

Allergens in Hymenoptera venom XI. Isolation of protein allergens from *Vespula maculifrons* (yellow jacket) venom

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Pure Vespula maculifrons venom was demonstrated to contain five major allergenic proteins, which were all isolated from commercial venom sac extract. The five proteins: Vmacl, MW 97,000; hyaluronidase, MW 46,000; Vmac3, MW 39,000; phospholipase A and B, MW 34,000; and antigen 5, MW 22,000 were all demonstrated to be biochemically and immunologically distinct. All five proteins had significant allergenic activity, with phospholipase and hyaluronidase demonstrating the most IgE binding with 39 sera from allergic patients. Sera from honeybee-reactive patients, who had weak cross-reactions with yellow jacket venom, demonstrated strong IgE binding to purified V. maculifrons hyaluronidase and Vmacl. Dose-dependent inhibition of RAST was observed by use of honeybee hyaluronidase and high-molecular-weight fraction to inhibit the binding to the corresponding yellow jacket allergen. (J ALLERGY CLIN IMMUNOL 74:93, 1984.)

Allergic reactions from insect venoms commonly result from stings by honeybees (*Apis mellifera*), yellow jackets (*Vespula* spp.), hornets (*Dolichovespula* and *Vespa* spp.), paper wasps (*Polistes* spp.), bumblebees (*Bombus* spp.), and fire ants (*Solenopsis* spp.).¹⁻³ Of these important venoms only the venom of the honeybee has been extensively studied, and many of the major components have been isolated.^{1, 4-10} Several investigators have reported on the partial separation and isolation of allergens from *Vespula* and *Dolichovespula* venoms.¹¹⁻¹⁵ These studies have been limited by the use of starting venom preparations that have either been prepared from venom sacs of frozen insects^{11-13, 15} or from venom collected by electrical stimulation.^{12, 14} In contrast to honeybee venom, *Vespula* venoms are not readily obtained in high purity and large quantity from mass electrical stimulation.¹² In addition, recent developments in protein chemistry such as the availability of highly sensitive silver stains that react with a wide variety of proteins,¹⁶ the high-resolving power of two-dimensional electrophoretic

Abbreviations used

HMW:	High-molecular-weight
HPLC:	High-performance liquid chromatography
MW:	Molecular weight
SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

techniques, the development of high performance ion exchangers,^{17, 18} and improved gel permeation chromatography supports now allow studies to be performed with much smaller amounts of material and at a much higher level of resolution than was previously possible. In this article we combine the use of these newer techniques with a method of obtaining high purity *Vespula* venom to isolate all major proteins of *V. maculifrons* venom and to immunochemically and physicochemically characterize the allergens among these proteins.

MATERIAL AND METHODS

Venoms

Reference venom was collected from individual live *V. maculifrons* workers and queens. The insects were chilled to 4°, pinned through the thorax, and warmed to room temperature. The insects were stimulated with metal forceps to

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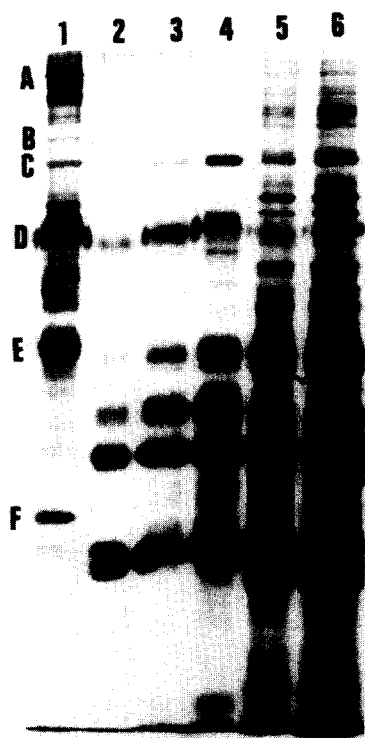


FIG. 1. SDS-PAGE of fresh *V. maculifrons* queen venom and commercial venom-sac extract from *V. maculifrons*. The first lane contains MW marker proteins: A-myosin (205,000), B- β -galactosidase (116,000), C-phosphorylase b (97,000), D-bovine-serum albumin (66,000), E-ovalbumin (45,000), and F-carbonic anhydrase (29,000). Lanes 2, 3, and 4 contain the fresh venom from a single queen; lane 3 contains about twice the protein of lane 2, and lane 4 about 10 times the protein or about 10 μ g. Lanes 5 and 6 contain 10 and 20 μ g, respectively, of *V. maculifrons* commercial venom-sac extract. The gel is stained with Gelcode silver stain.

sting into 6 by 50 mm test tubes. Each droplet of venom was carefully examined for fecal contamination. Most insects would sting four times. No differences were detected in venoms by SDS-PAGE between the first and fourth stings. Pure venom obtained in this manner contained no detectable protease activity but did contain leucine aminopeptidase activity. Further characterization of these preparations have been previously reported.^{10, 19} Approximately 1 mg of venom was collected.

Commercial venom-sac extract prepared from *V. maculifrons* was purchased from Vespa Laboratories, Spring Mills, Pa. Only lot 12118162YJ was used. Protein was estimated by use of the Lowry et al. procedure.²⁰ The reference protein was bee venom for Vespa Laboratories and bovine serum albumin in our own laboratory. The method of preparation of this material has been previously described in detail,^{11, 15} and we have previously reported on its relation to reference preparations of pure venom.¹⁹

Honeybee venom collected by electrical stimulation was

purchased from Champlain Valley Apiaries, Champlain, Vt. The HMW fraction was isolated by high resolution Sephadex G-75 superfine gel filtration as has been previously described.⁷ Hyaluronidase was prepared by gel filtration on Sephadex G-75 followed by ion-exchange chromatography on carboxymethyl-Sepharose.^{5, 6}

Human sera

Sera were obtained from individuals specifically allergic to yellow jackets as well as those with both yellow jacket and hornet reactivity. The sera were stored at -20° until use. Most yellow jacket-reactive sera used in this study have been previously described and used in other studies reported from this laboratory.^{21, 22} All sera were from patients who were both skin test and RAST positive to commercial yellow jacket venom with histories of allergic reaction within 6 mo of sampling. All samples were obtained before any venom treatment. Sera from patients who were highly reactive with honeybee venom and weakly reactive with yellow jacket venom were selected for the cross-reactivity studies. All patients experienced reactions from honeybee stings and were skin test positive to honeybee venom. Control sera were obtained from nonvenom allergic individuals and were RAST negative. Other negative controls included normal horse serum and human cord serum.

SDS-PAGE

SDS-PAGE was performed in slabs by use of a modified Laemmli technique²³ as previously described by our laboratory.^{10, 19} MW markers obtained from Sigma Chemical Co., St. Louis, Mo., were included in each slab. Protein was visualized by use of Gelcode polychromatic silver stain purchased from Upjohn Diagnostics, Kalamazoo, Mich.¹⁶ Reported MWs are the average of results from at least three separate determinations.

Two-dimensional electrophoresis

Protein mapping was performed by the use of a nonequilibrium stacking technique in the first dimension followed by SDS-PAGE in the second dimension. Proteins were visualized with Gelcode silver stain. We have previously described the method in detail.^{10, 19} MWs were run on the sides, and in some case purified proteins were added to the test samples to facilitate comparison of mobilities.

RAST

RAST was performed and scored as previously described by us.²⁴ Cyanogen bromide-activated paper disks were coupled with 2 μ g/disk of purified allergen in borate buffer, pH 8.0. Because of limited material only 1 μ g/disk was used for Vmac3. Test disks were prepared by use of the other allergens at three or four concentrations, and significant decreases in binding were observed with those prepared at less than 1 μ g.²⁵ All results are expressed as percent specific binding. Nonspecific binding was determined for each type of disk in each experiment.

Inhibition of RAST was performed as previously described.²⁶ All inhibition experiments were performed with

individual patient sera, and the range of inhibitor concentrations was 500-fold.

Gel filtration

Commercial venom-sac extract was fractionated on a 1.6 by 100 cm column of Fractogel TSK HW-55 (s) manufactured by EM Reagents (Gibbstown, N. J.). The sample was applied in 8M urea and was eluted with 4M urea in 0.1M NH_4HCO_2 , pH 5.0, at a flow rate of 16 ml/hr. Fractions of 3.2 ml were collected, and the absorbance at 280 nm was monitored. The compositions of fractions were monitored by SDS-PAGE and enzyme activities. Partially purified fractions were run through a 1.6 by 100 cm column of Sephadex G-75 superfine by use of an eluant of 0.1M NH_4HCO_2 , pH 5.0, at a flow rate of 9 ml/hr. Fractions of 2.25 ml were collected and analyzed. Proteins were concentrated by use of Amicon YM-10 membranes, Amicon Corp., Danvers, Mass.

Ion-exchange chromatography

Cation-exchange chromatography was performed by gradient elution by use of a Pharmacia Laboratories (Piscataway, N.J.) Mono-S FPLC column with a Spectraphysics model SP8700 ternary gradient HPLC pump, a Rheodyne 7125 injector (Rheodyne, Inc., Cotati, Calif.) and a Milton Roy LDC UVIII monitor (Riviera Beach, Fla.) at 280 nm. The monitor was connected to an automatic fraction collector by use of capillary tubing. The starting buffer was 0.05M sodium acetate, pH 5.2, at a flow rate of 1 ml/min. The terminating buffer was 0.05M sodium acetate containing 1M NaCl adjusted to pH 5.2. The gradient time was 42 min, producing a rate of 0.024M/min as recommended by the manufacturer. Elution positions were reproducible within a few percent. All eluants were degassed with helium. The injector valve was fitted with a 1 ml sample loop, and as many as five injections could be made before beginning gradient elution without affecting retention times. Pilot experiments with sulfoethylcellulose demonstrated a much lower resolution than the Mono-S column. In rechromatography experiments, recoveries based on absorbance at 280 nm generally exceeded 80%. A single run could handle up to 15 mg of protein without overload.

Enzyme activities

Phospholipase A and B activities were measured by the radial diffusion assays described by Habermann and Hardt²⁷ by use of egg yolk and lysolecithin prepared from egg yolk as substrates. No separation of phospholipase A and B activities were observed.

Hyaluronidase was measured by a radial diffusion assay by use of human umbilical cord-hyaluronic acid as substrate.⁸

Preparations were screened for a variety of other enzyme activities by use of API-ZYM strips purchased from Analytab Products, Chamblee, Ga. These strips included tests for a variety of proteases, peptidases, carbohydrate hydrolases, esterases, and phosphatases. Negative controls were run with all strips, since some gave spurious positive

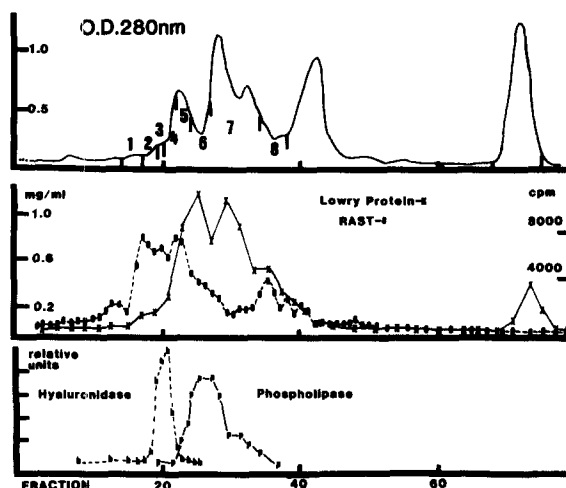


FIG. 2. Gel-filtration profile of the separation of 52 mg of commercial *V. maculifrons* venom-sac extract on Fractogel TSK HW-55 (s) as described in the text. The upper curve indicates the absorbance of the eluant at 280 nm. The vertical marks on the curve and large numbers indicate pools that were made on the basis of enzyme activities and SDS-PAGE. The middle curves indicate the protein content (x) as measured by Lowry protein assay and the RAST activity with pooled sera from yellow jacket-allergic patients (o) of each tube collected. The lower curve indicates hyaluronidase and phospholipase A and B activities. The points not indicated were negative. Phospholipase A and B activities did not separate.

reactions, particularly with phosphoamidase and eight carbon esterase. Proteases were also screened by use of methods from the Worthington manual.²⁸

Rabbit antisera

New Zealand white rabbits were immunized with partially purified fractions (first ion-exchange chromatography) of venom by injection of antigen at pH 7.4 emulsified with Freund's complete adjuvant. Injections were administered in multiple intradermal sites and were repeated after 1 mo. Rabbits were bled from the marginal ear vein 2 wk later. If a sufficient titer was not found by immunodiffusion, a third series of injections were administered. Total amount of antigen administered varied from 120 to 200 μg ; however, an additional 250 μg were administered to two rabbits, each in a third injection course. All rabbits responded. Polyvalent serum was prepared by mixing the individual sera from all of the animals.

Immunochemical techniques

Gel techniques were performed by use of a 1% solution of ME agarose purchased from Marine Colloids Inc., Rockland, Me., in a Tris-barbital buffer.²⁹⁻³¹ Immunodiffusion analysis, immunoelectrophoresis, and crossed immunoelectrophoresis were used to characterize the preparations. After washing and drying, the gels were stained with Serva violet 49 (Serva Fine Biochemicals, Garden City, N. Y.).³²

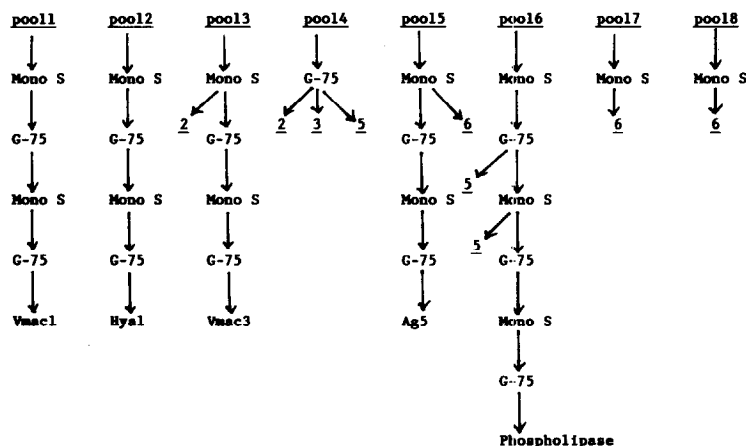


FIG. 3. Flow chart purification of *V. maculifrons*-venom allergens from the pools made from the separation in Fig. 2 to the final fractions.

RESULTS

Characterization of venom preparations

The reference preparations of fresh pure venom and the commercial venom-sac extract from *V. maculifrons* were compared and characterized by electrophoretic, enzyme, and immunochemical tests. The reference venom preparation contained high levels of hyaluronidase, phospholipase A, and phospholipase B activities. Leucine aminopeptidase, four carbon esterase, phosphatases, and β -galactosidase activities were all easily detectable. No tryptic, chymotryptic, or microprotease activity was detected by API-ZYM or spectrophotometric assays. The commercial venom-sac extract contained all of the activities found in the reference venom with a significant increase in phosphatase activities. In addition it contained easily detectable amounts of protease and a variety of other enzyme activities not found in the reference venom.

Both reference venom and venom-sac extract were examined by SDS-PAGE visualized with polychromatic silver stain as is demonstrated in Fig. 1. The reference venom contained five major protein bands of apparent MWs of 102,000, 44,000, 39,000, 34,000, and 26,000. When the gels were heavily loaded, at least 20 minor components could be detected. The venom-sac extract contained the same five major bands with apparent MWs of 98,000, 43,000, 37,000, 33,000, and 25,000 as well as numerous major and minor bands not observed in the reference venom preparation. In the two-dimensional electrophoretic studies we previously reported,¹⁹ there appear to be five major proteins in the pure *V. maculifrons* venom. Numerous proteins are found in the sac extract that are not found in the venom.

Immunoelectrophoretic studies by use of both regular and crossed immunoelectrophoresis demonstrated

that all major venom proteins were present in the sac extract and that the sac extract contained proteins not found in the reference venom preparation.

Preliminary separation experiments

Since it is extremely difficult to obtain milligram quantities of venom by the method used to obtain the reference preparation, we chose to attempt to separate the venom allergens from the venom-sac extract. In preliminary experiments it was found to be necessary to dissolve freshly the sac extract in 8M urea and to centrifuge it immediately before application to chromatography columns. Attempts to fractionate the extracts on Sephadex G-75, Fractogel TSK HW-55 (s), and sulfoethylcellulose were all unsuccessful because of the significant amount of aggregated protein in the extract. The use of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) did not significantly reduce the aggregation problems. However, the use of 4M urea was found to dissociate much of the aggregated protein. A preliminary separation in 4M urea in 0.1M ammonium formate buffer, pH 5.0, was carried out on a 1.5 by 160 cm column of superfine Sephadex G-75. The resolution appeared to be quite good, but the venom proteins were only partially separated when they were tested by enzyme activities and SDS-PAGE. The major problem with this technique was that 5 to 7 days were required for each 40 mg run. Fractogel TSK HW-55 was tested because it tolerates much higher pressures and flow rates than dextran gels. The separation was quite different from that observed with Sephadex requiring a much larger elution volume. SDS-PAGE studies indicated that many of the components were interacting with the plastic matrix. Although the resolution was not as good as the 160 cm G-75 superfine column, it was

TABLE I. Recoveries of purified *V. maculifrons* allergenic fractions from 620 mg of commercial *V. maculifrons* extract

Fraction	mg Recovered	% Of initial extract protein
Antigen 5	34.2	5.5
Phospholipase	10.6	1.7
Vmac1	1.8	0.3
Hyaluronidase	1.7	0.2
Vmac3	0.3	0.04
Total	48.6	7.8

adequate for first-stage purification, and 52 mg of venom-sac extract could be run each day. A typical gel filtration of 52 mg of *V. maculifrons* venom-sac extract is demonstrated in Fig. 2. The results illustrated in the figure include absorbance at 280 nm, Lowry protein, RAST activity with pooled yellow jacket-sensitive sera, phospholipase, and hyaluronidase activities. Fractions were also monitored by SDS-PAGE. Relatively good separation of the enzymes was observed.

Isolation of venom allergens

The material from 12 Fractogel TSK HW-55 (s) gel filtrations representing 620 mg protein starting material were pooled based on enzyme analysis and SDS-PAGE results. The eight pools demonstrated in Fig. 2 that contained almost all venom allergens were each subjected to ion-exchange chromatography on a Mono-S FPLC column as described in Material and Methods. One pool that contained hyaluronidase, antigen 5, and Vmac3 was first separated by gel filtration on a 1.6 by 100 cm column of Sephadex G-75. With use of both enzyme activities and SDS-PAGE as guides, the partially purified allergens were repooled. The fractions containing phospholipase, hyaluronidase, and antigen 5 were all gel filtered on Sephadex G-75, and then the appropriate fractions were chromatographed on the HPLC apparatus with the Mono-S column either once or twice additionally. A flow chart of the purification is demonstrated in Fig. 3. Elution profiles of the five allergens from some of the later purification runs on the Mono-S column are illustrated in Fig. 4. The fractions pooled are indicated by the horizontal arrows. Repeated purification cycles were terminated when no improvement between products of successive runs was seen in SDS-PAGE. The final yields are listed in Table I. Only 7.8% of the initial venom-sac protein was recovered. From enzyme activities we estimate about a 50% recovery, suggesting that the commercial material is about 15% venom protein.

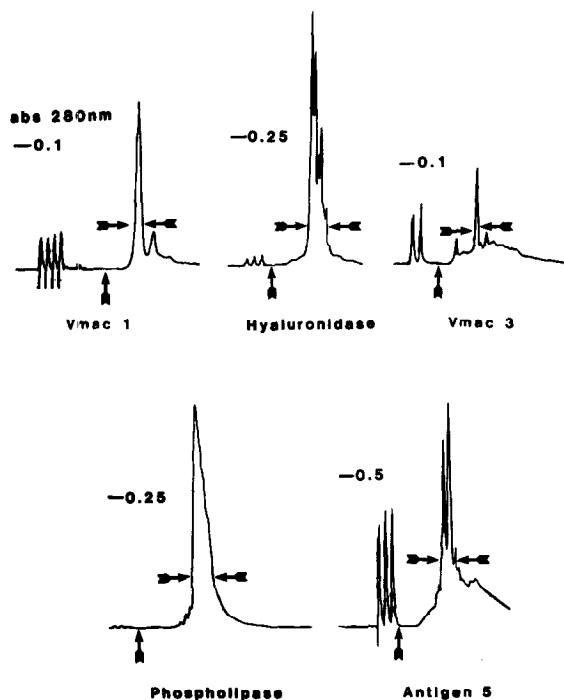


FIG. 4. Elution profiles from HPLC runs by use of a gradient of 0M to 1M NaCl in 0.05M sodium acetate buffer, pH 5.2, and a Pharmacia Mono-S FPLC column eluted at 1 ml/min. The last run in the purification scheme is indicated for each protein fraction. The vertical arrows indicate the start of the gradient, and the horizontal arrows indicate the pool that was made for each purified protein fraction. The elution positions in the gradient and the fine structures for each protein were high reproducible from run to run. Pools were made based on SDS-PAGE analysis.

Properties of venom proteins

Five proteins were isolated from the venom-sac extract and were identical to the five proteins found in the reference venom preparation by SDS-PAGE, two-dimensional electrophoresis, crossed immunoelectrophoresis, and enzyme activities. The largest protein revealed an apparent MW of 97,000 on SDS-PAGE and was only weakly basic; it is referred to as Vmac1. Hyaluronidase revealed a MW of 46,000 and appeared to exist in two forms slightly differing in size and charge. The third protein is present in much higher concentrations in queen venom than in worker venom and had an apparent MW of 39,000; it is referred to as Vmac3. The phospholipase contained both the A and B activities, which did not separate in any procedure we attempted. The apparent MW of phospholipase was 34,000. The fifth protein was identified as antigen 5 of King and associates^{11, 13, 15} by its size, lack of enzyme activity, and by its reactivity with an antiserum kindly provided by Dr. T. P. King. Its apparent MW was 22,000.

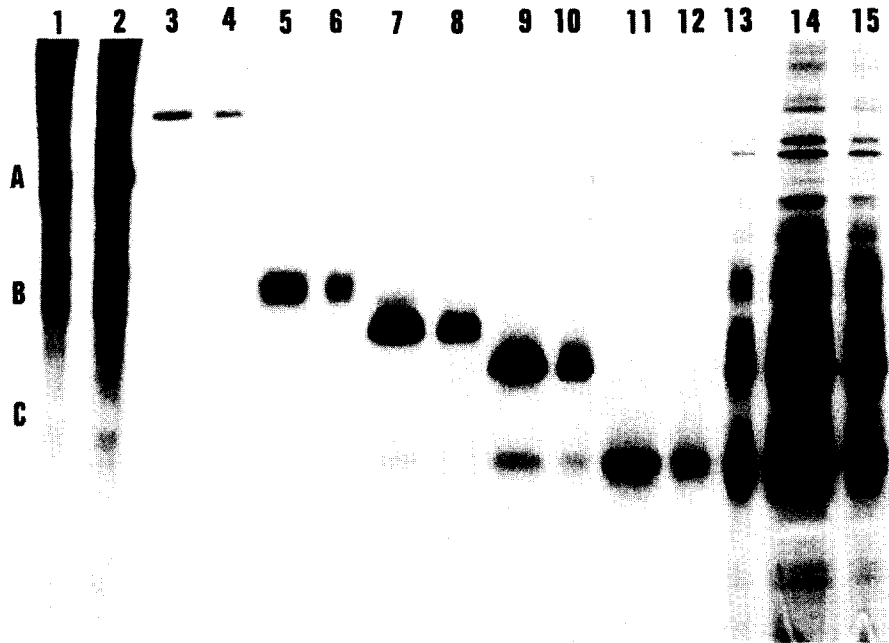


FIG. 5. SDS-PAGE of purified *V. maculifrons*-venom protein fractions. Lanes 1 and 2 contain MW marker proteins. A-66,000, B-45,000, and C-29,000. Lanes 3 through 12 contain the purified protein fractions at approximate protein contents of 0.6 and 0.2 μ g. Lanes 3 and 4 contain Vmac1, lanes 5 and 6 contain hyaluronidase, lanes 7 and 8 contain Vmac3, lanes 9 and 10 contain phospholipase, and lanes 11 and 12 contain antigen 5. Lanes 13, 14, and 15 contain commercial *V. maculifrons* venom-sac extract at loadings of 5, 20 and 10 μ g of protein, respectively. The gel is stained with Gelcode silver stain. Coomassie blue visualization required approximately 50 times the protein load and demonstrated many fewer components.

TABLE II. Estimates of purities of *V. maculifrons* allergenic fractions

Fraction	Vmac1	Hyaluronidase	Vmac3	Phospholipase	Antigen 5
Vmac1		Undet*	Undet†	Undet*	Undet†
Hyaluronidase	Undet†		Undet	0.1%	Trace
Vmac3	Undet	1% to 2%		0.3%	Trace
Phospholipase	Undet	<0.1%	Undet		2% to 4%
Antigen 5	Undet	Undet	Undet	0.9%	

Undet = undetectable.

*Hyaluronidase and phospholipase were estimated by radial diffusion enzyme assays by use of dilutions of purified bee venom enzymes as standards. Results are indicated as percent of enzyme activity per unit of protein referred to the purified *V. maculifrons* enzyme.

†Estimates are from analytical assays by use of a Mono S HPLC column, silver stained SDS-PAGE, and immunochemical analysis.

The purities of the venom protein fractions were estimated by enzyme activities, integration of the final HPLC profiles, SDS-PAGE, two-dimensional electrophoresis, and crossed immunoelectrophoresis. As is demonstrated in Table II, three of the proteins were detectably contaminated with each other. The Vmac3 preparation contained about 2% hyaluronidase; although it was probably possible to reduce greatly this level, the small amount of Vmac3 prohibited further purification cycles. The phospholipase contained up to 4% antigen 5; this was not reduced by G-75 gel filtration followed by ion-exchange chromatography.

The antigen 5 contains a small amount of phospholipase activity since no 34,000 MW band is visible on SDS-PAGE of antigen 5 as demonstrated in Fig. 5. This is probably caused by a fragment of phospholipase of similar MW to antigen 5. Traces of a few venom sac proteins are visible in the purified fractions.

RASTs

Each of the five purified *V. maculifrons*-venom protein fractions was coupled to activated paper disks as described in Material and Methods after optimization experiments. Each protein was tested against a

TABLE III. RAST results of 39 yellow jacket-reactive sera with the five purified protein fractions from *V. maculifrons*

Serum	Vmac1	Hyaluronidase	Vmac3	Phospholipase	Antigen 5
2597	1.1	11.1	11.6	13.4	5.1
2694	10.7	10.7	7.7	14.2	6.8
2762	11.2	11.6	14.1	6.5	7.9
2788	11.5	25.2	13.2	13.1	7.1
2794	2.0	4.5	4.6	2.6	1.8
2874	6.4	4.6	6.2	4.7	5.5
2883	0.2	9.7	5.8	7.7	11.0
2962	4.0	9.4	11.1	10.4	8.1
3016	3.6	5.3	5.6	4.6	2.8
3117	1.8	13.6	13.1	17.5	3.1
3351	3.1	14.9	5.9	8.1	7.3
3526	14.9	15.3	5.1	12.6	4.3
3644	18.7	25.5	25.7	21.0	33.7
3900	1.0	2.4	2.8	8.3	0.8
3926	3.9	5.5	2.0	21.6	13.5
4092	10.9	6.1	5.1	4.8	3.4
4127	3.3	4.6	3.5	1.9	2.8
4428	4.9	8.0	5.1	6.7	2.5
4661	1.9	3.8	7.3	18.3	1.6
4662	5.1	0.5	0.9	0.2	0.5
4670	5.0	9.5	3.3	6.9	2.2
5673	10.2	15.1	13.9	7.6	5.6
5675	5.2	7.6	5.7	10.7	3.7
5747	2.3	9.5	2.9	16.5	18.9
5865	10.9	10.5	11.2	15.9	6.7
5875	0.3	5.3	4.6	10.6	3.3
5934	3.9	7.0	5.7	12.7	12.5
6129	0.6	2.8	2.2	5.8	4.9
6250	1.5	12.2	4.1	13.9	7.6
6426	1.5	14.0	6.2	13.8	7.4
6603	5.3	3.3	3.2	3.9	0.7
6738	10.3	11.4	12.4	16.5	8.2
6797	1.4	2.5	1.6	5.3	1.0
10566	3.5	6.7	9.8	8.0	1.3
10655	2.2	3.0	3.0	8.6	1.9
10676	2.6	1.7	0.7	2.7	0.4
10873	10.9	17.9	14.2	16.0	5.1
10900	9.1	14.8	13.8	1.1	5.9
10980	3.4	4.9	2.8	15.8	4.1

Results are expressed in percent specific binding.

panel of 39 sera obtained from yellow jacket-sensitive patients as well as a panel of negative control sera. All negative controls bound from 0.2% to 0.7% of counts were added. Nonspecific binding was a function of the antigen with phospholipase exhibiting the highest level. The nonspecific binding has been subtracted from all of the RAST results presented in Table III. Of the 39 allergic sera in the panel, 33 sera were used in previous studies of yellow jacket allergens.^{21, 22} Significant binding was observed for all five venom protein fractions indicating that each

had allergenic activity. The strongest average binding was to phospholipase, followed by hyaluronidase, Vmac3, antigen 5, and Vmac1. Of the 39 sera, 18 or 46% were most reactive with phospholipase, 10 or 26% were most reactive with hyaluronidase, 4 or 10% were most reactive with either Vmac1 or Vmac3, and 3 or 8% were most reactive with antigen 5. Almost all sera demonstrated significant reactivity with all five fractions, although four sera exhibited negative or marginal binding to at least one fraction. Serum 4662 was relatively specific for Vmac1.

TABLE IV. Correlation coefficients between RAST results with the five purified allergenic fractions of *V. maculifrons* venom tested with the sera from the 39 allergic patients in Table III

	Hyaluron- idase	Vmac3	Phospho- lipase	Antigen 5
Vmac1	0.65	0.67	0.25	0.43
Hyaluronidase		0.80	0.50	0.58
Vmac3			0.43	0.55
Phospholipase				0.57

The correlation coefficients calculated from the RAST data in Table III are demonstrated in Table IV. All are in the range of 0.25 and 0.67 except that between hyaluronidase and Vmac3, which is 0.80. This higher association is not unexpected, since Vmac3 is contaminated with hyaluronidase. Although these values indicated a significant degree of association among the fractions, they are substantially below the values expected for very similar or identical allergens.^{21, 22, 23} These data indicate that all major proteins of *V. maculifrons* venom can be significant allergens. A similar association of reactivity to different allergens is observed in studies of highly purified egg white allergens.³³

Immunochemical analysis

Rabbit antisera were raised against four partially purified *V. maculifrons* fractions and against whole commercial venom-sac extract. Significant antibody titers were produced against four of the five of the major venom proteins, and lower titers were produced against some of the sac proteins. The antisera raised against venom-sac extracts were much less potent, and a polyvalent antiserum was prepared by pooling the antisera against the partially purified fractions. An additional polyvalent antiserum was kindly provided by Dr. T. P. King. The purified fractions were analyzed by double immunodiffusion, immunoelectrophoresis, and crossed immunoelectrophoresis. The rabbit antisera demonstrated that all five major proteins were noncross-reactive antigens. Results of immunoelectrophoresis for venom-sac extract and the five purified proteins are demonstrated in Fig. 6.

Cross-reactivity with honeybee venom proteins

A significant fraction of sera that are strongly reactive with honeybee venom by RAST demonstrate weak reactivity with yellow jacket venom. Similar results can be obtained with skin testing. In many cases

these patients only have histories of honeybee stings and come from areas where vespid stings are rare.³ Reports have appeared in the literature of cross-reactivity between bee and vespid allergens.^{34, 35} Nine sera were selected from patients with a history of allergic reactions to bee stings who demonstrated a strong positive RAST to bee stings with a weakly positive RAST (and in most cases skin test) to yellow jacket stings. These sera were tested with disks prepared from the five purified *V. maculifrons* proteins. The results are demonstrated in Table V. Seven of the sera reacted very strongly with hyaluronidase, four with Vmac1, and three with Vmac3. Reactivity with phospholipase and antigen 5 was much weaker. Although it is impossible to rule out sensitization from previous vespid stings, this suggests that there is cross-reactivity with hyaluronidase, Vmac1, and possibly Vmac3. There was insufficient Vmac3 for further testing.

RAST inhibition experiments were carried out with six of these sera. Binding to the purified yellow jacket allergens was inhibited by bee-venom allergens in six of the seven experiments presented in Table VI. The HMW fraction of bee venom containing acid phosphatase, allergen C, and some additional allergenic proteins but free of hyaluronidase, phospholipase A, and melittin revealed dose-dependent inhibition of RAST to Vmac1 with two of the three sera tested. Purified bee venom hyaluronidase free of phospholipase A revealed dose-dependent inhibition of RAST to *V. maculifrons* hyaluronidase with all four sera tested. The concentrations revealing 50% inhibition were comparable to those observed in comparisons of different venoms.^{5, 7, 21, 22, 26, 36} These results indicate that *V. maculifrons*-venom hyaluronidase and Vmac1 are cross-reactive in at least some patients with the homologous honeybee-venom proteins.

DISCUSSION

A method was developed for obtaining pure venom from vespid wasps, uncontaminated by sac proteins or fecal material. Pure venom from *V. maculifrons* (eastern yellow jacket) was demonstrated to contain five major proteins plus additional trace proteins. There was no detectable protease activity in the preparation. Enzymes detected included hyaluronidase, phospholipase A, phospholipase B, acid and alkaline phosphatases, leucine aminopeptidase, esterases, and β -galactosidase. Commercial venom-sac extract from the same species was demonstrated to contain all five major proteins plus a large number of proteins not found in pure venom.

The commercial venom-sac extract was difficult to separate because of aggregation and partial solubility

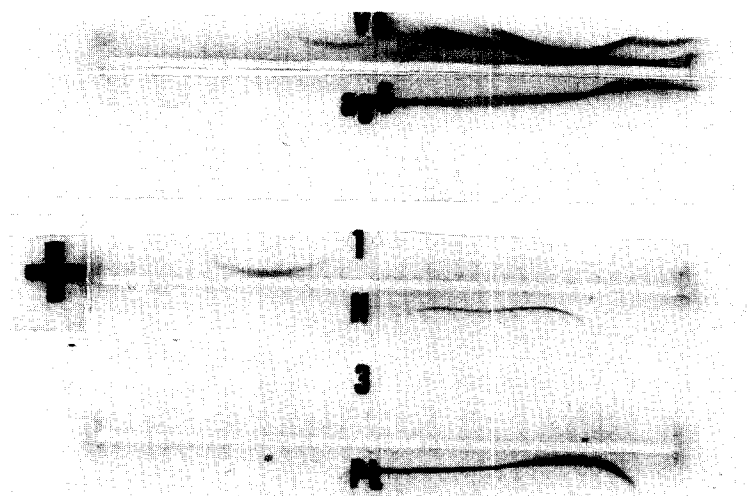


FIG. 6. Immunoelectrophoresis of *V. maculifrons* commercial venom-sac extract (*vs*) at 4 mg/ml, purified antigen 5 (*ag5*) at 1 mg/ml, Vmac1 (*1*) at 1 mg/ml, hyaluronidase (*H*) at 1 mg/ml, Vmac3 (*3*) at 0.3 mg/ml, and phospholipase (*PL*) at 1 mg/ml against pooled polyvalent anti-*V. maculifrons* venom-sac extract serum (see Material and Methods). The antiserum reacts only weakly with Vmac3.

TABLE V. RAST results for *V. maculifrons* allergens by use of sera from honeybee-allergic patients

Serum	Vmac1	Hyaluronidase	Vmac3	Phospholipase	Antigen 5
10586	5.7	9.9	6.8	0.1	2.1
10656	25.5	30.1	29.4	2.9	14.2
10657	7.9	5.9	10.3	2.5	3.4
10675	21.9	10.9	4.8	5.3	10.6
10703	14.1	21.5	20.6	3.8	9.2
10841	6.7	13.0	7.3	2.7	2.4
10874	4.5	13.8	9.6	0.2	1.6
10940	8.9	17.3	9.2	0.6	4.3
11246	3.8	5.6	4.6	0.5	1.2

Results are expressed in percent specific binding. All sera demonstrated at least three times the binding to honeybee venom that was found to yellow jacket venom. Binding to bee venom was from 10% to 30% and binding from 2% to 8% to yellow jacket venom.

of some of the proteins. In addition it contained protease that degraded the venom proteins. A method was developed by use of a plastic gel filtration material, Fractogel TSK HW-55 (s), with 4M urea in the eluant that could rapidly provide a preliminary separation of the venom proteins from most extraneous sac proteins and venom peptides. The fractions from the Fractogel column were pooled and purified by high-performance, ion-exchange chromatography on a Pharmacia Mono-S FPLC column and gel filtration by use of Sephadex G-75 superfine. All five major venom proteins were isolated and purified. Unlike the starting venom-sac extract, the isolated venom proteins were all stable in solution at 4° C for at least 2 mo without any additives when they were tested by SDS-electrophoresis, 2-dimensional electrophoresis, enzyme activities, and immunoelectrophoresis.

The five venom proteins were Vmac1, MW 97,000; hyaluronidase, MW 46,000; Vmac3, MW 39,000; phospholipase A and B, MW 34,000; and antigen 5 as described by King and associates,^{11, 13, 14} MW 22,000. By the use of immunochemical techniques with rabbit antisera, all five proteins were demonstrated to be immunologically unrelated. They were all distinct and corresponded to proteins found in the pure venom when they were tested by SDS-PAGE, two-dimensional electrophoresis, double immunodiffusion, immunoelectrophoresis, and crossed immunoelectrophoresis.

The allergenicity of the five venom protein fractions was tested by RAST by use of a panel of 39 sera from yellow jacket-sensitive individuals. All five fractions were demonstrated to have significant allergenic activity. Phospholipase was the strongest

TABLE VI. RAST inhibition of binding to yellow jacket allergens by honeybee allergens by use of individual honeybee-specific sera

Serum	<i>V. maculifrons</i> allergen	Bee inhibitor	50% Inhibition	Max % of inhibition
10675	Vmac1	HMW*	no inhibition	0
10656	Vmac1	HMW	280 µg/ml	91
10703	Vmac1	HMW	44	69
10703	Hyaluronidase	Hyaluronidase†	6	67
10841	Hyaluronidase	Hyaluronidase	40	63
10874	Hyaluronidase	Hyaluronidase	1.2	91
10940	Hyaluronidase	Hyaluronidase	1.3	82

*HMW fraction from honeybee venom separated by Sephadex G-75 gel filtration, which contains acid phosphatase, allergen C, and other minor allergens but is devoid of hyaluronidase and phospholipase activities. Inhibition was performed at from concentrations ranging from 20 to 3500 µg/ml.

†Inhibition was performed at five concentrations ranging from 1 to 65 µg/ml.

binding allergen, followed by hyaluronidase, Vmac1 and Vmac3, and antigen 5. At least three of the sera reacted most strongly with each one of the allergens, suggesting that all five proteins are significant allergens. Statistical analysis of the RAST results for the five fractions suggested that all are distinct, although there was a strong relation between reactivity to hyaluronidase and Vmac3. However, this is probably a result of the contamination of Vmac3 with hyaluronidase. The degree of association between pairs of allergens was comparable to that observed with egg white allergens³³ where all the proteins are unquestionably distinct. Most patients reacted to all of the allergens; only one serum was relatively specific for a single allergen.

In a small number of histamine-release experiments, King et al.¹⁵ reported three patients that had strong reactions with phospholipase from *Vespula* and one that reacted more strongly with antigen 5. They did not report results for hyaluronidase. King and associates^{13, 15} used affinity chromatography to isolate the phospholipase fractions from three species of *Vespula*. The phospholipase fraction they described differs from that described by us in that the King fraction contains proteins visible in SDS-PAGE not found in ours. The properties of the antigen 5 fractions and the hyaluronidase fractions are very similar in both studies. The fractions Vmac1 and Vmac3 have not been described previously. King and associates did not report on phospholipase B activity but did detect the presence of peptidase.

An immunologic cross-reactivity has been noted in some patients between honeybee and yellow jacket allergens.^{34, 35} A much larger fraction of patients react with both venoms than would be predicted, if the allergens were totally unrelated.¹⁻³ A panel of sera from honeybee-sensitive patients that exhibited weak reactivity with yellow jacket venom was used to de-

termine which of the purified yellow jacket allergens might be cross-reactive with bee allergens. Of the five allergens strong reactivity was observed with hyaluronidase and Vmac1 with much less reactivity to the other three fractions. RAST inhibition was performed by use of individual sera to demonstrate the dose-dependent inhibition of binding to *V. maculifrons* hyaluronidase and Vmac1 by, respectively, bee venom hyaluronidase and HMW allergens. This cross-reactivity was found in all four cases tested with hyaluronidase and with two or three tested with Vmac1. This demonstrates the immunological basis for the observed cross-reactivity and possibly the basis of much of the multiple reactivity to bee and vespids.

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