



PURIFICATION AND CHARACTERIZATION OF TWO FORMS OF ANTIGEN 5 FROM *POLYBIA SCUTELLARIS* VENOM

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(Received 12 April 1994; accepted 24 November 1994)

O. Cascone, V. Amaral, P. Ferrara, N. Vita, J. C. Guillemot and L. E. Díaz. Purification and characterization of two forms of antigen 5 from *Polybia scutellaris* venom. *Toxicon* **33**, 659–665, 1995.—Two polypeptides from the venom of *Polybia scutellaris* were purified to homogeneity by RP-HPLC. They differ very slightly in mol. wt (both are about 23,000) and hydrophobicity, and have isoelectric points greater than 9. Amino acid analyses show close similarity between them and with antigen 5 of vespids from different species. The two polypeptides have an identical N-terminal sequence (18 amino acids) which shows a high degree of homology with those of other vespids. Owing to the fact that the venom of this species is non-allergenic, the data for the mol. wt, isoelectric point, amino acid composition and N-terminal sequence allow us to identify the isolated polypeptides as two forms of antigen 5. Amino acids at positions 5 and 11 in *P. scutellaris* antigen 5 differ from those of the previously known sequences for antigen 5, suggesting that one or other might be responsible for the lack of allergenicity of the *P. scutellaris* venom.

INTRODUCTION

Most allergies to insect venoms are caused by bee, vespid and fire ant stings (Lichtenstein *et al.*, 1979; Reisman, 1985; Freeman, 1994). Phospholipase A₂ is the major proteic component in honeybee venom (Habermann, 1972; King *et al.*, 1976), but not in vespid venoms (King *et al.*, 1978). The venom composition of vespids, including hornets, polistine wasps and yellow jackets, is very similar. They contain three major proteins—hyaluronidase, phospholipase A₁ and antigen 5—which are active as allergens, the last being the most active on the basis of histamine-release assays using sensitized human leukocytes (King *et al.*, 1978, 1987). Antigen 5 is the major proteic component in vespid venoms, but is not found in honeybee venom. It does not, as yet, have a known biochemical function (King *et al.*, 1978, 1984).

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Most vespid-allergic patients show multiple reactions to more than one vespid venom (Lichtenstein *et al.*, 1979; Reisman, 1985; Hoffman, 1985), suggesting partial antigenic identity of the component proteins (King *et al.*, 1985, 1987).

Fang *et al.* (1988) isolated three forms of antigen 5 from *Dolichovespula maculata* venom by chromatography on SE-53 cellulose (King *et al.*, 1987) and determined the primary structure of two by cDNA and amino acid sequencing. They contain 204 and 205 amino acid residues, respectively, differ by 23% in sequences and have the same disulfide bonds (King *et al.*, 1990).

Hoffman (1993) determined the amino acid sequence of antigen 5 in eight different vespid venoms, including five species of yellow jacket, two variants of a European hornet and one species of paper wasp, and compared them with the seven previously published sequences from yellow jackets, hornets and paper wasps. The results provide the structural basis for the cross-reactivity patterns observed in clinical practice.

Polybia scutellaris subsp. *rioplatensis* (common name: camoati) is a polistine wasp from South America (mainly Argentina and Uruguay). *Polybia* and genus *Polistes* belong to the subfamily Polistinae. *Polybia* builds its nests, using vegetable fibre and mud, as a succession of organized hives in horizontal layers enveloped by a cover with conic protuberances, while the nests of *Polistes* sp. are non-covered hives hanging from a peduncle. The lack of anaphylactic activity of *P. scutellaris* venom is very curious. Although it is very common in South America and people are repeatedly stung, no hypersensitivity phenomena able to be confirmed by studies of the patients' sera have been reported.

Antigen 5 from three members of the genus *Polistes* (*P. annularis*, *P. exclamans* and *P. fuscatus*) has been sequenced, but none from the genus *Polistes*. To extend the knowledge of the vespid antigen 5 by studying it in a new genus, we describe in this paper the purification and characterization of two forms of this protein from *P. scutellaris* venom and compare their amino acid composition and N-terminal sequence with the data for antigen 5 from 15 other vespids.

MATERIALS AND METHODS

Polybia scutellaris venom

Venom was obtained by carefully squeezing sacs dissected from frozen insects and centrifuging the material at 15,000 rpm for 15 min. The pellet was discarded and the supernatant lyophilized. An average of 4 µl of venom per sac was collected.

Reagents

Isoelectric focusing gels (pH 3–9), 20% SDS–PAGE gels and silver-stain kits were obtained from Pharmacia (Uppsala, Sweden), guanidine chloride from Sigma Chemical Co. (St Louis, MO, U.S.A.), HPLC-grade acetonitrile from Prolabo (France), and reagents for amino acid and sequence analyses from Applied Biosystems, Inc. (U.S.A.). All other reagents were AR grade.

HPLC fractionation of the venom

In a typical experiment, 5–50 µl of venom was injected on to a Perkin Elmer HPLC system with a Vydac 218TP54 (25 cm length) column (Separation Group, Hesperia, U.S.A.). The elution was initially isocratic (20% B for 10 min) then a linear gradient (20–70% B in 80 min) was applied at a flow rate of 0.3 ml/min. Solvent A was aqueous 0.1% TFA and solvent B 0.1% TFA in acetonitrile. The effluent was monitored at 220 nm. Rechromatography of the isolated fractions was carried out under the same conditions, except that the elution gradient was 30–60% B.

SDS–polyacrylamide gel electrophoresis

SDS–PAGE was performed using a Pharmacia Phast System apparatus with 20% gels and silver staining according to the manufacturer's instructions.

Laser desorption mass spectrometry (LD-MS)

This was performed using a Lasermate device (Finnigan, San José, CA, U.S.A.), as previously described (Karas and Hillenkamp, 1988; Beavis and Chait, 1989).

Isoelectric point determination

This was performed using a Pharmacia Phast System apparatus with pH 3–9 gels and silver staining according to the manufacturer's instructions.

Amino acid analyses

An Applied Biosystems 420 A automatic amino acid analyser was used as per the manufacturer's instructions. Gas-phase hydrolysis was carried out using 6 N hydrochloric acid at 150°C for 90 min. The PTC amino acids obtained by derivatization with PITC were separated by RP-HPLC.

Sequence analyses

Automated gas-phase sequence determinations were carried out in an Applied Biosystems 470A sequencer following standard procedures. Before this step, proteins were pyridylethylated with 4-vinylpyridine (Henschen, 1986). PTH amino acids were determined using an Applied Biosystems 120A micro-HPLC system. The average repetitive yield ranged from 93 to 96% and the detection limit for PTH amino acids was 0.4 pmole.

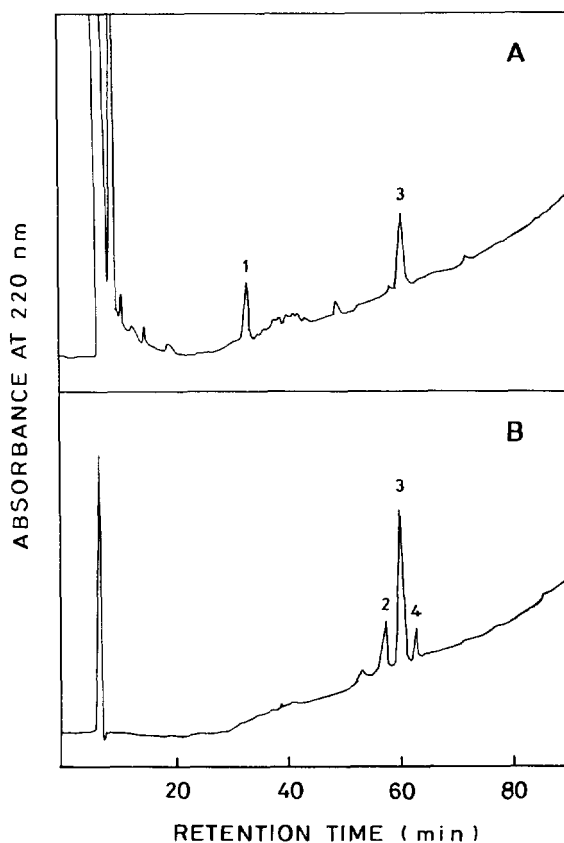


Fig. 1. RP-HPLC pattern of soluble and insoluble fractions from the *P. scutellaris* venom. Lyophilized venom was suspended to its original volume in water, then centrifuged at 10,000 *g* for 10 min. The supernatant was removed and the pellet dissolved in the original volume of 4 M guanidine hydrochloride. Five microlitres of the aqueous supernatant (A) or resolubilized pellet (B) was injected on to the HPLC. After 10 min at 20% B, an 80 min linear gradient from 20 to 70% B was applied at a flow rate of 0.3 ml/min. Solvent A was aqueous 0.1% TFA and solvent B 0.1% TFA in acetonitrile. The effluent was monitored at 220 nm.

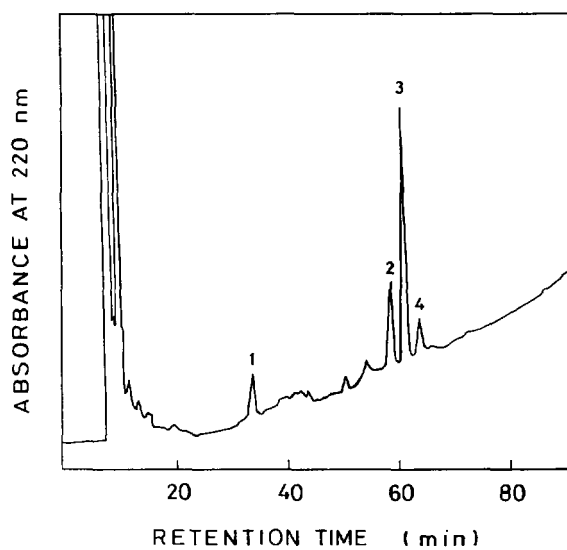


Fig. 2. RP-HPLC pattern of guanidine hydrochloride solubilized *P. scutellaris* venom. Lyophilized venom was dissolved in its original volume of 4 M guanidine hydrochloride and 5 μ l was chromatographed under the same conditions as in Fig. 1.

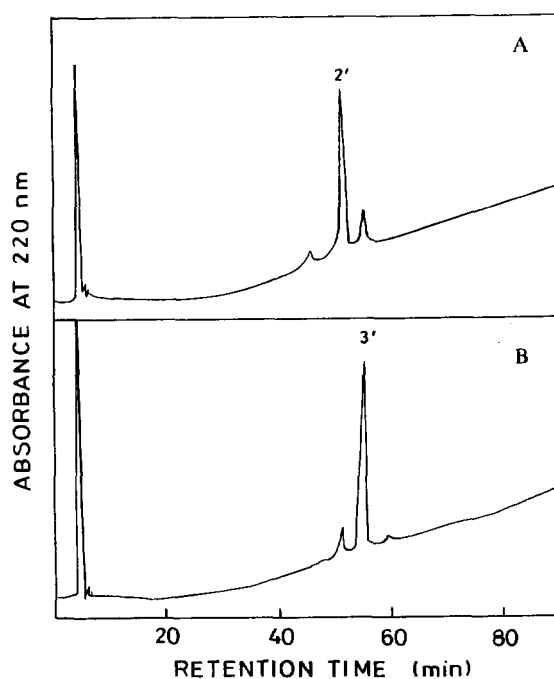


Fig. 3. Rechromatography of fractions 2 and 3. Each fraction was concentrated to 10 μ l and injected on to the HPLC. After 10 min at 30% B, an 80 min linear gradient from 30–60% B was applied at a flow rate of 0.3 ml/min. Solvent A was aqueous 0.1% TFA and solvent B 0.1% TFA in acetonitrile. The effluent was monitored at 220 nm. (A) Fraction 2, (B) fraction 3.

Table I. A comparison of the amino acid composition of *Polybia scutellaris* venom antigen 5 fractions and those from other vespids

Amino acid	<i>Polybia scutellaris</i> *		<i>Polistes annularis</i> †	<i>Vespula germanica</i> ‡	<i>Dolichovespula maculata</i> § form	
	2'	3'			2	3
Lys	18	19	21	24	21	19
His	5	5	7	6	5	7
Arg	6	6	6	5	9	5
Asp	23	23	24	26	32	29
Thr	10	10	11	9	13	12
Ser	16	14	12	8	6	10
Glu	28	28	25	26	21	21
Pro	6	7	8	7	8	7
Gly	20	18	20	16	16	18
Ala	14	14	14	13	9	9
Val	15	16	12	13	11	14
Met	2	3	3	2	3	6
Ile	10	10	12	7	11	9
Leu	11	10	12	12	9	7
Tyr	8	10	10	11	10	9
Phe	6	6	4	5	7	5
Cys	ND	ND	9	7	8	8
Trp	ND	ND	ND	ND	5	5

*This work.

†King *et al.* (1984).‡King *et al.* (1983).§Fang *et al.* (1988).

ND, Not determined.

RESULTS AND DISCUSSION

Lyophilized *P. scutellaris* venom was not completely soluble in water, but was soluble in 4 M guanidine hydrochloride.

Figure 1 shows the RP-HPLC pattern of the water-soluble and water-insoluble fractions after solubilization in 4 M guanidine hydrochloride. Peak 1 was found only in the water-soluble material, peaks 2 and 3 only in the guanidine hydrochloride sample and peak 3 in both. This suggests that much of the material may have been denatured on lyophilization.

Figure 2 shows the RP-HPLC fractionation pattern of guanidine chloride-solubilized venom. As expected, the chromatogram displays all the peaks seen in Fig. 1A and B; all further fractionations were performed using guanidine chloride solubilized material.

SDS-PAGE analysis of the material in the four peaks seen in Fig. 2 showed that peak 1 contained a single 1.8 kDa peptide, peaks 2 and 3 both contained a protein of about 23 kDa, and peak 4 contained a 10 kDa polypeptide. No bands were seen in the peak eluting with the solvent front (not shown).

Rechromatography of the material in peaks 2 and 3 (Fig. 2) is shown in Fig. 3. Each gave one symmetrical main peak eluting at 45 (2') and 47% (3') acetonitrile, respectively; SDS-PAGE of these peaks yielded a single band at 23 kDa.

To determine more accurately the mol. wt of the 23 kDa proteins, laser desorption mass spectrometry of material from fractions 2' and 3' was performed; this showed a small but significant difference in mol. wt with values of $22,904 \pm 21$ and $23,418 \pm 32$ for peaks 2' and 3', respectively ($n = 3$).

Table 2. A comparison of the N-terminal sequence of *Polybia scutellaris* antigen 5 and those from 15 previously known vespid antigens 5

Antigen 5 source	N-terminal sequence														
	1	5	10	15											
<i>Polybia scutellaris</i> *	N	K	Y	C	N	I	K	C	S	K	V	—	A	H	T
<i>Polistes annularis</i>	V	D	Y	C	K	I	K	C	P	S	G	—	I	H	T
<i>Polistes exclamans</i>	V	D	Y	C	K	I	K	C	P	S	G	—	I	H	T
<i>Polistes fuscatus</i>	V	D	Y	C	K	I	K	C	S	S	G	—	I	H	T
<i>Vespula flavopilosa</i>	N	N	Y	C	K	I	K	C	L	K	G	G	V	H	T
<i>Vespula germanica</i>	N	N	Y	C	K	I	K	C	L	K	G	G	V	H	T
<i>Vespula maculifrons</i>	N	N	Y	C	K	I	K	C	L	K	G	G	V	H	T
<i>Vespula pensylvanica</i>	N	N	Y	C	K	I	K	C	L	K	G	G	V	H	T
<i>Vespula squamosa</i>	V	D	Y	C	K	I	K	C	L	K	G	G	V	H	T
<i>Vespula vidua</i>	K	V	N	Y	C	K	I	K	C	L	K	G	G	V	H
<i>Vespula vulgaris</i>	N	N	Y	C	K	I	K	C	L	K	G	G	V	H	T
<i>Dolichovespula arenaria</i>	N	N	Y	C	K	I	—	C	P	K	G	—	T	H	T
<i>Dolichovespula maculata</i> form 2	N	N	Y	C	K	I	K	C	R	K	G	—	I	H	T
<i>Dolichovespula maculata</i> form 3	N	N	Y	C	K	I	K	C	S	R	G	—	I	H	T
<i>Vespa crabro</i> form 1	N	N	Y	C	K	I	K	C	R	S	G	—	I	H	T
<i>Vespa crabro</i> form 2	N	N	Y	C	K	I	K	C	S	R	G	—	I	H	T

*This work.

All other sequences were taken from Hoffman (1993).

Dashes indicate gaps inserted to maximize homology.

Both methods of mol. wt determination (SDS-PAGE and LD-MS) showed the material to be pure. The isoelectric point of both 23 kDa proteins was greater than 9.0.

Table 1 shows the amino acid composition of fractions 2' and 3' compared with those of antigen 5 from a representative of the genera *Dolichovespula* (*D. maculata* forms 2 and 3), *Vespula* (*V. germanica*) and *Polistes* (*P. annularis*). The comparison indicates some similarity in composition between antigen 5 previously characterized in different vespids and fractions 2' and 3' from *P. scutellaris*. Fractions 2' and 3' are remarkably similar, and differ from the other already-known antigens 5 only in a slightly lower content of basic amino acids and a higher content of hydroxylated amino acids.

Taken together, the data for mol. wt, amino acid composition and isoelectric point, allow us to say that fractions 2' and 3' correspond to two different forms of antigen 5 from *P. scutellaris*, which differ slightly in mol. wt and hydrophobicity (fractions 2' and 3' eluted from a RP-HPLC column at 45 and 47% acetonitrile, respectively).

Further evidence for the identity of fractions 2' and 3' and for their characterization as antigen 5 was provided by N-terminal sequence determination. The N-terminal sequence of fractions 2' and 3' was identical. As shown in Table 2, which compares their sequence with those of 15 antigen 5 (Hoffman, 1993), a high degree of sequence homology can be seen.

Of these first 18 amino acids, the sequence of the *P. scutellaris* antigen 5 differs from these previously known antigens 5 at two positions (5 and 11). It is possible that one or other of the differences may account for the lack of allergenicity of the *P. scutellaris* venom.

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