Allergens in Hymenoptera venom XIII: Isolation and purification of protein components from three species of vespid venoms

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Allergens in Hymenoptera venom XIII: Isolation and purification of protein components from three species of vespid venoms

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Pure venoms were collected from individual insects of the species Dolichovespula maculata, *white-faced hornet,* Vespula squamosa, *southern yellow jacket, and* Polistes exclamans, *paper wasp (one species). The venoms were first fractionated by high-resolution gel filtration on a 1.6 m column of Sephadex G-75 superfine, and the components were then purified by high-performance, ion-exchange chromatography on a Mono-S cation exchange column followed by a further gel filtration step. The isolated components were evaluated for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis by use of two different types of silver stains, by assays for enzyme activities, and by immunodiffusion with the use of rabbit antisera. The protein components were isolated in highly purified states by these techniques. Only three significant proteins were found in* V. squamosa *venom: phospholipase (PL) A and B, hyaluronidase (HYAL), and antigen 5 (Ag 5).* D. maculata *venom contained HYAL, Ag 5, two isozymes of PL A and B, a high-molecular-weight protein, and several trace proteins. No significant amounts of proteases were found in* D. maculata *venom.* P. exclamans *venom contained HYAL, PL A and B, Ag 5, a high-molecular-weight protein, and several minor proteins. In all three venoms the PL A and B activities were found to be in the same molecule and did not separate. Trace components with apparent PL A activity were observed in the venoms. The venoms were screened for a variety of esterases, proteases, peptidases, glucosidases, and phosphatases, and none were detected in more than trace amounts. Vespid venoms do not appear to contain significant amounts of acid phosphatases as bee venoms do. (J ALLERGY CLIN IMMUNOL 75:599-605, 1985.)*

Previous articles describing the isolation and allergenic activities of protein components from vespid venoms have used commercial venom sac extracts as the starting materials.[1-6] In a recent abstract one group of investigators has claimed to separate proteins from pure *D. maculata* venom,[7] but they found large amounts of at least one component that we could not detect. In the separation of *V. maculifrons,* eastern yellow jacket, venom recently reported from our laboratory, the five components isolated from the commercial sac extract were all demonstrated to be present in a reference preparation of pure venom.[6] The separation of these components from the sac proteins was laborious and required repeated cycles of conventional chromatographies or the use of affinity methods.[1, 4, 5]

Abbreviations used

SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
BSA:	Bovine serum albumin
HPLC:	High-performance liquid chromatography
PL:	Phospholipase
HYAL:	Hyaluronidase
Ag 5:	Antigen 5

Recently we have reported on the collection of usable amounts of pure venom by milking individual live insects.[8] In this article we describe the separation of three of these venoms into the individual protein components by the use of high-resolution gel filtration and high-performance cation exchange chromatography. The availability of purified vespid venom proteins allows the determination of the importance of the various proteins as allergens and definitive study

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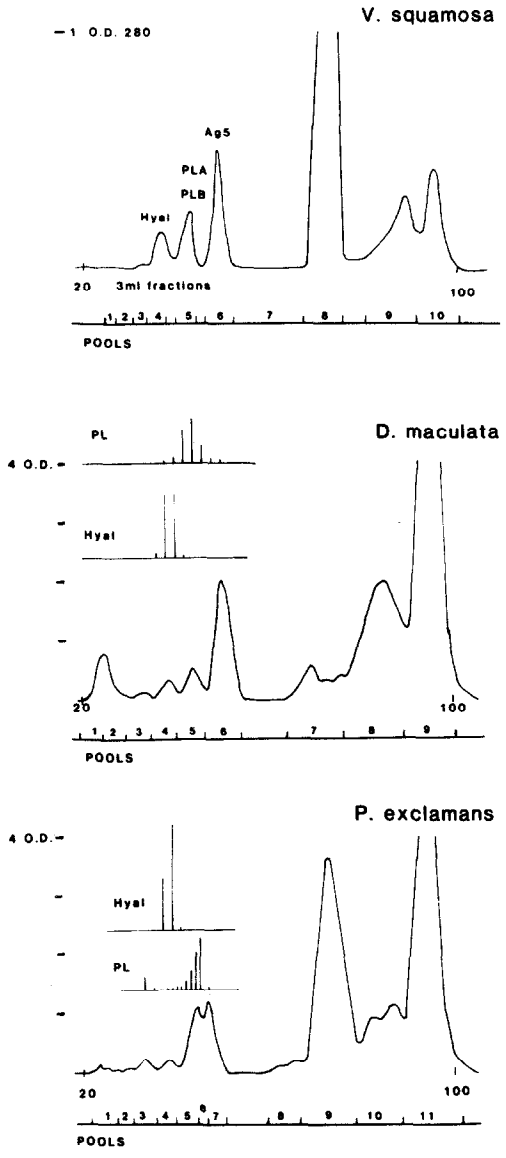

FIG. 1. Gel filtration profiles from a 1.5 by 1600 cm column of Sephadex G-75 superfine of 12.5 mg of *V. squamosa* venom, 50 mg of *D. maculata* venom, and 45 mg of *P. exclamans* venom. The columns were eluted with 0.1 mol/L ammonium formate, pH 5.0, at a flow rate of 6 ml per hour. Fractions of 3 ml were collected. The compositions of the pools based on SDS-PAGE and enzyme activities are illustrated on the *horizontal lines below* the *absorbance curves*. Enzyme activities are illustrated *above* the *absorbance curves*. *V. squamosa* antigen 5 was identified by immunodiffusion against antiserum to *V. maculifrons* antigen 5.

of the immunologic basis of vespid venom cross-reactivity.[9, 10]

MATERIAL AND METHODS

Venoms. Pure venoms were obtained from individual live insects by milking with and without electrical stimulation as has been described previously by our laboratory.[8]

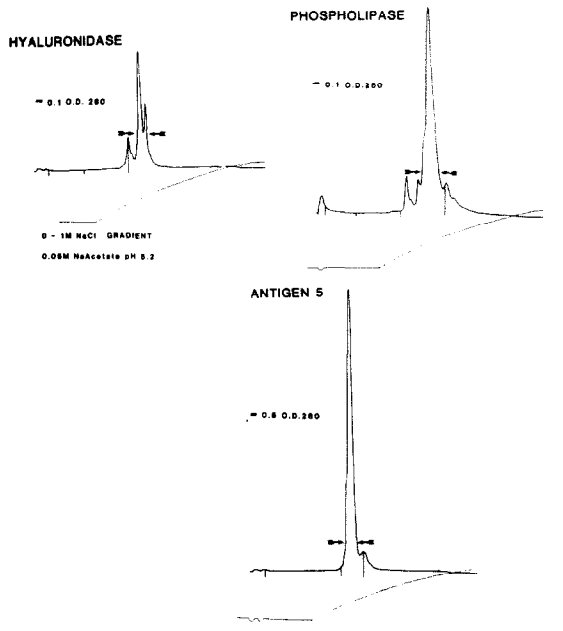

FIG. 2. High-performance, ion-exchange chromatography of *V. squamosa* venom proteins on a Mono S column. The *heavy curves* are the absorbance at 280 nm and the *lighter curves* below are the conductivity of the eluting buffer. Columns were eluted with a gradient of 0 to 1 mol/L NaCl in 0.05 mol/L sodium acetate, pH 5.2. The *horizontal arrows* indicate the fractions pooled. The flow rate was 1 ml per minute, and 2 ml fractions were collected. Pools were made based on SDS-PAGE and enzyme activity measurements.

Protein concentrations. The protein concentrations of venoms and purified fractions were estimated by a Coomassie blue dye binding assay with BSA as a standard.[8, 11] Reagents were obtained from Pierce Chemical Company, Rockford, Ill.

Enzyme activities. The methods for estimating PL A and B, HYAL, acid phosphatase, proteases, and the API-ZYM screen for 19 enzyme activities have been previously described in detail.[6]

Gel filtration. Separations based on molecular weight were carried out with a 1.6 by 160 cm column of Sephadex G-75 superfine. The column was eluted with 0.1 mol/L ammonium formate, pH 5.0, at a flow rate of 6 ml per hour. Fractions of 3 ml were collected. The fractions were pooled based on results of SDS-PAGE and enzyme activity measurements. Column effluent was monitored at 280 nm for protein. It took approximately 48 hr to complete a single run. The column was calibrated, and the resolution was tested with honeybee venom. Pools were concentrated by use of Amicon YM-10 membranes (Amicon Corp., Danvers, Mass.) and/or freeze drying.

HPLC cation exchange chromatography. Fractions from the gel filtration were further separated as previously described[6] by use of a Pharmacia (Piscataway, N. J.) Mono-S FPLC cation exchange column with a gradient from 0 to 1 mol/L NaCl in 0.05 mol/L sodium acetate, pH 5.0. Absorbance was monitored at 280 nm as well as effluent con-

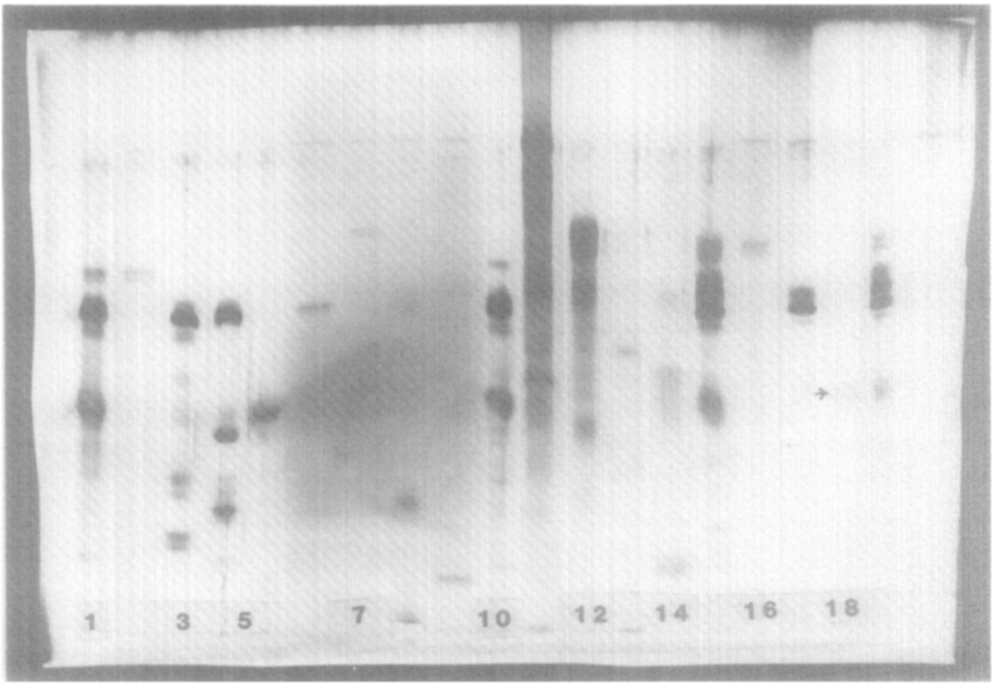

FIG. 3. SDS-PAGE under reducing conditions of *D. maculata* and *V. squamosa* venom proteins in a 10% T, 2.7% C gel stained with Gelcode polychromatic silver stain. Tracks *1* and *10* contain *D. maculata* venom, and its components are in lanes *2* through *9*: HYAL in lane *2*, PL_1 in lane *3*, PL_2 in lane *4*, Ag 5 in lane *5*, pool 3 in lane *6*, high molecular weight in lane *8*, minor fractions are in lanes *7* and *9*. Markers in tracks *11* to *14* are BSA, ovalbumin, carbonic anhydrase, and cytochrome c. *V. squamosa* venom is in tracks *15* and *19*, and its fractions HYAL in track *16*, PL in track *17* and Ag 5 in track *18*. Each lane contains from 0.15 to 0.5 μg of protein.

ductivity. Data were collected in a computerized system as well as on a chart recorder.

SDS-PAGE. SDS-PAGE was carried out in single concentration gels as previously described.[12, 13] Duplicate gels were run. One was stained with a polychromatic silver stain,[14] Gelcode (Upjohn Diagnostics, Kalamazoo, Mich.), and the other with a Merril-type silver stain[15] purchased from Bio-Rad Laboratories, Richmond, Calif.

Antisera and immunochemical assays. Antisera against the individual purified venom components were raised in New Zealand white rabbits by subcutaneous injections of antigen emulsified in Freund's complete adjuvant by the protocol we have previously described.[6] Immunodiffusion and immunoelectrophoresis were performed as previously described.[6]

RESULTS

The venoms collected from *V. squamosa*, *D. maculata*, and *P. exclamans* were separated by gel filtration on a 1.6 m column of Sephadex G-75 superfine. The absorbance profiles are illustrated in Fig. 1 for all three venoms. Large amounts of HYAL activity and PL A and B activities were detected in the fractions illustrated in Fig. 1. No significant amounts of phosphatases, proteases, peptidases, or glucosidases were found in any of the fractions or in the unseparated venoms. The acid phosphatase previously reported in yellow jacket venom sac extract[2] appears to be of tissue rather than venom origin.

The fractions from gel filtration were pooled based on the results of enzyme assays and SDS-PAGE, and the pools were concentrated by ultrafiltration and/or freeze drying. The protein fractions were further separated by high-performance cation exchange chromatography on a Mono-S column. HPLC results are illustrated for the *V. squamosa* proteins in Fig. 2. The fractions from HPLC were again analyzed for enzyme activities and by SDS-PAGE and pooled. The pools were finally purified by gel filtration on the Sephadex G-75 superfine column and were then freeze dried and reconstituted. The final fractions were again analyzed for enzyme activities and by SDS-PAGE as is illustrated in Figs. 3 and 4. The purities as estimated from enzyme assays are illustrated in Table I. The yields of the various components are illustrated in Table II.

V. squamosa venom contained significant amounts of only three proteins. HYAL had a molecular weight of 45,000. There were two closely spaced bands of molecular weight 37,000 and 35,000 that expressed both PL A and B activities. These activities did not separate by any technique used. The third band of

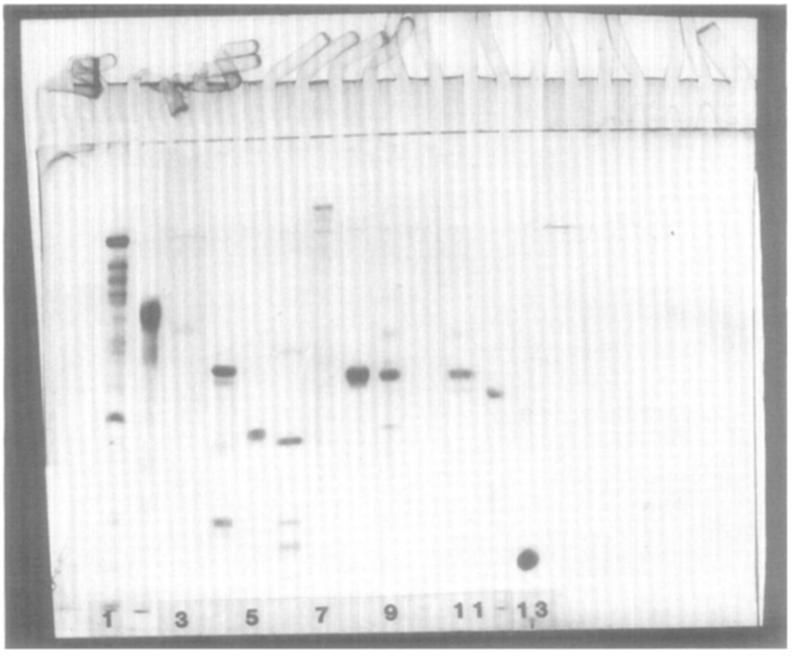

FIG. 4. SDS-PAGE of *P. exclamans* venom fractions stained with a Merril type silver stain. The fractions were applied under reducing conditions. The samples are HYAL in track *3*, PL in track *4*, Ag 5 in track *5*, pool 3 in track *6*, high molecular weight in track *7*, four minor fractions are in tracks *8* to *11*. Marker proteins are BSA in track *1*, ovalbumin in track *2*, carbonic anhydrase in track *12*, and cytochrome c in track *13*. From 0.5 to 1.5 μg of protein was applied to each track.

TABLE I. Purities of vespid venom fractions

	HYAL activity	PLA activity
V. squamosa		
HYAL	375*	4×10^{-5}†
PL	0.75	0.01
Ag 5	undet	$<10^{-5}$
D. maculata		
High molecular weight	10	5×10^{-6}
HYAL	10,000	2×10^{-6}
PL	20	0.0011
PL	5	0.0006
Ag 5	undet	10^{-6}
P. exclamans		
78000 + 76000	<0.3	10^{-4}
HYAL	3060	undet
PL	6	0.01
Ag 5	0.6	2.5×10^{-4}
Pool 3‡	1.5	Apparent A but no B activity

Undet = undetectable.
*National formulary units per microgram with use of testicular HYAL as standard.
†In micrograms bee venoms A per microgram of protein.
‡Composition discussed in text.

molecular weight 24,000 was identified as Ag 5[1] by its size and cross-reaction with *V. maculifrons* Ag 5.[6]

D. maculata venom was found to be more complicated. It contained a high-molecular-weight protein of about 100,000 MW, HYAL of 41,000 MW, two isozymes of PL A and B of 34,000 and 33,000 MW, Ag 5 of 22,000 MW, and small amounts of a number of other proteins and peptides. Traces of aminopeptidase activity, acid phosphatase activity, β-galactosidase, and α-glucosidase were found in the highly concentrated fractions that were excluded from the Mono-S column. Some of these enzymes are probably of lysozomal origin rather than venom proteins. The two isozymes of PL could be separated readily by ion exchange chromatography as is illustrated in Fig. 5. Both PL A and B activities were present in both forms of the enzyme. The more basic form, PL_2, had the higher molecular weight.

P. exclamans venom was somewhat more difficult to separate because the proteins had similar charges and did not resolve as well on the initial gel filtration. In addition there appeared to be a number of minor components present. The proteins isolated included a pair of high-molecular-weight proteins at 78,000 and 76,000, HYAL at 42,000 MW, PL A and B at 34,000 MW, and Ag 5 at 24,500 MW. A pool containing at least five peptides between 10,000 and 38,000 MW

TABLE II. Yields of vespid venom protein fractions

Fraction	Protein (mg)	% Of protein
V. squamosa	6.38*	
HYAL	0.30	4.7
PL	1.46	22.9
Ag 5	1.05	16.5
Incompletely separated and other	0.20	3.1
Total	3.01	47.2
D. maculata	27.76	
High molecular weight	0.36	1.3
HYAL	0.60	2.2
PL	1.36	4.9
PL	2.11	7.6
Ag 5	10.52	37.9
Incompletely separated and other	0.37	1.3
Total	15.32	55.2
P. exclamans	20.03	
78000 + 76000	0.18	0.9
HYAL	0.31	1.6
PL	4.59	22.9
Ag 5	3.51	17.5
Pool 3	0.37	1.9
Incompletely separated and other	1.19	5.9
Total	10.15	50.7

*Protein more than 10,000 MW on Sephadex G-75 determined by Coomassie blue dye binding assay.

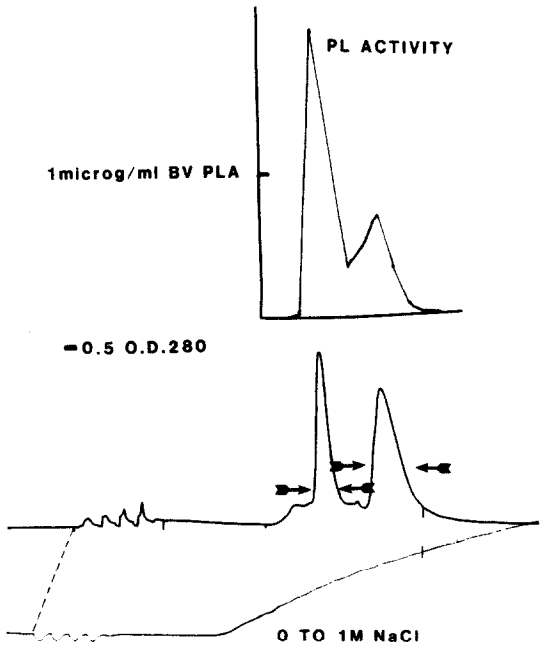

FIG. 5. High-performance, ion-exchange chromatography of *D. maculata* PL demonstrating the separation of the two isozymes, PL_1 and PL_2. Conditions are the same as Fig. 2. The *dotted line* indicates the offset of the conductivity measurement. PL A and B activity are illustrated *above* the *absorbance curve. Horizontal arrows* indicate the two pools made.

was also prepared. This pool elicited a single broad peak on Mono-S HPLC and a single narrow peak in gel filtration. It is probably an aggregate of several of the proteins and peptides in the venom, since one component appears to be PL.

In Figs. 3 and 4 the PLs from all three venoms appear to be contaminated by lower molecular weight peptides. These peptides are not removed by further purification steps, and similar peptides are present in *V. maculifrons* PL[6] and have been reported by King et al.[1, 4, 5] in all of the vespid venom PLs that they have prepared by either affinity or conventional chromatographies. These bands are not present in SDS-PAGE in the absence of reducing agents such as mercaptoethanol or dithiothreitol. Although in some cases one of the bands appears to be the same molecular weight as Ag 5, it is immunologically distinct. Apparently some of the PL has been cleaved at various points by the traces of peptidases and proteases present in the venom. Only trace amounts of any proteolytic activity could be detected in any of these venoms.

DISCUSSION

The three known proteins isolated from *D. maculata* venom in this study were similar in properties to the corresponding proteins isolated by King et al.[1] The molecular weights of HYAL, PL, and Ag 5 were found to be 41,000, 33 to 34,000, and 22,000 MW by our use of SDS-PAGE, and were found to be 45,000, 35,000, and 25,000 MW by King et al. King et al. isolated only a single form of PL but did note some heterogeneity of HYAL, which was probably caused by its isolation from venom sac extract rather than pure venom. The high-molecular-weight protein at about 100,000 MW has not been reported in this venom previously but may be an analogue of Vmac1 previously isolated in our laboratory from *V. maculifrons* venom and venom sac.[6] Our results contrast strikingly with those reported by Littler et al.[7] who claim that they detected large amounts of protease in *D. maculata* venom collected by mass electrical stimulation. We could not detect more than trace amounts of aminopeptidase in either the pure venom or its fractions. No casein hydrolytic activity could be detected. It appears that venom collected by mass electrical stimulation is probably contaminated with either fecal or digestive material. Hornets unlike yellow jackets tend to suck back venom into their abdominal

segments and spray it rather than sting. The sprayed venom is many times contaminated with fecal and other foreign materials. This can be readily observed if one disturbs a nest on a warm day.

V. squamosa belongs to a different subgenus of yellow jackets from that of the other common species whose venoms have been studied.[16, 17] Its venom is the simplest yet described with only three major protein components. The HYAL, PL, and Ag 5 are similar in molecular weight to those from other species of *Vespula*.[4, 6] No proteins corresponding to Vmac1 or Vmac3 were observed in the *V. squamosa* venom. No significant proteinase or peptidase activity was found in either the venom or its fractions in agreement with the suggestion of King et al.[4] that it is probably of sac origin.

A separation of *P. exclamans* proteins from venom sac extract has recently been reported[5] in which HYAL of 44,000 MW, PL of 37,000 MW, and Ag 5 of 27,000 MW were isolated. In our study the molecular weights were 42,000, 34,000, and 24,500. In addition we obtained a fraction of two bands of 76,000 and 78,000 MW size, as well as a fraction containing a number of peptide chains from 10 to 38,000 MW.

In their recent report King et al.[5] demonstrated that *P. exclamans* and the other vespid PLs are of A_1 and B specificities not A_2 and B as had previously been supposed. He also demonstrated a significant substrate dependence of activity. It is also important to note that these venoms contain peptides that are specific modifiers of PL activity, as melittin is for honeybee PL. These peptides are named mastoparans in the wasps and do not modulate bee PL.[18] The presence of these peptides does not allow one to quantitatively follow PL activity through a purification, if one uses micellular substrates. Our studies are consistent with these observations.

No substance was found in any of these three venoms corresponding to the protein between HYAL and PL named Vmac3 isolated from *V. maculifrons* venom. This was not unexpected, since no corresponding protein was observed on SDS-PAGE gels of the other venoms.

The yields of the components were relatively low after high-performance cation exchange and a second gel filtration. A significant amount of material appeared in the low-molecular-weight fractions. Reexamination of the SDS-PAGE gels from the original gel filtration demonstrated a large amount of material running at the buffer front (less than 10,000 MW). This suggests that a large amount of peptide is associated with the venom proteins in the native state.

This is the first article of the isolation of the protein components from pure vespid venoms. The use of pure venoms allows one to use conventional chromatography techniques to isolate the proteins and makes it unnecessary to use extensive recycling techniques. As we have previously demonstrated, commercial venom sac extracts contain a large amount of substances that are not a natural part of the venom.[6, 12, 13] These make the isolation of venom components relatively difficult in many cases. Although the collection of pure venom from live insects is laborious, it is the only method that establishes the venom origin of the proteins. A comparison of our results with the one report of venom separation from a product collected by mass electrical stimulation[7] suggests that the product of mass stimulation is highly contaminated with digestive and/or fecal material. In the following articles the venom proteins isolated in this study are demonstrated to have significant IgE binding activity when these were tested with the sera from allergic individuals,[9] and the immunologic basis of vespid cross-reactivity is investigated by use of these purified materials.[10]

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