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Crystal Structure of the Major Allergen from Fire Ant Venom, Sol i 3

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Fire ant venom is an extremely potent allergy-inducing agent containing four major allergens, Sol i 1 to Sol i 4, which are the most frequent cause of hypersensitivity reactions to hymenoptera in the southern USA. The crystal structure of recombinant (*Baculovirus*) major fire ant allergen Sol i 3 has been determined to a resolution of 3.1 Å by the method of molecular replacement. The secondary-structure elements of Sol i 3 are arranged in an α - β - α sandwich fold consisting of a central antiparallel β -sheet surrounded on both sides by α helices. The overall structure is very similar to that of the homologous wasp venom allergen Ves v 5 with major differences occurring in the solvent-exposed loop regions that contain amino acid insertions. Consequently, the limited conservation of surface chemical properties and topology between Sol i 3 and Ves v 5 may explain the observed lack of relevant cross-reactivity. It is concluded that Sol i 3 recognizes immunoglobulin E antibodies with a distinct set of its own epitopes, which are different from those of Ves v 5. Indeed, the molecular area in Sol i 3 covered by non-conserved residues is large enough to accommodate four unique Sol i 3 epitopes.

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Introduction

The venom of fire ants of the genus *Solenopsis* is a major cause of serious anaphylactic reactions in the southern United States and many areas in Latin America as well as in areas of the Pacific and south-east Asia where they have been introduced.¹ The red imported fire ant, *Solenopsis invicta*, is the dominant species in North America. The venom of fire ants is composed primarily of water-insoluble piperidine alkaloids.² These alkaloids cause the characteristic hives and sterile pustules.³ Immunoglobulin E (IgE)-mediated allergic reactions result from a sensitization to nanogram amounts of water-soluble protein allergens, Sol i 1, 2, 3, and 4.⁴ The primary structures of these protein allergens have been determined^{5–7} and Sol i 2 and 3 have been expressed as recombinant proteins in native form using a *Baculovirus* expres-

sion system.^{8,9} Sol i 1 is a phospholipase A₁B and shows some cross-reactivity with phospholipases from wasp venoms^{7,10}. Sol i 2 is the most abundant protein in the venom and is a member of a protein family not found in other insect venoms along with Sol i 4⁵. Sol i 3 is a member of the antigen 5 (Ag5) family with an unknown biological role. Immunotherapy with insect venoms is highly successful in preventing future anaphylactic reactions.¹¹ Since fire ant venom is not commercially available, immunotherapy for preventing anaphylactic reactions induced by fire ant venom is performed using whole-body extracts, some of which may be deficient in allergen content.¹² Although pure venom is known to be more specific and potent,¹³ its collection is laborious and expensive.⁴ It is also very difficult to prepare natural venom that is free from alkaloids. The use of recombinant allergens may be a practical method of overcoming the problems related to natural venom.¹⁴

Sol i 3 belongs to large family of eukaryotic extracellular proteins that have been shown to be evolutionary related. Proteins belonging to this family are pathogenesis-related (PR) proteins of plants (PR-1), synthesized during pathogen infection or other

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Abbreviations used: IgE, immunoglobulin E; Ag5, antigen 5; PR, pathogenesis related; CE, conformational epitope; SE, sequential epitope; His₆, hexahistidine.

stress-related responses, and include rodent sperm-coating glycoprotein, which is thought to be involved in sperm maturation; mammalian testis-specific protein (Tpx-1); mammalian cysteine-rich secretory proteins; glioma pathogenesis-related protein; reptile toxins, Stecrisp from snake venom (*Trimeresurus stejnegeri*); SP-2 secretory proteins from nematode parasites (*Necator americanus*) such as hookworms; and insect venom allergens.¹⁵ Cysteine-rich secretory proteins comprise three domains: an N-terminal PR domain, a hinge region, and a C-terminal cysteine-rich domain. Cysteine-rich domain is found in mammalian proteins but is absent in insect, plant, and fungal proteins. These allergens from insect venoms, including Ag5 allergens from wasp (Ves v 5) and fire ant (Sol i 3), belong to the PR-1 protein family.¹⁶ Allergens from the plant PR-1 family include Bermuda grass pollen allergen Cyn d 24, the mugwort pollen allergen Art v 2, and the muskmelon allergen Cuc m 3.

The Ag5 proteins from various *Vespula* species are highly cross-reactive and share a high level of sequence identity (about 95%), whilst homologous Ag5 proteins from *Dolichovespula* and *Polistes* have a 58–67% sequence homology and display only partial cross-reactivity within the Vespidae family.¹⁷ It has been suggested that the conserved surface patches of the yellow jacket Ves v 5 and homologous allergens in various yellow jackets, hornets and paper wasps are involved in their antigenic cross-reactivity.¹⁵ The cross-reactivity between fire ant and yellow jacket venoms is primarily from the phospholipase and cross-reactive carbohydrate determinants.^{7,10} However, none of the patients studied have shown IgE cross-reactivity between Ves v 5 and Sol i 3,^{5,10} which share 44% sequence identity. The mature Sol i 3 molecule consists of 212 amino acids with a molecular mass of 23,968 Da with three potential N-linked glycosylation sites and four disulfide bonds.^{5,9} The natural molecule does not show any glycosylation, but some of the *Baculovirus*-produced recombinant proteins are glycosylated at Asn124.⁹

The structure of an antigen determines its epitopes that are recognized by antigen-specific IgE antibodies of a susceptible patient during the type I allergic reaction. Only a few three-dimensional structures of allergens from an insect venom are known to date: the X-ray structure of the major bee venom allergens, phospholipase A2,¹⁸ melittin,¹⁹ hyaluronidase,²⁰ and the hyaluronidase in complex with specific IgG Fab.²¹ The crystal structure of Ag5 from *Vespula vulgaris* has been determined¹⁵ and a three-dimensional model of Sol i 3 has been predicted by computer modeling.⁹ Here we report the crystal structure of a major allergen of fire ant venom, Sol i 3, and compare it to that of Ves v 5. The analysis of the surface properties of the two allergens showed that the conserved surface patches are too small to constitute a common epitope, in agreement with the very low cross-reactivity among the two species. Yet, the non-conserved surface area could accommodate few surface patches that are large enough to represent a few epitopes unique for Sol i 3.

Results and Discussion

Structure determination

The three-dimensional structure of the recombinant major allergen from fire ant venom, Sol i 3, containing hexahistidine (His₆) tag at the C-terminus, was determined by X-ray crystallography to a resolution of 3.05 Å (Table 1). The protein crystallizes in the space group *P*6₁ with two monomers in the asymmetric unit. The structure was solved by molecular replacement using the structure of Ves v 5, a major allergen of yellow jacket [Protein Data Bank (PDB) code 1QNX],²² as the search model yielding orientation and location of the two copies in the asymmetric unit. Refinement with strong non-crystallographic symmetry constraints yielded the final model, which consists of two monomers composed of residues 2–212 plus the His₆ tail at the C-terminus and has an *R* value of 20.1% and an *R*_{free} value of 23.9%. The N- and C-termini of chain A have been unambiguously defined by electron density and are components of α1 helix and β6 strand, respectively. Strong electron density is observed for six C-terminal histidine resi-

Table 1. Crystallographic data for Sol i 3, a major fire ant venom allergen

<i>Data collection</i>	
Space group	<i>P</i> 6 ₁
Unit cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	93.3, 93.3, 144.0
α, β, γ (°)	90.0, 90.0, 120.0
X-ray source	SLS-PX
Detector type	MAR CCD
Wavelength (Å)	0.978
Resolution range (Å)	80.8–3.05 (3.21 – –3.05) ^a
No. of total observation	35,130 (4835)
No. of unique observation	13,121 (1919)
Completeness (%)	96.5 (96.6)
Multiplicity	2.7 (2.5)
<i>I</i> /σ(<i>I</i>)	6.1 (1.8)
<i>R</i> _{sym} ^b (%)	9.8 (40.1)
<i>Refinement</i>	
<i>R</i> _{factor} / <i>R</i> _{free} ^c (%)	20.1/23.9
Protein atoms	3235
Average <i>B</i> factor (Å ²)	
Protein all atoms	47.9
rms Δ <i>B</i> of bonded atoms (Å ²)	
Main chain	0.692
Side chain	1.559
rmsd from ideal values	
Bond lengths (Å)	0.008
Bond angles (°)	1.016
Ramachandran plot	
Most favoured region (%)	88.8
Additionally allowed region(%)	10.1
Generously allowed region (%)	0.8
Disallowed region (%)	0.3

^a Numbers in parentheses are statistics for the highest-resolution shell.

^b $R_{\text{sym}} = \sum \sum |I(h)_i - \langle I(h) \rangle| / \sum \sum I(h)_i$, observed intensity in the *i*th data set and $\langle I(h) \rangle$, mean intensity of reflection *h* over all measurements of *I*(*h*).

^c *R* factor is the conventional *R* factor and *R*_{free} is the *R* factor calculated with 5% of the data that were not used in refinement.

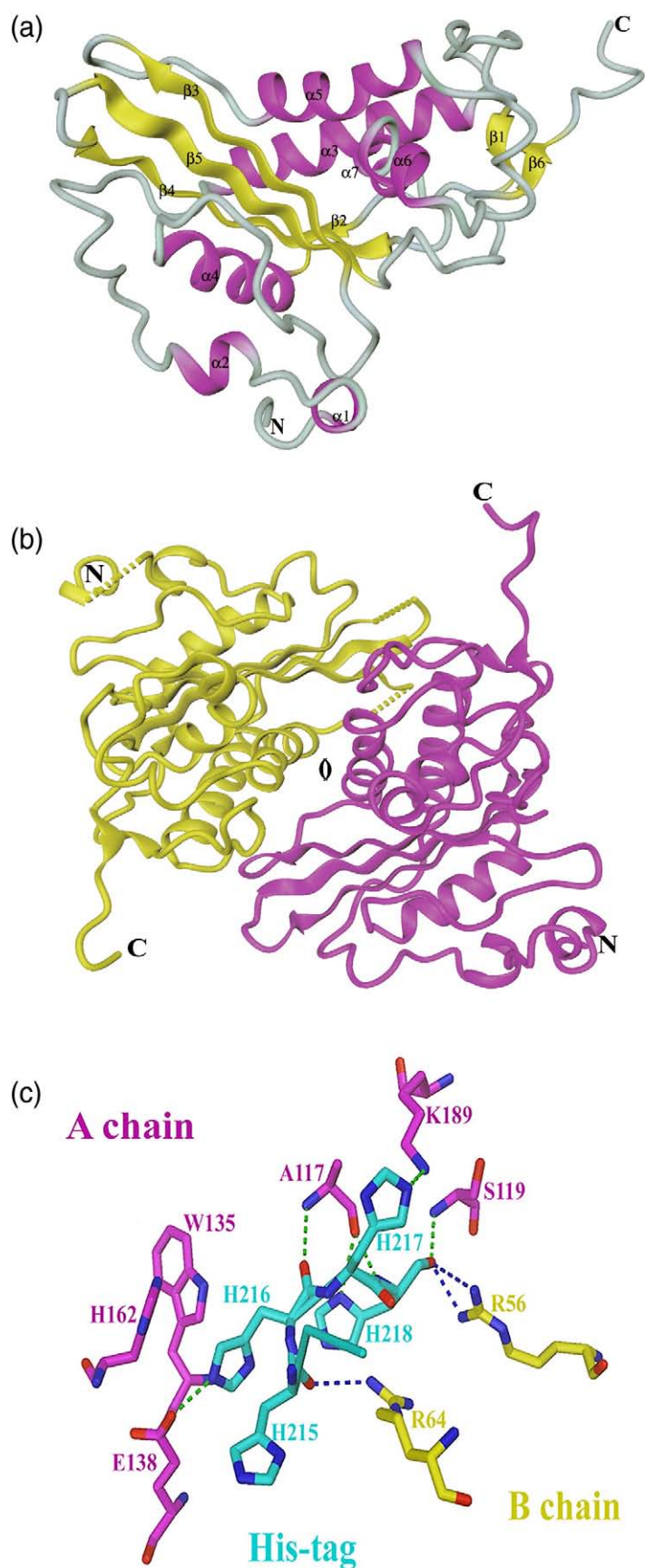


Fig. 1. The crystal structure of Sol i 3, a major allergen from fire ant venom. (a) Overall structure, represented by a ribbon diagram, comprises seven helices ($\alpha 1$ – $\alpha 7$) and six beta strands ($\beta 1$ – $\beta 6$), which are arranged as three stacked layers giving rise to an α – β – α sandwich. (b) The two chains that form a local twofold dimer are held together by isologous contacts involving residues from helix $\alpha 5$. The dashed lines indicate disulfide bridges and the polypeptide termini are labelled with N and C. (c) Interaction of the His-tag residues shown as a stick model (cyan) with the residues from chain A (magenta) and B (yellow) of the Sol i 3 dimer.

dues (H213–H218) in chain A. Electron density is missing for residues 1 and 123 in chain A and for the residues 1, 5–6, 9–13, 122–124, 182–185, and 217–218

in chain B. Both chains are very similar and superimpose with an rmsd of 0.47 Å for all C α positions. The following discussion is restricted to chain A.

The overall structure of Sol i 3 and comparison to the structure of Ves v 5

The overall structure of Sol i 3 monomer (Fig. 1a) consists of seven helices $\alpha 1$ (7–10), $\alpha 2$ (17–20), $\alpha 3$ (40–58), $\alpha 4$ (83–94), $\alpha 5$ (128–140), $\alpha 6$ (155–158), $\alpha 7$ (162–166) and six strands $\beta 1$ (72–74), $\beta 2$ (80–82), $\beta 3$ (112–120), $\beta 4$ (172–183), $\beta 5$ (187–197) and $\beta 6$ (209–211). The two chains that form a local twofold dimer are held together by isologous contacts involving residues from the helix $\alpha 5$ (Fig. 1b). Interestingly, the His₆ tag of one Sol i 3 dimer interacts with the residues from the interface of neighboring symmetry related dimer (Fig. 1c). The secondary structure elements of a Sol i 3 are arranged in an α - β - α sandwich consisting of central β -sheet formed by antiparallel strands $\beta 3$ – $\beta 5$ – $\beta 4$, surrounded, on one side, by helices $\alpha 1$, $\alpha 2$ and $\alpha 4$ and on the other by helices $\alpha 3$, $\alpha 5$, $\alpha 6$, $\alpha 7$ (Fig. 1a). The strands $\beta 3$ and $\beta 4$ form the edges of an antiparallel β -sheet. The

secondary structure elements are connected by 11 loops that comprise 49% of the entire 212 amino acids of Sol i 3. Only one loop is β -hairpin, connecting the consecutive β -strands 4 and 5 of the β -sheet. Sol i 3 contains 4 disulfide bridges (Fig. 1b) that stabilize its structure: C4–C19, C30–C96, C176–C194 whereby the disulfide bridge C9–C103 is somewhat distorted.

The overall structures of Sol i 3 and Ves v 5 (44% sequence identity) are very similar (Fig. 2a) and could be superimposed with an rmsd of 1.0 Å for 187 C α atoms. Major structural differences are observed in the loop regions where structural alignment (Fig. 2a) necessitated the insertion of several residues in the following Ves v 5 loops:¹⁵ α III– α IV (4 residues), β C– β D (1 residue), β B– α III (1 residue) and 2 residues were inserted in the N-terminal part of Ves v 5 between helices I' and I'' (Fig. 2b). In Sol i 3, a single residue insertion is found in the loop $\beta 3$ – $\alpha 5$. Loops containing insertions were excluded from the

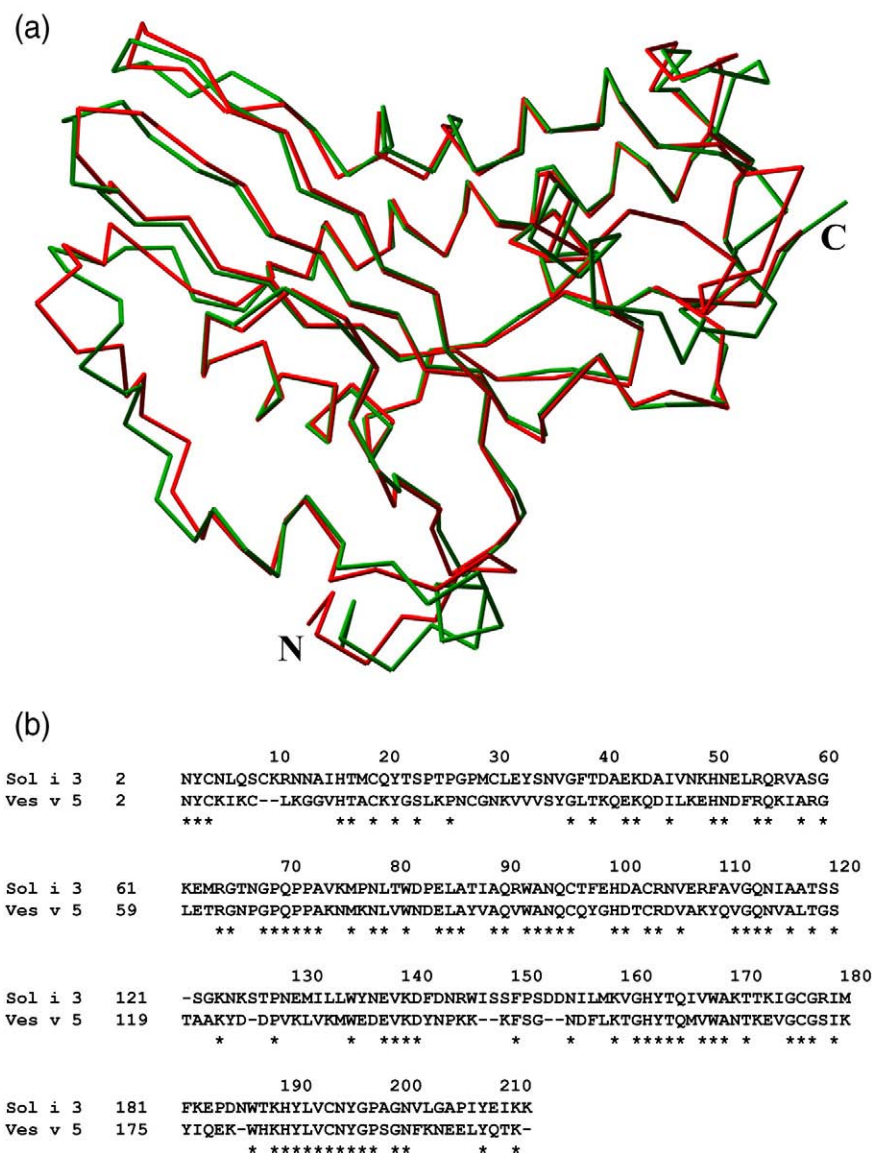


Fig. 2. (a) Superposition of carbon C α chain (PR-1 domain) of Sol i 3 (green) and Ves v 5 (red, PDB code 1QNX), a major allergen from yellow jacket venom. (b) Structure-based sequence alignment of Sol i 3 and Ves v 5.

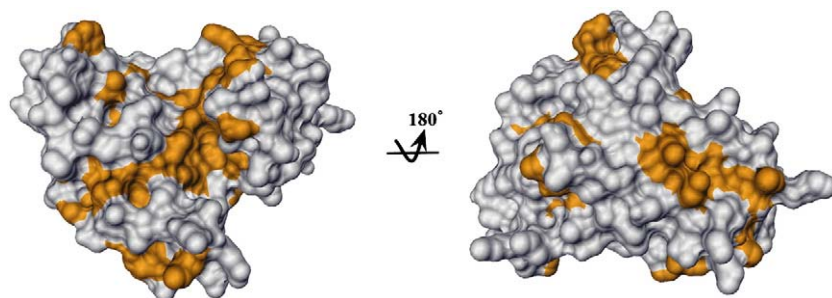


Fig. 3. Solvent-accessible surface of Sol i 3. Brown colour denotes amino acids identical with Ves v 5, whereas non-identical residues are shown in gray. The molecular view in the top left is as in Fig. 1a.

structural superposition and the calculation of the rmsd. Further differences, revealed by comparison of Sol i 3 and Ves v 5, are: (i) Sol i 3 has a seven-residue extension at the C-terminus comprising residues G204–K211 (Fig. 2b), (ii) a central β -sheet of Ves v 5 is composed of four β -strands A, B, C, D¹⁵ as compared to three beta strands in Sol i 3 because the strand A (30–35) of Ves v 5 corresponds to the loop region in Sol i 3, (iii) helix α 6 of Sol i 3 corresponds to the loop region in Ves v 5, and (iv) β -strand A of Ves v 5 correspond to the loop region in Sol i 3.

Structural basis of the weak cross-reactivity between Sol i 3 and Ves v 5

Patients allergic to insect venom frequently display sensitivity to various venoms, indicating that sensitization to one venom may lead to sensitivity to multiple insect venoms. Significant cross-reactivity has been observed between Ag5 proteins from various *Vespula* species that share a high level of sequence identity (about 95%), whereas there is only very weak human IgE cross-reactivity between Ag5 allergens from *Solenopsis* (Sol i 3) and yellow jacket (Ves v 5) despite considerable sequence identity (44%).^{5,10} It has been suggested that the dominating IgE-binding epitopes are preferentially located in the

surface areas conserved among the species and are responsible for the cross-reactivity.^{15,22,23} Although a high level of sequence homology may lead to allergen cross-reactivity, as in tree pollens^{22,23} and lipid transfer proteins,²⁴ in some cases high level of homology does not cause allergen cross-reactivity, as shown for birch and carrot cyclophilins.²⁵

Mapping of the Sol i 3 surface with residues fully conserved between Sol i 3 and Ves v 5 revealed a few conserved surface patches (Fig. 3) that are relatively small and cover areas smaller than the average surface area of 600–900 Å², which is usually occupied by an epitope, as revealed by structural studies of antigen–antibody complexes.²⁶ Thus, a highly similar protein fold and sequence identity of 44% are not sufficient to provide the basis for cross-reactivity between Sol i 3 and Ves v 5. The protein allergenicity is determined by the side-chain properties of the exposed amino acids that define the antigen surface that is recognized by Abs. Thus, the chemical character of the amino acid side chains together with the constraints imposed by protein folding determine the surface properties, such as electrostatic potential, hydrophobicity, and hydrogen-bonding potential, which are determinants of antigen recognition by Abs. Figure 4 shows that Sol i 3 and Ves v 5 have significantly different chemical

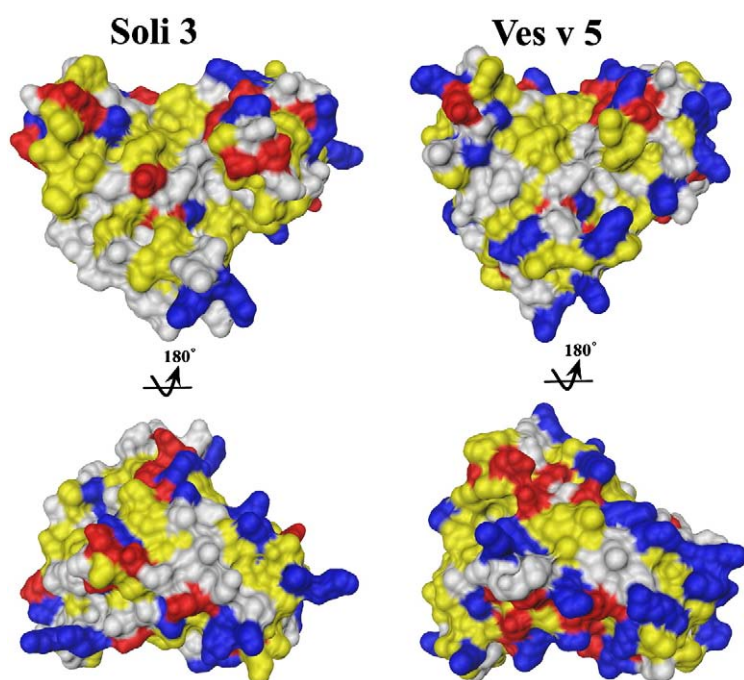


Fig. 4. Molecular surfaces of Sol i 3 and Ves v 5. The surface is colour coded according to residue type with red and blue representing acidic and basic residues, respectively; the yellow colour represents aliphatic and aromatic residues. The molecular view at the top row is as in Fig. 1a. In the bottom row, the molecule has been rotated by 180° along the horizontal axis with respect to the view in the top row.

properties of surface residues. This, together with the different surface topology that reflects the different conformations of the solvent-exposed loops of the two allergens, may explain the minor, or absent, cross-reactivity between Sol i 3 and Ves v 5. It is most likely that Sol i 3 recognizes IgE antibodies with a distinct set of its own epitopes that are different from those of Ves v 5. Indeed, the molecular area in Sol i 3 covered by non-conserved residues is large enough to accommodate four unique Sol i 3 epitopes, as shown in Fig. 5. The surface patches, coloured blue, green, red, and yellow, occupy an area of 715, 1172, 1503, and 1604 Å² whereby each of these surfaces, or part of them, may represent a distinct Sol i 3 epitope.

In an attempt to predict the B-cell epitopes of Sol i 3, we have submitted coordinates of Sol i 3 to the CEP server, which first predicts antigenic determinants and, based on this, the conformational (CE) and sequential (SE) B-cell epitopes.²⁷ CEP algorithms predicted a surface area of exposed antigen residues that define the protein interface interacting with the antibody (epitope) using the knowledge of the 3-D structure of an antigen and the accessibility of amino acids in an explicit manner. CEP predicted 10 antigenic determinants as well as four CEs (CE1 to CE4) and four SEs (SE1 to SE4) that do not overlap. Two distinct surface patches are defined by CEs. The larger surface patch is defined by three overlapping CEs (CE1, CE2, and CE4) that include three-residue segments P28–V36, T118–E130, and K182–K189; this epitope coincides with a larger part of the green-coloured epitope shown in Fig. 5. The smaller surface patch (CE3) is defined by the residues' segments S152–K159 and V202–P206 that coincide with part of the red-coloured epitope in the C-terminal part of Sol i 3 (Fig. 5). The conformation of each of the CEP-predicted Sol i 3 segments is significantly different from those in Ves v 5; in addition, most of the involved residues are not conserved, suggesting that the predicted epitopes are unique for Sol i 3.

Immunologic results from previously reported studies

The structure presented here and the epitope prediction are consistent with results of previously reported immunological studies. Two types of mouse monoclonal antibodies reacting with Sol i 3 were produced.¹² The first type was reactive with the Sol 3 antigens from the related species *Solenopsis richteri*, but was not reactive with Ag5 from vespid wasp. The second type of monoclonal antibody (AMS32), raised with Ribi adjuvant, reacted only with Sol i 3 and not Sol r 3. Since the amino acid sequence of Sol i 3 and Sol r 3 differ only in the C-terminal part of the molecule it has been concluded that AMS32 is directed towards the C-terminus of Sol i 3.⁹ Indeed, AMS32 did not react with recombinant Sol i 3 containing a C-terminal His₆ tag, but did react with r-Sol i 3 without the tag. AMS32 did not react with reduced Sol i 3. The specificity of AMS32 appears to correspond to the red and/or green epitopes illustrated in Fig. 5.

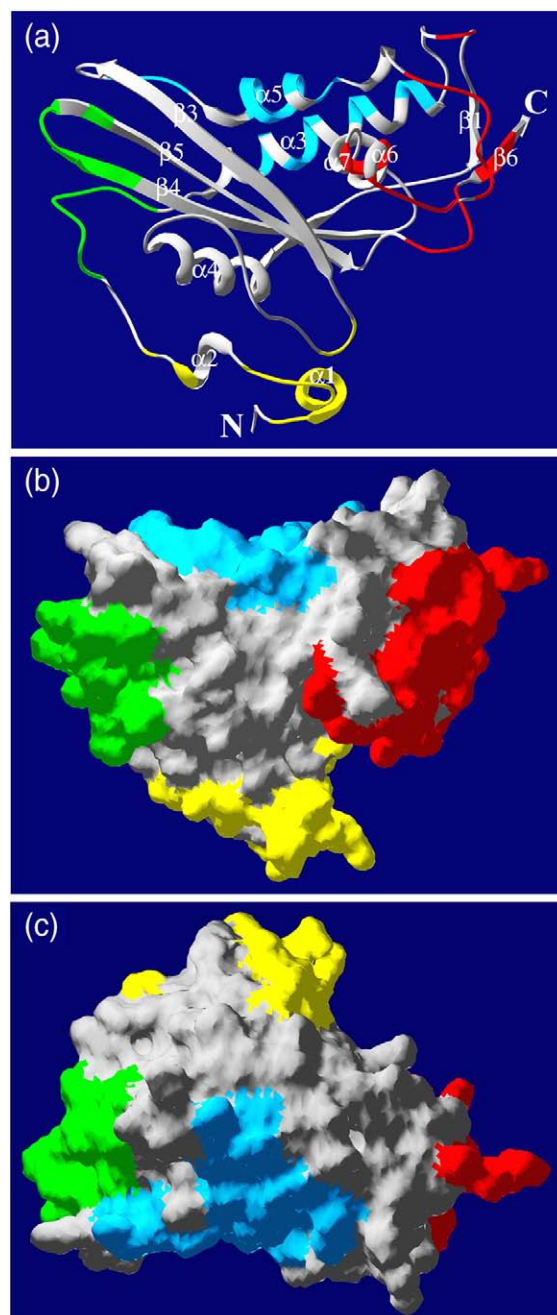


Fig. 5. (a) Ribbon presentation of the Sol i 3. Residues belonging to one of the four unique non-conserved CEs of Sol i 3 are yellow (5–15, 20, 22, 107–110), green (27–36, 180–186, 188), blue (44–45, 47–49, 52–53, 56–57, 124–127, 129–134, 136–137), and red (66–67, 142–149, 151–154, 156–158, 202–207, 209, 212). All amino acids constituting the proposed epitopes, except K111 and F150, are not conserved with respective residues of Ves v 5. (b) Surface presentation of Sol i 3 with the same viewing direction as (a). The coloured surface patches define four non-conserved epitopes characteristic for Sol i 3 (side-chain and backbone atoms of respective residues are coloured). (c) The rear view of the Sol i 3 surface, i.e., molecular orientation, in (b) has been rotated by 180° along the horizontal axis.

Rabbit antibodies were raised using Freund's complete adjuvant against purified antigen Ag5 from *Vespula squamosa*, *Dolichovespula maculate*, and *Polistes exclamans*. All of the antisera demonstrated cross-reactivity with vespid Ag5 from other species.²⁸ None were reactive with Sol i 3 in precipitin tests. One antiserum, C3, produced by immunization with Ves s 5, was tested by immunoblot analysis against a panel of Ag5-related molecules. It bound strongly to Ves s 5, Ves m 5, and Dol m 5, moderately to Pol e 5, but not to Sol i 3. Interestingly, the antibody C3 bound to secretory protein 2 produced by helminth *Ancylostoma caninum* strongly enough to be useful in following the purification of the antigen.²⁹ These results are consistent with the results of human IgE antibody testing that demonstrated no significant cross-reactivity between *Vespula* Ag5 and Sol i 3.¹⁰

Conclusions

The structure of Sol i 3 presented here confirms the folding pattern established by the structure of the major allergen of yellow jacket venom, Ves v 5,¹⁵ with major differences occurring in the surface exposed loops. The limited conservation of surface chemical properties and topology between Sol i 3 and Ves v 5 may explain the lack of relevant cross-reactivity revealed by immunochemical²⁸ and serological studies with human IgE antibodies.¹⁰ Four contiguous surface patches, composed of residues non-conserved between Sol i 3 and Ves v 5, are proposed to be epitopes unique for Sol i 3. Two of the four epitopes, coloured red and green, correspond to the specificity of AMS32 antibody that is directed to the C-terminal part of Sol i 3.⁹ Further structural, immunological, and clinical studies are required to validate the proposed B-cell epitopes of Sol i 3.

Materials and Methods

Expression and purification of recombinant Sol i 3

The recombinant virus containing Sol i 3 cDNA described previously⁹ was used for mass production in insect cells Sf9. Approximately 5 l of insect cell culture was inoculated with high-titer viral stock (80 ml/l) and the supernatant was harvested 5 days post-transfection. The supernatant was dialysed, concentrated, and purified with Talon[®] metal resin followed by cation-exchange chromatography.⁹

Crystallization, data collection, and structure determination

The rodlike crystals were grown by the hanging-drop vapor-diffusion method at 20 °C in 7 days under the following condition: the solution of Sol i 3 in milli-Q water (9.9 mg/ml) was mixed with reservoir solution [19% (w/v) polyethylene glycol 1000, 100 mM Tris-HCl, pH 8.5] at a ratio of 1:1. Crystals were cryoprotected with 23% polyethylene glycol 1000, 100 mM Tris, pH 8.5, and 15% glycerol prior to data collection. Diffraction data were collected to 3.05 Å from a single crystal at the Swiss Light Source with a MAR CCD detector ($\lambda = 0.9762$ Å). All

measurements were performed at 100 K. The images were indexed and integrated in space group $P6_1$ with the program MOSFLM.³⁰ There are two monomers of Sol i 3 per asymmetric unit, resulting in a solvent content of 70% ($V_m = 4.2$ Å³). The Sol i 3 structure was solved by molecular replacement with the program PHASER³¹ using the crystal structure of Ves v 5 from yellow jacket (PDB code 1QNX) as a search model. Manual adjustment of the model and replacement of the model amino acid sequence with that of Sol i 3 was performed with program O.³² Refinement was performed using the program REFMAC³³ and a few water molecules were added with program ARP. The final model includes two independent full-length molecules of Sol i 3 (residues N2–K112) and had an R/R_{free} value of 20.1%/23.9%. Data statistics are given in Table 1. The stereochemistry of the refined structure was validated with the program PROCHECK,³⁴ which showed that 88.8% of residues were within the most favoured region of the Ramachandran plot. The program SPDBV was used for structure superposition³⁵.

Protein Data Bank accession numbers

Coordinates and structure factors have been deposited in the PDB with accession numbers 2vzn and r2vznsf, respectively.

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