

Allergens in *Hymenoptera* venom

XX. Isolation of four allergens from imported fire ant (*Solenopsis invicta*) venom

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Commercial *Solenopsis invicta* (Sol i) venom was fractionated by gel filtration and high-performance cation exchange chromatography. Four proteins were isolated and purified to homogeneity. The four proteins were tested with a panel of sera from patients allergic to fire ant venom; all proteins had significant allergenic activity. These proteins corresponded to four of the bands we previously reported to be allergenic by immunoblot analysis. Sol i I has an apparent molecular weight of 37,000 daltons and yields bands of 18,000, 16,500 and 14,000 daltons in sodium dodecyl sulfate-polyacrylamide gel electrophoresis; cation-exchange chromatography indicates that there are three charge forms. Sol i II has a native molecular weight of 28,000 daltons and appears to be easily cleaved into half molecules; it is a phospholipase structurally unlike either bee or wasp phospholipases. Sol i III has a native and denatured molecular weight of 26,000 daltons. Sol i IV has an apparent native molecular weight of 20,000 daltons and gives a single chain of 15,000 daltons in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sol i II and III are the major proteins in the venom; there are only small amounts of Sol i I and IV. All are significant allergens, and patients are found who react most strongly with each. Regression analysis of RAST data with highly purified allergens indicated that the IgE responses to the allergens were not related to each other. Amino acid compositions indicated that the four allergens were distinct and that the allergens were structurally different from each other. Four proteins identical to Sol i I to IV were isolated from hand-milked pure venom. (J ALLERGY CLIN IMMUNOL 1988;82:818-27.)

Allergic reactions to stings of the imported fire ant, *Solenopsis invicta* and *S. richterii*, are the most common form of venom allergy in much of the southeastern United States.¹ Imported fire ants were introduced accidentally into North America and the dominant species, *Sol i*, has been spreading northward and westward. The ants are relatively resistant to pesticides and their large mounds are a serious problem to agriculture. Imported fire ants are aggressive and will sting almost any animal en masse. The imported fire ant is also a major problem in urban areas including New Orleans, Tampa, and San Antonio.

Previous studies of imported fire ant venom have been limited by the extreme difficulty in obtaining

Abbreviations used

PMSF: Phenylmethylsulfonyl fluoride
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sol i: *Solenopsis invicta*, imported fire ant

significant amounts of venom. The venom is composed of approximately 90% to 95% water-insoluble piperidine alkaloids,² which are not allergenic but are responsible for the typical pustule that forms at sting sites. A small amount of aqueous phase venom was studied by Baer et al.³ in 1979. They reported that the venom was 0.1% protein and separated into three protein fractions, one of which had phospholipase activity. They also reported that the venom contained hyaluronidase activity. The three protein fractions all exhibited IgE binding activity as determined by RAST. Our laboratory recently used nondenaturing electrophoresis with immunoblotting to examine *Sol i* venom.⁴ Five IgE binding bands were demon-

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strated in both commercial and pure venom extracts. Individual patient serum samples showed significant variation in IgE reactivity with the five bands.

In the studies reported herein, four allergenic proteins have been isolated from both commercial and pure hand-milked venom, purified, and characterized. These proteins correspond to the four strongest bands observed in immunoblot analysis.⁴

MATERIAL AND METHODS

Venoms

Commercial *Sol i* venom was obtained from Vespa Laboratories Inc., Spring Mills, Pa. Pure venom was hand milked from worker ants collected in Craven and Jones Counties, N.C., by the method we described previously.⁴ Alkaloid was removed from the pure venom preparation by three extractions with ether.

Sera

Control serum samples were drawn from adult subjects with no history of stinging insect allergy who resided in an area free from imported fire ants. Studies of other populations' reactivity with fire ant venom are presented elsewhere.⁵ Serum samples were also drawn from patients with a recent history of systemic allergic reactions to fire ant stings who resided in North Carolina, Georgia, South Carolina, Florida, Texas, or Alabama. All samples were stored frozen at -20°C until used. Allergic sera were screened by RAST with whole fire ant venom before use.

Gel filtration

Sephadex G-75 column chromatography was performed as we have described.⁶ The column was calibrated with molecular weight markers and was periodically recalibrated by separating venoms that have been previously studied. Bee and vespine venom proteins are primarily highly basic, as are fire ant venom proteins.

Cation-exchange chromatography

Fractions from gel filtration were further purified by ion-exchange on a Pharmacia (Piscataway, N.J.) Mono S high-performance column. The method has been described in detail.⁷ All separations were monitored by a computerized data acquisition system.

SDS-PAGE

SDS-PAGE was performed in minigels by a method previously described.⁸ Routine samples were reduced with dithiothreitol. In nonreducing SDS-PAGE, the dithiothreitol was omitted from the sample buffer. Gels were stained with ICN Rapid-Ag silver stain (ICN Radiochemical Division, Irvine, Calif.).

Immunoblot analysis

Nondenaturing acidic gel electrophoresis was performed by the method we recently described.⁴ Electrophoretic transfers were made to Immobilon PVDF (Millipore Corp., Bed-

ford, Mass.). Blots were incubated with human serum and then radioactive anti-IgE, and autoradiographs were prepared.⁴ A gel was prepared with both purified venom proteins and whole venom to identify the bands.

RAST

RAST was performed by our standard method.⁹ Disks were coupled to purified allergens at limiting quantities to minimize effects of small amounts of contaminating allergens.¹⁰ RAST results with purified allergens were analyzed by linear regression analysis with a Tadpole computer program (Elsevier Biosoft, Cambridge, U.K.).

Enzyme assays

Phospholipase activity was analyzed by the method of Habermann and Hardt.¹¹ Hyaluronidase activity was determined by a radial diffusion assay with human umbilical cord hyaluronic acid as substrate¹²; assays were performed both with and without 1,10-diaminodecane added to the gel. Total protease content was measured by the Bio-Rad protease detection kit (Bio-Rad Laboratories, Richmond, Calif.), which uses casein as substrate. Other activities were screened with Api-Zym strips (Analytab Products, Plainview, N.Y.). The Api-Zym panel consists of 19 activities, which include a variety of proteases and peptidases.

Protein determination

Protein was routinely determined by the coomassie blue dye binding reagent (purchased from Pierce Chemical Company, Rockford, Ill.), with bovine serum albumin as a standard.¹³ Weights of the purified proteins were also determined by amino acid analysis.

Amino acid analysis

Three 6N HCl hydrolyses were performed on each purified protein, and each hydrolysate was analyzed in duplicate by a Dionex D502 single-column amino acid analyzer (Dionex Corp., Sunnyvale, Calif.) with triketohydrindene hydrate detection. Compositions were normalized to the molecular weight determined by SDS-PAGE. Tryptophan content was not determined. Cysteine was determined as cysteic acid after oxidation of *Sol i* II.

RESULTS

Enzyme activities in fire ant venom

Samples of commercial and hand-milked aqueous phase *Sol i* venoms were tested for enzyme activities. Both preparations contained a significant amount of phospholipase, with primarily A activity. Hyaluronidase activity was not detectable in either preparation, with or without addition of the activator 1,10-diaminodecane. No activity hydrolyzing casein was detected. Further enzyme screens with Api-Zym strips showed the presence of small amounts of acid phosphatase and glucosidase in both preparations. These activities did not copurify with any of the major components and were present only in trace amounts.

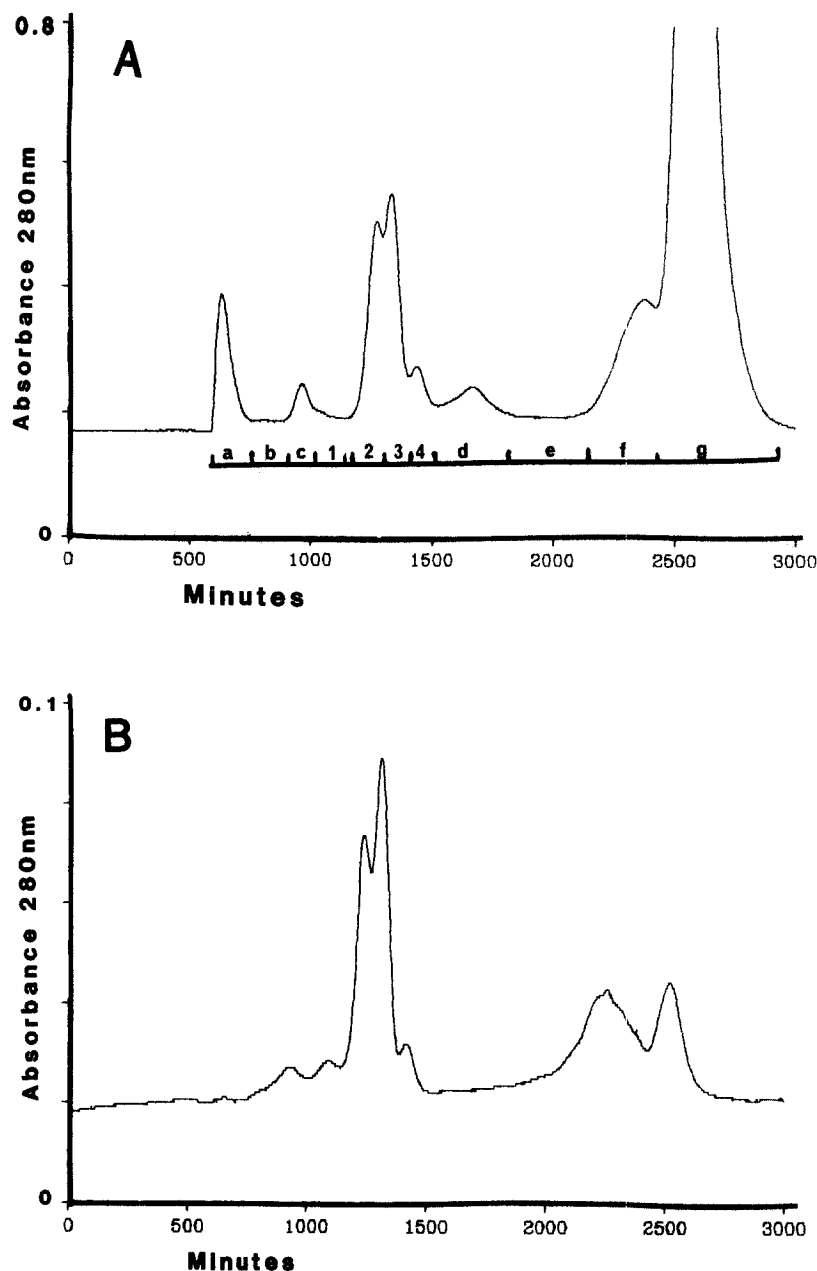


FIG. 1. Gel filtration profiles of *Sol i* venom extracts on a 1.5×1600 cm column of Sephadex G-75. The column was eluted with 0.1 mol/L ammonium formate, pH 5.0, at a flow rate of 7 ml/hr. Fractions were collected every 30 minutes. **A**, Approximately 18 mg of commercial *Sol i* venom dissolved in 0.03 mol/L acetic acid. Fraction pools are indicated on the horizontal line. Those indicated by *numbers* were further purified by cation-exchange chromatography. All pools were evaluated by SDS-PAGE and RAST with a pool of sera from patients allergic to fire ant venom. **B**, Profile of pure hand-milked venom from 9000 worker ants. Alkaloid was removed by ether extraction. Fractions were pooled as in **A**.

Separation of commercial venom and characterization of components

Commercial *Sol i* venom was separated in batches of 17 to 18 mg protein on a 1.5×1600 cm column of Sephadex G-75, with 0.1 mol/L ammonium formate, pH 5.0, at a flow rate of 7 ml/hr and was

collected in fractions of 3.5 ml. The separation profile is shown in Fig. 1, A. Pool 2 contained the phospholipase activity. Aliquots were taken from fractions along the entire length of the separation, pooled, concentrated, and analyzed by silver-stained SDS-PAGE. In addition, RAST disks were prepared from each of

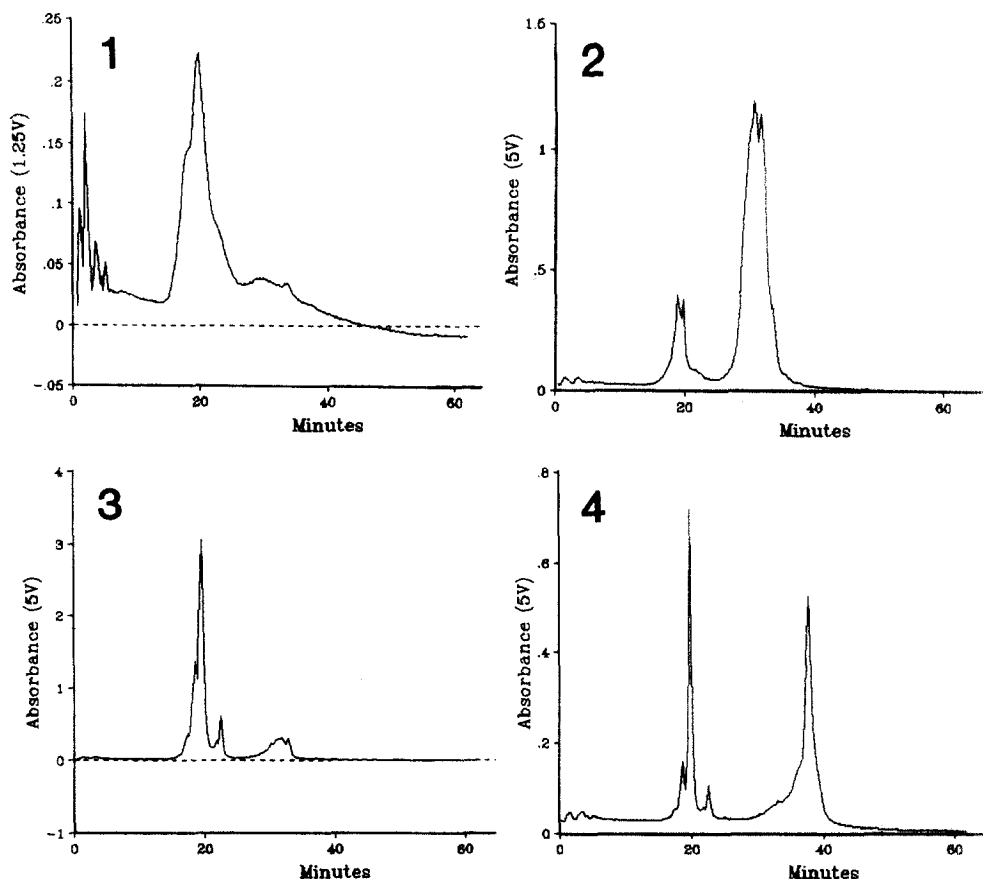


FIG. 2. Purifications of pools 1 to 4 from separation shown in Fig. 1, A by a Mono S high-performance cation exchanger. The column was eluted with a gradient of 0.05 mol/L sodium acetate, pH 5.2, to 1 mol/L sodium chloride in sodium acetate buffer from 10 to 42 minutes at a flow rate of 1 ml/min. Absorbance was monitored at 280 nm and 1 ml fractions were collected. Fractions were analyzed by SDS-PAGE before pooling. The patterns are described in the text and in Table I.

the pools in Fig. 1, A. The disks were tested with a pool of sera prepared by mixing equal amounts from six highly reactive serum samples with various patterns of reactivity by immunoblot analysis. Only the four numbered fractions in Fig. 1, A bound significant amounts of IgE. The weakly binding fractions contained small amounts of bands identical to those seen in the highly active fractions as determined by SDS-PAGE.

Significant amounts of protein were found only in four areas, labeled pools 1 to 4 in Fig. 1, A. Each of the four pools was concentrated by ultrafiltration with an Amicon YM-10 membrane (Amicon Corp., Danvers, Mass.) and then further separated by cation exchange on a Mono S column. The column was eluted with a gradient from 0.05 mol/L sodium acetate, pH 5.2, to 1 mol/L sodium chloride in acetate buffer (Fig. 2). The gradient was monitored by conductivity. The linear gradient was 42 minutes in duration, beginning 10 minutes into the run. One-minute fractions

were collected. The areas under the peaks were sampled and analyzed for phospholipase and by SDS-PAGE.

Four different proteins were found. Pool 1 contained protein I with polypeptide chains in SDS-PAGE at 18,000, 16,500 and 14,000 daltons molecular weight (Fig. 3). The ion-exchange separation in Fig. 2, A indicates that there are three charge forms. Rechromatography of protein I on the Sephadex G-75 column showed an apparent average molecular weight of 37,000 daltons. Pool 2 contained mainly protein II, which had strong phospholipase A activity. On SDS-PAGE (Fig. 3), bands were seen at 28,000 and 14,000 daltons. The band at 14,000 daltons seemed to increase with storage time, while the 28,000 dalton band decreased. SDS-PAGE without reducing agent showed very little of the 14,000 dalton band. A 6 mg aliquot of *S. invicta* venom was treated with 5 mmol/L PMSF to inactivate serine protease, and protein II was purified from this preparation by gel

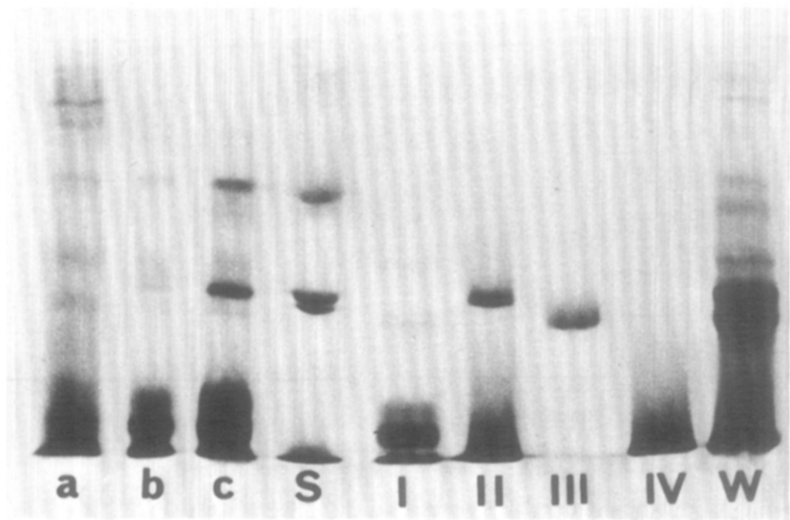


FIG. 3. SDS-PAGE separation of the purified proteins I, II, III, and IV from *Sol i* venom stained with silver. Also shown are whole venom (*W*) and three pools from the initial Sephadex G-75 separation (*a*, *b*, and *c*). *S*, Molecular standards (ovalbumin, carbonic anhydrase, chymotrypsinogen, and lysozyme).

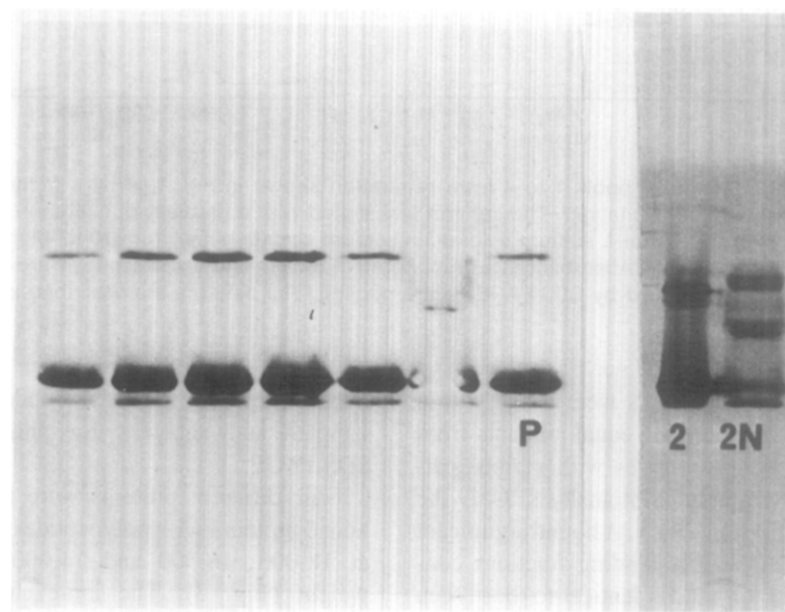


FIG. 4. SDS-PAGE of *Sol i* II, a phospholipase. The five samples on the left are fractions from the Mono S separation of pool 2 collected at minutes 31, 32, 33, 34, and 35. The concentrations have not been equalized. *P* represents *Sol i* II prepared from pure hand-milked venom. The five samples on the left were prepared from venom treated with PMSF to inactivate serine protease. The right panel shows an SDS-PAGE gel of pool 2 with (2) and without (2N) the reducing agent dithiothreitol. Equal amounts of protein were loaded in both lanes of this gel. Note that in the nonreduced sample, there is very little of the 14,000 dalton component.

filtration and ion exchange. The fractions from across the ion exchange peak were subjected to SDS-PAGE (Fig. 4). The two bands were found proportionally in the fractions, as they were in the protein II prepared

without PMSF. The natural fragments of protein II were separated on a special 20% peptide SDS-PAGE gel and the two bands were barely resolved (results not shown). Rechromatography of protein II on the

TABLE I. Properties of *Sol i* venom proteins

Protein	Apparent native molecular weight (daltons)	Recovery (mg) from 18 mg venom		Peak elutes from Mono S (mol/L NaCl)	Phospholipase
		By dye	By amino acid		
I	37000	0.11	0.13	0.16, 0.24, 0.31	Undetectable
II	30000	3.33	1.95	0.53, 0.56	+ + + +
III	26000	0.77	0.58	0.18, 0.22	Undetectable
IV	20000	0.44	0.25	0.68	Undetectable

TABLE II. Amino acid analyses of *Sol i* venom allergens

	<i>Sol i</i> I	<i>Sol i</i> II	<i>Sol i</i> III	<i>Sol i</i> IV
Asparagine plus aspartic acid	45	36	36	15
Threonine	22	14	19	7
Serine	29	10	17	7
Glutamic acid plus alutamine	29	22	24	9
Proline	13	14	14	5
Glycine	26	13	20	8
Alanine	27	27	19	4
Cystine		14		
Valine	22	24	12	9
Methionine	0	6	8	1
Isoleucine	18	9	12	13
Leucine	22	13	12	12
Tyrosine	14	0	9	5
Phenylalanine	15	0	6	2
Histidine	10	3	6	0
Lysine	22	32	18	20
Arginine	9	23	11	7
Residues	323	260	243	124
Molecular weight (daltons)	35058	28234	26111	13983

Residue values are the nearest integer to the mean of duplicate determinations from three separate hydrolyses. Total number of residues was normalized to the approximate molecular weight calculated from SDS-PAGE. Standard deviations were $\pm 6\%$. Cysteine was determined for *Sol i* II by oxidation to cysteic acid.

G 75 column showed an apparent molecular weight of 30,000 daltons. Protein II was also found in pool 3.

Pool 3 contained a protein peak that eluted earlier than protein II on the Mono S column. On SDS-PAGE (Fig. 3), a single band of 26,000 daltons was observed. The same molecular weight was found by rechromatography on the G-75 column. Protein III did not have any of the enzyme activities that we tested. Pool 4 showed two major components in ion-exchange chromatography, protein III and another strongly charged protein, IV. Protein IV gave a molecular weight by SDS-PAGE of 15,000 daltons and by G-75 rechromatography of 20,000 daltons.

The two other large peaks seen in Fig. 1, A, the exclusion peak and the very large inclusion peak, consist primarily of an alkaloid that becomes soluble in 0.1 mol/L acetic acid. There was only a trace of protein in the exclusion peak.

The physicochemical properties and yields of proteins I to IV are summarized in Table I. The four proteins were subjected to amino acid analysis after 6N HCl hydrolysis; normalized results are given in Table II. The compositions show a number of interesting features. Protein I does not contain methionine and has little or no cysteine. Protein II contains no phenylalanine or tyrosine and only a small amount of histidine; it does contain about 14 cysteines. Protein

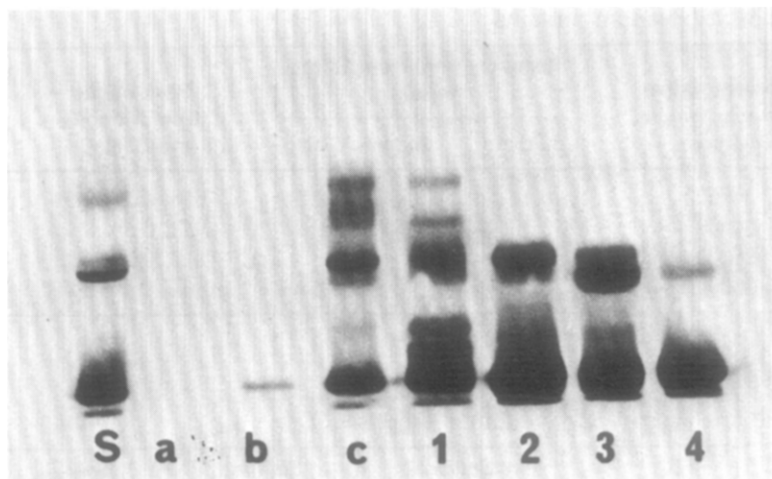


FIG. 5. SDS-PAGE of the pools from the Sephadex G-75 separation of pure hand-milked *Sol i* venom shown in Fig. 1, *B*. The lane labeled *S* contains standards as in Fig. 3. A few minor components can be seen in pools *c* and 1.

III contains all of the common amino acids measured, whereas protein IV lacks histidine. Analysis of the ratios of various pairs of amino acids suggests that these four proteins are not related to each other.

Separation of pure venom and comparison with commercial venom

Alkaloid was removed by ether extraction from the venom hand collected from 9000 *Sol i* worker ants. The aqueous phase pure venom was separated on the same Sephadex G-75 column used previously (Fig. 1, *B*). When the profile is compared with that of the commercial venom in Fig. 1, *A*, it can be seen that the exclusion peak is now only a trace and that the final peak is greatly reduced in size. Analysis of these peaks from commercial venom indicated that they both contained a large amount of alkaloid. Integration of the pure venom scan from the exclusion peak to 1700 minutes gave a total protein estimate of 1.1 mg. This was similar to estimates obtained by dye binding assay. The venom protein content was 0.12 μ g per ant. This is probably larger than a typical sting, because larger ants were chosen for milking and they were milked until no more venom was expressed. The G-75 fractions from the exclusion limit through fraction 50 (1500 minutes) were combined into seven pools and concentrated. Results of SDS-PAGE of these fractions are shown in Fig. 5. The last four fractions corresponded to commercial venom pools 1 to 4. The third fraction appeared to be a small amount of dimer of the fifth fraction, with some additional minor components. The peptide chain molecular weights were the same as those from commercial venom. The pools corresponding to commercial venom pools 1 to 4 were analyzed by cation exchange

on the Mono S column. Each pool gave a profile similar to that of the corresponding commercial venom pool. Proteins I, II, III, and IV were found. Parts of the pure venom pools were mixed with the corresponding pools from commercial venom and analyzed by Mono S chromatography. The proteins from both venom sources coeluted. The apparent molecular weights of the proteins I to IV isolated from pure venom were identical to those isolated from commercial venom on Sephadex G-75 rechromatography. The four proteins isolated from commercial venom are identical to proteins found in pure hand-milked venom.

IgE binding activities of purified venom proteins

Purified proteins I to IV were each coupled to cyanogen bromide-activated RAST disks at about 20 μ g/100 disks. RAST was performed with negative control sera and 23 sera from individuals reactive to fire ant venom. All sera from reactive individuals were positive to whole venom by RAST, and those identified by two-letter codes were also known to be positive by skin test. RAST results for the purified allergens are shown in Fig. 6. Average binding of negative control sera has been subtracted from all results. The reactivity patterns are highly variable, with six combining most strongly with protein I, four with protein II, four with protein III, six with protein IV, two equally with proteins I and II, and one equally with proteins I and IV. Overall, 20 serum samples reacted with protein I, 14 with protein II, 14 with protein III, and 17 with protein IV. Linear regression analysis was performed on the RAST results (Table III). Correlation coefficients between the allergen

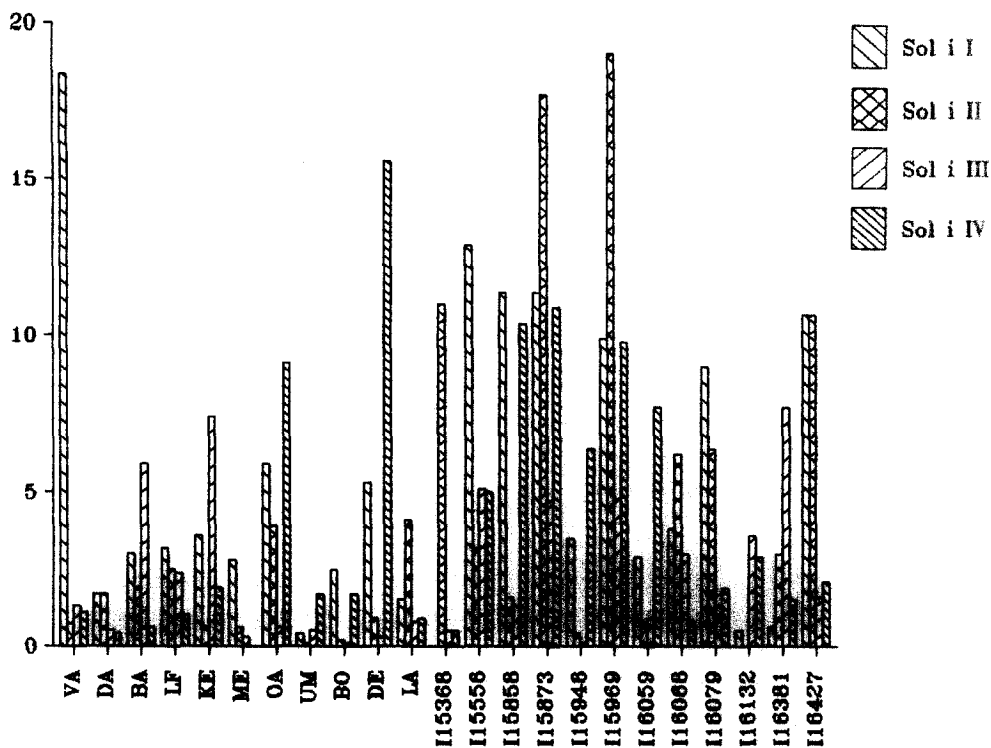


FIG. 6. RAST analysis of a panel of 23 sera with the four highly purified allergens from *Sol i* venom. The mean binding of a group of negative sera has been subtracted before the values were plotted. The ordinate is in percent of counts added. The sera studied exhibit a wide variety of reactivity patterns. Results of regression analysis of the data shown are given in Table III.

TABLE III. Linear correlation coefficients for RAST results for the four highly purified *Sol i* venom allergens tested with a panel of 23 serum samples from allergic patients

	<i>Sol i</i> I	<i>Sol i</i> II	<i>Sol i</i> III
<i>Sol i</i> II	0.317		
<i>Sol i</i> III	0.097	0.191	
<i>Sol i</i> IV	0.258	0.350	0.286

None are statistically significant at the $p = 0.05$ level.

pairs run from 0.10 to 0.35, and none are statistically significantly different. For comparison, linear correlation coefficients for the egg white allergens ovalbumin, ovomucoid, conalbumin, and lysozyme, all of which are structurally unrelated to each other, run from 0.12 to 0.55.¹⁴

Because all four of the purified venom proteins have been demonstrated to have significant IgE binding activity with sera from patients allergic to *Sol i* venom, they can be assigned designations according to the World Health Organization/International Union of Immunologic Societies system.¹⁵ Thus protein I is *Sol i* I, protein II is *Sol i* II, protein III is *Sol i* III, and protein IV is *Sol i* IV.

The four purified allergens and whole *Sol i* venom were tested in a nondenaturing acidic electrophoresis

gel that was electrophoretically transferred to a covalent binding membrane and developed with a pool of sera from individuals allergic to fire ant venom. Purified allergens *Sol i* I to IV corresponded, in order, from the top down to the four bands seen on the left strip of Fig. 5 in reference 4. The same patient serum pool was used in both studies.

DISCUSSION

Four protein allergens—*Sol i* I, II, III, and IV—were isolated from commercial *Sol i* venom. Four identical proteins were also isolated from pure hand-milked *Sol i* venom. No other fraction of the venom contained any significant IgE binding activity. The four purified protein allergens were tested by RAST with a panel of 23 sera from patients reactive to im-

ported fire ant venom. All were shown to be important allergens. As has been shown via nondenaturing immunoblot analysis,⁴ patients' sera exhibit varying patterns of reactivity with fire ant allergens. None of the allergens were dominant. A number of individual sera were found that did not bind to each of them. Similarly, each of the four allergens gave the highest binding for at least four sera from the panel. Linear regression analysis indicated that the allergens were not immunologically related to each other. The correlation coefficients for most pairs were lower than those of the structurally distinct egg white allergens¹⁴ and were similar to those of other highly purified venom allergens.¹⁶ This is supported by amino acid analysis, which showed major differences in composition, indicating that the proteins were not derived from each other.

Baer et al.^{3, 17} have reported on partial separations of *Sol i* venom allergens. They isolated three fractions with IgE binding activity. Their fraction I contained phospholipase activity and appears to be equivalent to a crude mixture of *Sol i* I and II. Fraction II of Baer et al. appears to be crude *Sol i* III, possibly including *Sol i* IV. It is not possible to evaluate their fraction III in relation to ours. Unlike Baer et al., we have not been able to demonstrate the enzyme hyaluronidase in either commercial or pure hand-milked fire ant venom. If the alkaloid is not removed completely, a small amount of hyaluronic acid solubilization occurs, which could be mistaken for hydrolysis.

The four allergens reported have been shown to be four of the five allergenic bands found by immunoblot analysis.⁴ The fifth unidentified band, found in electrophoresis of pure venom, appears to consist of aggregates of the other four components. RAST with highly purified allergens demonstrated the same degree of pattern heterogeneity seen with immunoblot studies.

Two other groups of investigators have studied allergens in *Sol i* venom.¹⁸⁻²⁰ However, both groups used crossed immunoelectrophoresis and did not determine any physical or chemical properties of the IgE binding substances. All four of the allergens are strongly basic; thus interpretation of crossed immunoelectrophoresis and crossed radioimmuno-electrophoresis patterns can be very difficult because individual allergens may give multiple precipitates and because complexes of pairs of allergens may also occur.^{21, 22} None of these investigators has tested pure hand-milked venom.

Sol i II is a phospholipase with A and some B activity. Phospholipases are found in other *Hymenoptera* venoms.²³ The molecular weight of *Sol i* II is 30,000 daltons on Sephadex G-75 chromatography and 28,000 daltons on SDS-PAGE. This is different

from honeybee phospholipase, which has an apparent native molecular weight of 16,000 and pure A₂ activity. Vespid phospholipases have molecular weights on SDS-PAGE of about 34,000 to 37,000⁶ and have A₁ and B specificities for phosphatidylcholine.²⁴ Rabbit antisera raised against bee and vespid phospholipases were not reactive in immunoblot analysis with *Sol i* II. The amino acid composition of *Sol i* II is very different from those reported for either honeybee²⁵ or vespid^{26, 27} phospholipases. *Sol i* II appears to be easily cleaved into fragments of about 14,000 daltons. In the native molecules, these fragments are held together by disulfide bonds (Fig. 4). The cleavage of *Sol i* II was not prevented by pretreatment of the venom with the serine protease inhibitor PMSF. It was not possible to separate the cleaved molecules from the intact molecules by either ion-exchange chromatography or gel filtration of the native protein. It appears that *Sol i* venom phospholipase is significantly different from venom phospholipases of both the honeybee and vespid types.

The work reported here demonstrates unequivocally for the first time that the important allergens in *Sol i* are of venom origin. There are four distinct allergens, one of which is a phospholipase. The existence of purified allergens from the venom allow biochemical, immunologic, and physicochemical methods to be developed for the standardization of imported fire ant venom extracts. With the purified allergens, it is possible to perform detailed studies of the immune response in the allergic patient and as a function of immunotherapy. Purified allergens also allow investigation of the reported cross-reactivity between fire ant and other stinging insect venoms.^{18, 28}

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