega Inc.) was added to each tube, after which the tubes were placed at 37 °C for 1 h. For polymerase chain reaction (PCR), 5 μ l of each sample was added to a new microtube. Touchdown PCR was used to amplify the gene of interest. The primers used were based on the previously published Sol i 2w and Sol i 4.01w, and Sol i 4.02w allergen sequences (GenBank ID: P35775; GenBank ID: AAC97370; GenBank ID: AAC97369, respectively). Amplification was carried out in an Eppendorf Mastercycler Gradient in 2, 15 cycle increments: 30 s at 94 °C, 40 s at 59 °C, and 1 min at 72 °C for 15 cycles. The second set of 15 cycles was run at 15 s at 94 °C, 15 s at 50 °C and 1 min at 72 °C. The final polymerization was extended for 2 min. PCR products were analyzed by agarose gel electrophoresis and were ligated into the pGEM[®]-T Easy Vector System (Promega Inc.) and cloned in *E. coli* JM109 competent cells (Promega Inc.). For each transformation, plasmid DNA was isolated from three colonies positive for inserts and sequenced at the Texas Tech University Biotechnology Core Facility on a PE Biosystems 310 Genetic Analyzer.

2.3. Protein expression & purification

S. invicta allergen cDNA, without the leader sequence, were sub-cloned into pENTR[™]/D-TOPO[®] entry vector (Invitrogen) and then recombined into pEXP1-DEST vector expression vector (Invitrogen). Expression constructs were transformed in SHuffle[™] T7 Express *lysY* competent cells (New England BioLabs). SHuffle[™] T7 Express *lysY* competent cells were assessed because these cells are designed to promote disulfide bridge formation and misfolding. Cultures were grown overnight at 30 °C at 250 rpm. After 24 h, fresh media was inoculated with the overnight culture and grown for three hours at 30 °C at 250 rpm, based on SHuffle[™] T7 Express *lysY* growth curves to reach on OD₆₀₀ of ~0.3. After three hours the temperature was reduced to 16 °C and induced using 0.1 mM IPTG. The induced cultures were grown overnight. Venom protein was then chemically extracted using BugBuster[®] Protein Extraction Reagent and Lysonase[™] Bioprocessing Reagent (Novagen).

High-pressure liquid chromatography (HPLC) was performed using a POROS HS/M (strong cation exchanger – high density sulfopropyl) 4.6/100 column on a BioCAD Vision Workstation using BioCAD Perfusion Chromatography software, version 3.0.1 (PerSeptive BioSystems Inc.). HPLC was performed using 50 mM sodium acetate buffers (Hoffman et al., 1988). Using the method editor program the following method was developed, based on the isoelectric point of the allergens (worker 9.4 & queen 9.16): NaCl was increased rapidly from 0 M to 0.6 M over 1.5 column volumes (CV = 1.7 ml) (~1 min). NaCl was then raised slower from 0.6 M to 1.0 M, over 7CVs (~4.5 min) and maintained at 1.0 M for 2CVs (~2 min). Protein absorbance was monitored at 220 nm instead of 280 nm, because *S. invicta* venom proteins have low

amounts of phenylalanine, tyrosine, and tryptophan (Hoffman, 2010).

2.4. Conformation

Two different type assessments were performed to partially understand the conformation of the expressed venom proteins: 1) disulfide bond determination and 2) monoclonal antibody testing. Following Habeeb (1972), the Ellman's Reagent was prepared by adding 39.6 mg of 5,5'-dithiobis-(2-nitrobenzate) (DNTB) to 10 ml of 50 mM PBS, pH 7. Ellman's reagent is light sensitive, so it was prepared directly before use with the lights off. Absorbance was monitored at 412 nm on a Shimadzu Spectrophotometer UV-2401-PC using UV Prove version 1.1 Addendum software (Shimadzu Scientific Instruments Inc., Columbia MA). The spectrophotometer was blanked using 1 ml of PBS and then using 100 μ l of the Ellman's Reagent in 900 μ l of PBS. Directly following, an equivalent sample amount was removed and ~7 μ M RIFA venom gland protein was added to the cuvet (so if 20 μ l of sample needed to be added to have the correct amount of protein, then 20 μ l was removed was removed from the cuvet). The change in absorbance at 412 nm was observed for 20 min. Based on 7 μ M protein samples, a protein with one free thiol group should have an absorbance of 0.10 at 412 nm. Bovine serum albumin (BSA) was used as a positive control because BSA has 35 cysteines (34 in disulfide bonds and one free cysteine).

Expressed Sol i 2q, Sol i 2w, Sol i 4q, and Sol i 4.01w venom proteins, as well as native Sol i 2w and Sol i 4w proteins were tested against: 1) G260, 2) A4, 3) Mab4, and 4) Mab6 mouse IgG monoclonal antibodies to assess protein conformation. G260 is designed to confirm denatured Sol i 2 and 4 venom proteins (but is not conformation or species specific). A4 is conformation and species specific for Sol i 2. Mab4 and Mab6 confirms conformation in Sol i 4 venom proteins; where Mab4 cross-reacts with Sol i 2w, but Mab6 is Sol i 4w specific. For monoclonal antibody assays, 0.2 μ g of protein was used per dot. The strips were rocked in 1:3000 ascites fluid in Pierce Superblock-TBS (Thermo Fisher Scientific Inc., Rockford IL) for four hours. The strips were then washed three times in TBST while shaking. The strips were then rocked in 1:10,000 Anti-mouse IgG-alkaline phosphatase (Sigma-Aldrich, St. Louis MO) in Pierce Superblock-TBS for three hours. While shaking, the strips were washed three times in TBS and rinsed with water. The strip was then developed with BCIP/NBT. To stop the color development the strips were rinsed with water, then allowed to dry.

2.5. Immuno-reactivity

Immunoblots were accomplished on 0.45 μ m nitrocellulose (BioRad Inc.). The nitrocellulose was cut into strips and 1 μ l of each allergen (0.1–1 μ g) was spotted onto the nitrocellulose and allowed to dry. Human immuno-reactivity assays were tested for immunoglobulin E (IgE) using four treatments: two groups of sera from patients allergic to RIFAs (each group pooled from sera of five allergic patients), one group of sera pooled from patients allergic to yellow jacket venom and not to RIFA venom, and

a negative control (East Carolina University; UMCIRB#10-0102). The strips were rocked in 1:10 sera in Superblock-TBS (Pierce) overnight, washed three times in TBST while shaking, and then rocked in 1:5000 biotinylated Anti-human IgE (epsilon) (Kirkegaard and Perry Laboratories, Inc.) in Superblock-TBS overnight. The strips were washed three times in TBS and then were then rocked in 1:10,000 streptavidin-alkaline phosphatase (Vector Laboratories, Inc.) in Superblock-TBS for two hours. The strips were washed three times in TBS, rinsed with water, and then developed with BCIP/NBT. To stop the color development the strips were rinsed with water, then allowed to dry.

3. Results

3.1. Protein Identification & sequencing

A clear protein band was isolated at about 17 kDa in both the alate and dealate queen venom samples and the whole body rinses of alate and dealate queens (Fig. 1). A second clear band was isolated at about 28 kDa in the venom and whole body samples (Fig. 1). Partial N-terminus protein sequences were determined for both bands at 17 and 28 kDa size from the venom samples and the whole body rinse. Queen venom protein sequencing indicated the 17 kDa band was likely composed of more than one protein with two predominant protein sequences present. The more predominant of the two proteins showed 45% similarity to Sol i 4w within the first 20 residues. The less predominant sequence showed 45% similarity to Sol i 2w within the first 20 residues. The 28 kDa band showed 60% similarity to the first 15 residues of Sol i 2w and 92% similarity to the less predominant 17 kDa band.

Cloning of cDNA was used to determine cDNA and protein sequences for queen venom proteins similar to Sol i 2w (GenBank ID: P35775), and Sol i 4.01/Sol i 4.02w allergens (GenBank ID: AAC97370; GenBank ID: AAC97369) (Fig. 2). Sol i 2q was found to be 119 amino acids in length and Sol i 4q 117 amino acids in length. The queen venom protein (28 kDa) shares 74.8% amino acid identity with the Sol i 2w allergen; therefore, to differentiate between the

two proteins we called the queen venom protein Sol i 2q (GenBank ID: AAY32928) (Fig. 2). The queen venom protein (17 kDa) shares 95.7 and 97.4% identity with the previously published *S. invicta* worker allergen IV sequence; therefore, we called this queen venom protein Sol i 4q (GenBank ID: AAY32929) (Fig. 2).

When compared to the other published *Solenopsis* 2 and 4 venom proteins, only 30 out of 117 amino acids line up between the two protein types, illustrating that the two groups are distinct yet distantly related (Fig. 2). Another key find during this analysis was the conservation of the cysteines among all *Solenopsis* 2 and 4 venom proteins (Fig. 4).

The identity between all of the previously sequences *Solenopsis* 2 proteins ranged from 72% identity to 91%, the Sol 2 proteins were very diverse and on average had 78.5% identity among all Sol 2 venom proteins. Among the Sol 2 proteins, at 91%, the queen proteins (Sol i 2q and Sol g 2q (GenBank ID: AAY32926) had the most identity. Sol s 2w (GenBank ID: ABC58726) had the greatest divergence from Sol i 2w and Sol r 2w (GenBank ID: P35776), 72% and 73% respectively, yet showed a 90% and 93% identity with Sol g 2q and Sol i 2q, respectively. The identity between all of the previously sequences *Solenopsis* 4 proteins was high. The Sol i 4q is 96% and 97% similar to Sol i 4.01w and 4.02w, respectively. Interestingly, Sol g 4q (GenBank ID: AAY32927) is more similar to Sol i 4.01w (100%) than to either Sol g 4.01w and 4.02w (both 88%).

3.2. Protein expression & purification

Sol i 2q, Sol i 2w, Sol i 4q, and Sol i 4.01w were expressed using pEXP1-DEST in SHuffle™ T7 Express lysY *E. coli*. Expression was carried out at both 37 °C and 16 °C. Expression at 37 °C resulted in insoluble protein, where expression at 16 °C resulted in soluble protein. Growth patterns looked significantly different between non-induced and induced trials and between Sol i 2 (worker and queen) and Sol i 4 (worker and queen), where the Sol i 2 cultures grew much slower than the Sol i 4 protein cultures. An ANOVA test established that non-induced cultures were statistically similar at zero hours (starting point) ($F_{4,46} = 0.68$; $P = 0.608$) and differences after 24 h were significant ($F_{4,47} = 2.89$; $P = 0.034$). Tukey's multiple comparison test further established that the control (empty pEXP1-DEST vector) differed from the venom protein cultures, but the venom protein cultures did not differ from each other. For induced cultures, an ANOVA established that induced cultures were statistically similar at zero hours ($F_{4,49} = 1.28$; $P = 0.291$) and differences after 24 h were significant ($F_{4,49} = 23.06$; $P < 0.001$). Further, *t*-tests established that after 24 h the Sol i 2w and q were significantly different from Sol i 4w and q ($t_{35} = -4.46$; $P < 0.001$).

Venom proteins were purified using HPLC cation exchange. Venom proteins were eluted at ~1 M NaCl. The proteins were then concentrated using an iCon protein concentrators with a 9 K molecular weight cut-off (Thermo Scientific) and the buffer exchanged into 50 mM sodium acetate with 0 M NaCl (Fig. 3). Final protein yields resulted in mg amounts. Sol i 2 proteins were expressed as homodimers and Sol i 4 proteins as monomers (Fig. 3).

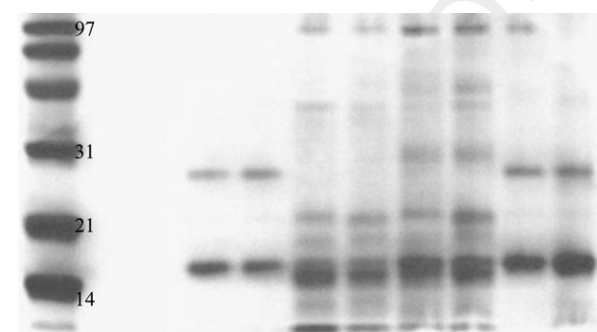


Fig. 1. SDS-PAGE analysis of protein extracts from alate and dealate queen segments (left to right): 1) protein standard (97, 66, 45, 31, 21, and 14 kDa), 2) blank, 3) control – PBS, 4) alate whole body, 5) dealate whole body, 6) alate head and thorax, 7) dealate head and thorax, 8) alate gaster, 9) dealate gaster, 10) alate venom, 11) dealate venom. Two bands were detected at ~28 kDa and ~17 kDa in both the venom and the whole body extracts.

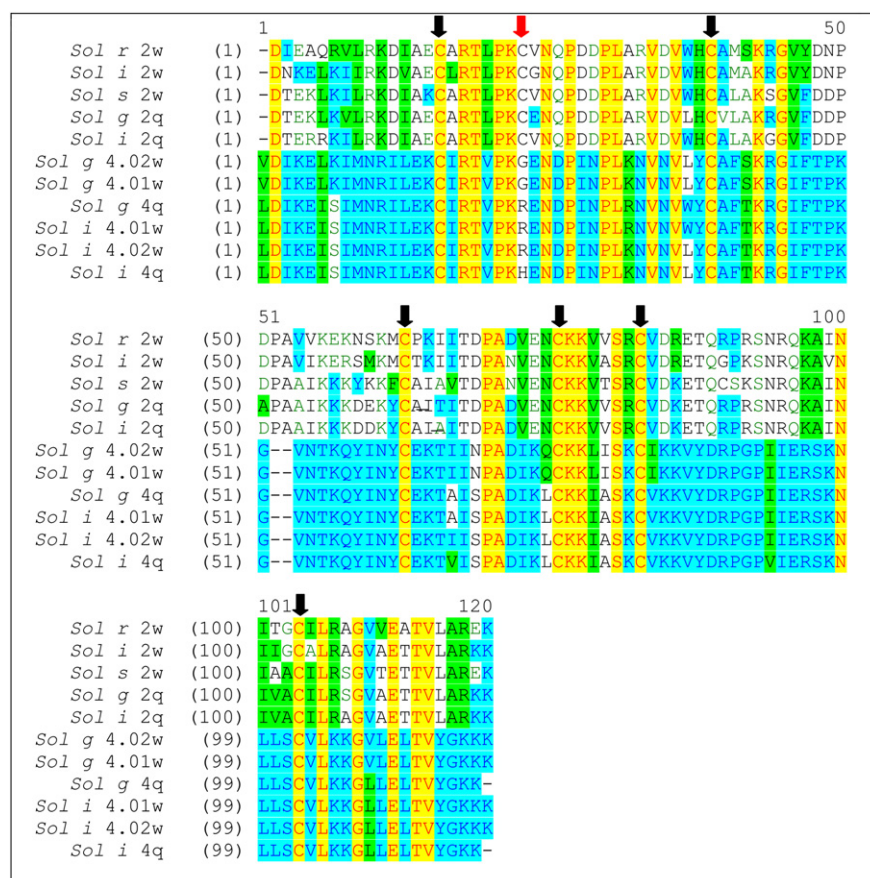


Fig. 2. Protein sequence alignment of *Solenopsis* 2 and 4 venom protein from *S. invicta*, *S. geminata*, *S. savessima*, and *S. richteri*: non-similar (black letters, no background), conservative (blue letters, blue background), block of similar (black letters, green background), identical (red letters, yellow background), weakly similar (green letters, no background). Note the alignment of the six cysteines (black arrows) between all Sol 2 and 4 genes and the 7th cysteine alignment in the Sol 2 genes (red arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Conformation

No detectable thiol groups were identified in the RIFA venom gland protein samples. Sol i 2q had a max absorbance (at 412 nm) of 0.009 at 11.5 min, Sol i 2w had an absorbance of 0.019 at 20.0 min, Sol i 4q had an absorbance of 0.023 at 20.0 min, and Sol i 4.01w had an absorbance of 0.027 at 20.0 min. All of the Sol 2 and 4 venom gland proteins were much less than the absorbance of 0.10 at 412 nm, based on 7 μ M protein samples, for a protein with

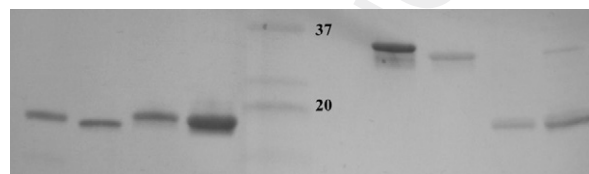


Fig. 3. Western blotting of tagged venom proteins (left to right): denatured (with β -mercaptoethanol) Sol i 2q (2q), Sol i 2w (2w), Sol i 4q (4q), and Sol i 4.01w (4w); protein standard (Std 37, 25, 20, and 15 kDa); non-denatured (without β -mercaptoethanol) Sol i 2q (2q), Sol i 2w (2w), Sol i 4q (4q), and Sol i 4w (4w). Venom proteins can be seen at ~20 kDa on the denatured side, where on the non-denatured Sol i 2q and w can be seen at ~37 kDa (as a dimer) and Sol i 4q and w at ~20 kDa.

one free thiol group. The BSA (control) had a max absorbance of 0.057 at 20 min, which was less than the 0.10 for a protein with one free thiol group, however absorbance curve followed the typical curve for thiol detection (Dr. Masakazu pers. comm.).

Native Sol i 2w and Sol i 4w and expressed Sol i 2q, Sol i 2w, Sol i 4q, and Sol i 4.01w venom proteins bound similarly to G260 confirming the presence of IFA venom protein; however, G260 is not conformation or species specific. Native and expressed Sol i 2w bound significantly to A4 monoclonal antibody, but the expressed Sol i 2q weakly bound and there was some binding of the Sol i 4.01w and Sol i 4q proteins. Native Sol i 4w and both expressed Sol i 4q and Sol i 4.01w bound to Mab4 and Mab6 monoclonal antibodies.

3.4. Immuno-reactivity

Sol i 2w, Sol i 4.01w, and Sol i 4q strongly bound to RIFA IgE, where Sol i 2q bound weakly (Fig. 4). One of the patient pools was more reactive than the other pool, and there is some background stickiness in the RIFAV-/YJV+ and negative strips. The background stickiness is probably

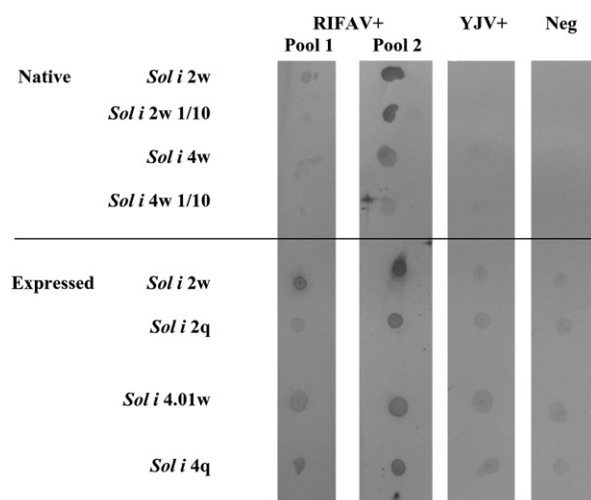


Fig. 4. Immuno blot of expressed and native venom protein tested against sera from patients allergic to: 1) RIFA venom (RIFAV+, two pools), 2) yellow jacket venom but not RIFA venom (YJV+), and 3) neither (Neg). Anti-human IgE antibodies were used to identify RIFA IgE binding in pooled sera. Expressed venom proteins reacted similarly to native venom proteins; however, the expressed tags make them stickier than the natural proteins (YJV+ and Neg strips). Sol i 2w binds strongly to RIFA IgE, where Sol i 2q binds weakly and binding was stronger in the Sol 2 venom proteins compared to the Sol 4 proteins.

a result of the expression tags and background protein in the partially purified samples.

4. Discussion

4.1. Protein Identification & sequencing

N-terminus sequence data suggests the 17 kDa band found in the queen venom is actually a mixture of two or more proteins (Fig. 1; Sol i 2q and Sol i 4q), and at least two of the proteins are similar to previously sequenced allergens found in fire ant worker venom (Sol i 2w and Sol i 4w). This finding is consistent with the fact that Sol i 2w is a disulfide-linked homodimer with a molecular weight of 28,234. The band at about 28 kDa with a sequence similar to Sol i 2w is Sol i 2q, where the 17 kDa band is the monomer version of Sol i 2q (Fig. 1). Sol i 4w is a monomer with a molecular weight about 13,340, although Sol i 4q was identified in the 17 kDa band (Fig. 1). However, it was interesting that no queen equivalents of Sol i 1 (37 kDa) and Sol i 3 (24 kDa) were not identified (Fig. 1).

Cloning of full-length cDNA from *S. invicta* queens allowed for the comparison of the Sol i 2 and Sol i 4 translated sequences between queens and the conspecific workers. Perhaps the most significant comparison was the 74.8% amino acid identity found between the Sol i 2w and Sol i 2q proteins (Fig. 2). The translated cDNA sequence obtained for Sol i 2q is identical to the first 13 residues of the N-terminus partial amino acid sequence obtained, confirming its validity. There is higher identity between the Sol i 4q sequences and the Sol i 4w sequence (Fig. 2). *Solenopsis* 4 venom proteins have been found to be highly conserved across species.

4.2. Protein expression & purification

Sol i 2q, Sol i 2w, Sol i 4q, and Sol i 4.01w venom proteins were expressed using pEXP1-DEST in SHuffle™ T7 Express *lysY E. coli*. Because of the lack of carbohydrates (Hoffman, 2010) and amount of cysteines and potential disulfide bridge the SHuffle™ T7 Express *lysY* were selected for optimal expression. These cells are engineered for disulfide bridge formation: oxidizing cytoplasm (allows for correct oxidative folding of disulfide proteins) and DsbC (a disulfide bridge isomerase that enhances the capacity to fold multi-disulfide bridged proteins correctly). Other *E. coli* cells (BL21 Star™ (DE3) pLysS One Shot® (Invitrogen) and BL21-AI™ One Shot® (Invitrogen)) were assessed and resulted in insoluble protein. Native proteins were purified using cation exchange; therefore, it was utilized as the purification method of expressed recombinant Sol i 2 allergens (Hoffman et al., 1988). Over 1 mg of partially purified venom protein was produced per 800 ml expression cultures. The availability of large amounts protein, will considerably help the future study of these extremely potent allergens.

4.3. Conformation

In this study we identified all seven cysteines as conserved within all of the *Solenopsis* 2 venom proteins and all six (same six of Sol i 2's seven cysteines) as conserved within the *Solenopsis* 4 venom proteins (Fig. 2). Using an Ellman's test, we identified the lack of free -SH groups in the expressed venom gland proteins, confirming that all of the cysteines in the expressed proteins were oxidized and are forming disulfide bridges internally. We further compared the expressed proteins to native proteins using monoclonal antibodies to assess if the conformation of the expressed protein as the same as native venom protein. Native and expressed Sol i 2w bound significantly to A4 monoclonal antibody confirming the correct conformation of the expressed Sol i 2w, but the expressed Sol i 2q weakly bound. Native Sol i 4w and both expressed Sol i 4q and Sol i 4.01w bound to Mab4 and Mab6 monoclonal antibodies confirming the correct conformation during protein expression.

Borer et al. (2012) determined that the overall crystal structure of Sol i 2w to be based stabilized with three intramolecular disulfide bridges with one intramolecular disulfide bridge creating a hydrophobic cavity. Because expressed proteins reacted similarly to native worker proteins in the immuno-reactivity and conformation assays, we would conclude that the expressed Sol i 2w, Sol i 4.01w, and Sol i 4q venom proteins have the same structural features as native proteins. However, we would conclude because of the lack of free -SH groups during the Ellman's test of Sol i 2q, that it forms similar hydrophobic cavities with a different overall structure. This could be true if the queen and worker venom proteins have evolved differently due to different ecological roles within the colony. Diverging protein structure would not uncommon in *Solenopsis* venom proteins, since *Solenopsis savessima*'s Sol s 2w protein has eight cysteines and could potentially have a different structure as well.

4.4. Immuno-reactivity

The results of the immunoblots, Sol i 2w, Sol i 4.01w, and Sol i 4q bound more strongly to RIFA IgE than Sol i 2q (Fig. 4), were as hypothesized and were consistent with Baer et al.'s (1979) results several patients allergic to fire ant stings reacted more to the worker venom than the queen venom with respect to Sol i 2q. Considering the 75% identity between Sol i 2q and Sol i 2w a difference in the immuno-reactivity could be due to a different conformation of the queen protein to that of the worker protein. Similarities in the Sol i 4 immuno-reactivity could be explained by the highly conserved 96/97% identity between Sol i 4.01w/Sol i 4.02w and Sol i 4q proteins.

5. Conclusion

The difference in allergic patients' reactions between queen and worker RIFA stings can be explained by the differences in the alkaloid and protein makeup of the venom. The alkaloid component is responsible for the burning sensation and blister (Chen and Shang, 2010), and the protein component is responsible for the allergic reaction Hoffman (1987). Whereas many different alkaloids comprise worker RIFA venom, there are only two main alkaloids in queen RIFA venom, (Brand et al., 1973; Chen and Shang, 2010), suggesting that the burning sensation and blister from a queen sting would differ substantially from that of a worker sting. Further, we hypothesize that allergic reactions to queen venom would be less than allergic reactions to worker venom based on protein differences between the queen and worker venom composition. The queen venom is already missing two (Sol i 1 and Sol i 3) venom proteins, where the Sol i 1 is cross-reactive between bee and wasp venoms (Hoffman et al., 1988). The loss of Sol i 1 and Sol i 3 coupled with the reduced identity of Sol i 2q, leaves most of the allergic response to Sol i 4q. The highly conserved identity of the Sol i 4q protein could explain why allergic patients still react to RIFA queen stings, but less significantly than worker stings.

Functional expression of these venom proteins in *E. coli* will save researchers money and time, as opposed to expression in *Baculovirus*-infected insect cells. This research determined a rapid and cheaper protocol for the expression of significant amounts of venom protein that could be used for allergenic testing of these venom proteins, and thus could reduce the cost of such testing procedures.

Chen and Shang (2010) stated that the molecular features of these proteins must be determined in order to understand why these venom gland proteins are so important. They further stated that sequence information aid in T- and B-cell determinants, which can be further used to regulate specific immune responses to the venom allergens by reducing specific IgG blocking antibody production (Hoffman, 1993). Based on the dramatic differences in protein sequencing and immuno-reactivity it appears that the conformation of Sol i 2q is different than that of Sol i 2w, Sol i 4.01w, and Sol i 4q despite conservation of the important disulfide bridge forming cysteines. Is the reduced immuno-reactivity activity of Sol i 2q a consequence of the

evolution of the function or conformation of the protein. One might expect such a course if the queen's proteins evolved into a pheromone that regulates colony development and that workers have a different role in nest dynamics as suggested by Klobuchar and Deslippe (2002). Venom contents of RIFAs are used and deposited in different ways depending on the caste. Queens deposit their venom gland contents on eggs, an action that serves two functions: (1) stimulation of brood care by workers and (2) prevention of contamination by microbes (Vander Meer and Morel, 1995). In contrast, workers primarily deposit their venom through stinging for defensive purposes, but also release venom into the air (a term defined as gaster flagging) for anti-microbial, brood care, and defensive purposes (Obin and Vander Meer, 1985; Glancey and Dickens, 1988; Storey et al., 1991). Perhaps these proteins are ligand transporter proteins as suggested by Borer et al. (2012), and Sol i 2q is responsible for transporting a different ligand based on the different uses of queen versus worker venom. Future studies need to assess whether the conformation of Sol i 2q is indeed different from that of Sol i 2w, Sol i 4w, and Sol i 4q, as well as the role of these venom proteins role as a pheromonal source or a transporter of ligand pheromones. Identification of a colony regulation pheromone could have a great impact on the control of these devastating insects.

Acknowledgements

We thank Michael San Francisco, Brian Sutton, and Mohamed Fokar for helpful discussions, Susan San Francisco for DNA and protein sequence analyses, and Hirasawa Masakazu for the Ellman's Test. This research was supported by the Department of Biological Sciences, Texas Tech University, as well as grants from the Association of Biologists at Texas Tech University, Texas Imported Fire Ant Research, and Management Program, and Norman Hackerman Advanced Research Program.

Conflict of interest

None.

References

- Baer, H., Liu, T.Y., Anderson, M.C., Blum, M., Schmid, W.H., James, F.J., 1979. Protein component of fire ant venom (*Solenopsis invicta*). Toxicon 17, 397–405.
- Blum, M.S., Fales, H.M., Leadbetter, G., Leonhardt, B.A., Duffield, R.M., 1992. A new dialkylpiperidine in the venom of the fire ant *Solenopsis invicta*. J. Nat. Toxins 1, 57–63.
- Borer, A.S., Wassmann, P., Schmidt, M., Hoffman, D.R., Zhou, J., Wright, C., Schirmer, T., Marković-Housley, Z., 2012. Crystal structure of Sol i 2: a major allergen from fire ant venom. J. Mol. Biol. 415, 635–648.
- Brand, J.M., Blum, M.S., Ross, H.H., 1973. Biochemical evolution in fire ant poisons. Insect Biochem. 3, 45–51.
- Chen, J., Shang, H., 2010. Advances in research on the venom chemistry of imported fire ants. In: Liu, T.X., Kang, L. (Eds.), Recent Advances in Entomological Research: From Molecular Biology to Integrated Pest Management. Higher Education Press, Beijing, China, pp. 251–260.
- Chen, J., Shang, H., Jin, X., 2010. Interspecific variation of $\Delta 1,6$ -piperideins in imported fire ants. Toxicon 55, 1181–1187.
- Drees, B.M., 2002. Medical problems and treatment considerations for the red imported fire ant. Fire Ant Plan Fact Sheet #023. Texas Imported Fire Ant Research and Management Project.

- Glancey, B.M., Dickens, J.C., 1988. Behavioral and electrophysiological studies with live larvae and larval rinses of the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). *J. Chem. Ecol.* 14, 463–473.
- Habeeb, A.F.S.A., 1972. Reaction of protein sulfhydryl groups with Ellman's reagent. *Method. Enzymol.* 25, 457–464.
- Hoffman, D.R., 1987. Allergens in Hymenoptera venom XVII: allergenic components of *Solenopsis invicta* (imported fire ant) venom. *J. Allergy Clin. Immunol.* 80, 300–306.
- Hoffman, D.R., 1993. Allergens in Hymenoptera poison XXIV: the amino acid sequences of imported fire ant poison allergens *Sol i* II, *Sol i* III, and *Sol i* IV. *J. Allergy Clin. Immunol.* 91, 71–78.
- Hoffman, D.R., 1995. Fire ant venom allergy. *Allergy* 50, 525–544.
- Hoffman, D.R., 2010. Ant venoms. *Curr. Opin. Allergy Clin. Immunol.* 10, 342–346.
- Hoffman, D.R., Dove, D.E., Jacobson, R.S., 1988. Allergens in Hymenoptera poison XX: isolation of four allergens from imported fire ant (*Solenopsis invicta*) poison. *J. Allergy Clin. Immunol.* 82, 818–827.
- Klobuchar, E.A., Deslippe, R.J., 2002. A queen pheromone induces workers to kill sexual larvae in colonies of the red imported fire ant (*Solenopsis invicta*). *Naturwissenschaften* 89, 302–304.
- Lockey, R.F., 1974. Systemic reactions to stinging ants. *J. Allergy Clin. Immunol.* 54, 132–146.
- Obin, M.S., Vander Meer, R.K., 1985. Gaster flagging by fire ants (*Solenopsis* spp.): functional significance of poison dispersal behavior. *J. Chem. Ecol.* 11, 1757–1768.
- Rhoades, R.B., 1977. Medical Aspects of the Imported Fire Ant. The University Presses of Florida, Gainesville, FL.
- Stafford, C.T., Hoffman, D.R., Rhoades, R.B., 1989a. Allergy to fire ants. *Southern Med. J.* 82, 1520–1527.
- Stafford, C.T., Hutto, L.S., Rhoades, R.B., Thompson, W.O., Impson, L.K., 1989b. Imported fire ant as a health hazard. *Sothorn Med. J.* 82, 1515–1519.
- Storey, G.K., Vander Meer, R.K., Boucias, D.G., McCoy, C.W., 1991. Effect of fire ant (*Solenopsis invicta*) in vitro germination and development of selected entomogenous fungi. *J. Invertebr. Pathol.* 58, 88–95.
- Tankersley, M.S., 2008. The stinging impact of the imported fire ant. *Curr. Opin. Allergy Clin. Immunol.* 8, 354–359.
- Tschinkel, W.R., 2006. The Fire Ants. The Belknap Press of Harvard University, Cambridge.
- Vander Meer, R.K., Morel, L., 1995. Ant queens deposit pheromones and antimicrobial agents on eggs. *Naturwissenschaften* 82, 93–95.
- Yaeger, W., 1978. Frequency of imported fire ant stinging in Lowndes County, Georgia. *J. Med. Assoc. Ga.* 67, 101–102.