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IMMUNOCHEMICAL STUDIES OF YELLOWJACKET VENOM PROTEINS*

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Abstract—The major proteins of yellowjacket venoms have been isolated and characterized immunochemically. They consist of hyaluronidase, phospholipase, and antigen 5. Venoms from three species of yellowjacket were studied, *Vespula germanica*, *V. maculifrons*, and *V. vulgaris*. The phospholipases could be isolated in good yield only when affinity chromatography was used to minimize limited proteolysis. A kallikrein-like peptidase was found present in the yellowjacket venom. Phospholipases from these three species were immunochemically indistinguishable from each other, as were their antigen 5s. Sera from individuals sensitive to yellowjacket venom contained IgE and IgG specific for antigen 5 and phospholipase.

INTRODUCTION

Bee and vespid stings can cause allergic reactions in sensitized humans. The vespids include hornets, yellowjackets and wasps. We have shown (King *et al.*, 1978b) that the venom sac extracts of hornets and yellowjackets are composed of similar proteins. Three basic proteins of hornet venoms were identified as allergens in humans. Two of these have hyaluronidase and phospholipase activities and a third one, antigen 5, has no presently known biochemical function. Antigen 5 may be a presynaptic toxin active at the neuromuscular junction of crustaceans because its physicochemical properties are similar to those reported for a neurotoxin isolated from an Asian species of hornet, *Vespa mandarina* (Abe *et al.*, 1982).

In our previous study with yellowjacket venom, we succeeded in purifying the antigen 5 component, but the other components, hyaluronidase and phospholipase, were not purified. During isolation, a nearly complete loss of these two enzymatic activities was noted. Accordingly, we have re-investigated the separation and characterization of venom proteins from three species of yellowjackets: *Vespula germanica*, *V. maculifrons*, and *V. vulgaris*.

MATERIALS AND METHODS

Venom sac extracts of *V. maculifrons* (Lot

No. 110792 YJ), *V. germanica* (Lot No. 12038047 YJ), and *V. vulgaris* (Lot No. 12038048 YJ) were prepared by Vespa Laboratories, Spring Mills, Pennsylvania, in the following manner. Collected insects were stored frozen at -20°C until use. For venom extraction, about 50 dissected venom sacs in 5 ml of 25 mM β -alanine-acetic acid buffer (pH 4.6) containing 0.45% NaCl at 4 $^{\circ}\text{C}$ were homogenized for 5–10 sec. The homogenized sacs were collected by centrifugation and re-extracted overnight at 4 $^{\circ}\text{C}$. The combined supernatants were filtered through 0.45 μm Millipore filters, lyophilized, and stored frozen. Two small samples of venoms from *V. maculifrons* and *V. germanica* were collected by milking of the dissected venom sacs, i.e. by gentle squeezing, and the venom collected in the β -alanine buffer was filtered and lyophilized.

In this study, the venom weights were determined by amino acid analysis following acid hydrolysis of about 10 μg samples in 50 μl of 6 *N* HCl at 110 $^{\circ}\text{C}$ in sealed and evacuated tubes (<0.1 torr.). The venom weights as determined by amino acid analysis (Liao *et al.*, 1973) were approximately 73% of those found by Vespa Laboratories, where venom weights were determined by Folin color analysis with bee venom as the standard. Half-cystine residues of purified proteins were determined with their reduced and carboxamidomethylated derivatives. About 5 μM protein was reduced with 30 mM dithiothreitol in 8 *M* guanidine hydrochloride at pH 8.6 for 4 hr at room temp.

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alkylated with 60 mM iodoacetamide for 30 min, then dialyzed.

The absorbent for affinity chromatography of phospholipase (Rock & Snyder, 1975) was prepared by a carbodiimide-promoted coupling of racemic 1-(8-carboxyoctyl)-2-hexadecylglycerol-3-phosphorylcholine (PC) to AH-Sepharose 4B (Pharmacia, Uppsala, Sweden). The required PC compound was prepared by oxidation of racemic 3-(octa-dec-9-enyl)-2-hexadecylglycerol-1-phosphorylcholine (Vega Biochemicals) (Shimojo *et al.*, 1974). A sorbent containing concanavalin A was prepared by coupling the lectin (10 mg) to CNBr-activated Sepharose 4B (Pharmacia) following the directions supplied by the manufacturer.

Protein solutions were concentrated by ultrafiltration through Amicon membrane UM-2. Dialysis was carried out in Visking 8 32 tubing. Sodium dodecyl sulfate slab gel electrophoresis (Laemmli, 1970) was carried out using the published procedures. The protein bands in the gels were detected with silver stain (Oakley *et al.*, 1980) or with Coomassie Blue R-250 stain (Reisner *et al.*, 1975).

Phospholipase activity was determined titrimetrically either with 10% egg yolk in 0.1 N NaCl containing 0.5% Triton X-100 or with a 1 mg per ml suspension of purified egg lecithin (Type VI-E, Sigma) in 1 mM Tris-HCl buffer (pH 8) containing 10 mM CaCl₂, 20 mM KCl, and 0.5% Triton X-100. One unit of phospholipase activity is defined as the amount of enzyme which catalyzes the release of 1 μ mole of acid per minute at pH 8 and 25°C in 3 ml of substrate solution. Identical specific activities of the venoms or purified enzymes ($\pm 30\%$) were observed with these two substrates.

Hyaluronidase activity was measured turbidimetrically (Tolksdorf *et al.*, 1949). One unit of activity is defined as the amount of enzyme required to hydrolyze 1 μ g of hyaluronic acid (U.S. Biochemical) per min at 25°C in 200 μ l of a 200 μ g per ml solution of hyaluronic acid at pH 5.3. Peptidase activity was determined spectrophotometrically with a 2 mM solution of D-valyl-L-leucyl-L-arginyl *p*-nitroanilide (Ortho S-2266 peptide) in 0.1 M sodium phosphate of pH 8. One unit of activity is defined as the amount of enzyme required to hydrolyze 1 μ mole of substrate per min in 1 ml of the above solution at 25°C. Proteinase activity (Rinderknecht *et al.*, 1968) was determined using an insoluble colored substrate, hide powder azure (CalBiochem, Los Angeles, CA),

as follows. To test tubes (0.9 $\times$ 7.5 cm) were added the enzyme and the substrate (5 mg) in 0.75 ml of 0.1 M phosphate at pH 8 together with a glass bead of 3 mm dia. to aid in mixing. The tubes were rotated on a shaker in a slanted position for the desired time, then the released blue dye was measured at 595 nm. A standard curve correlating the absorbance at 595 nm to the amount of substrate digested was established on complete solubilization of known amounts of substrate by trypsin digestion. One unit of activity is defined as the amount of enzyme required to hydrolyze 1 mg of the substrate in 20 hr under the above conditions.

Immunosorbents containing different proteins were prepared in the same manner as described for lectin-containing columns; they were used for isolation of specific antibodies (King *et al.*, 1978a). Immunodiffusion was carried out in agarose gel.

Rabbit anti-sera specific for the venom sac extraction of *V. maculifrons* were prepared in three New Zealand white rabbits (King *et al.*, 1978b). Mouse antisera specific for purified venom proteins were prepared in groups of three mice of BALB/c strain (20–25 g weight) by intraperitoneal immunization with 5 μ g of protein and 1 mg of alum in 0.2 ml of 5 mM sodium phosphate buffer (pH 6.4) + 0.15 M sodium chloride. Human sera specific for yellowjacket venom were obtained from allergic patients who were receiving venom therapy (Lichtenstein *et al.*, 1979). These sera were reported to contain venom-specific IgG in the range of 20–70 μ g per ml on radioimmunoassay with venom protein-coupled Sepharose beads as the solid-phase antigen (Hamilton & Adkinson, 1981).

Enzyme immunoassays were used to measure the binding of human, mouse, or rabbit antibodies to solid-phase antigen (Giallongo *et al.*, 1982), or the inhibition of antibody binding to solid-phase antigen by test antigens. The reagents included (1) peroxidase-labeled sheep antibodies specific for rabbit IgG, (2) rabbit antiserum specific for mouse IgG (γ -chain), (3) rabbit antibodies specific for human IgG (Fc), and (4) rabbit antibodies specific for human IgE (Fc). The first two reagents have been described previously (Giallongo *et al.*, 1982). The third reagent was prepared by affinity chromatography of an antiserum sample (Cappel Laboratory) on an immunosorbent containing human IgG. The

fourth reagent, obtained from Research Reagents Branch, National Institute of Allergy and Infectious Diseases, was further purified by passage through a human IgG-immunosorbent. The level of cross-contamination of the third and the fourth reagents was established by immunoassay (Giallongo *et al.*, 1982) to be less than 0.4 and 0.06%, respectively.

Allergic activities of the venom fractions were studied by the histamine release method from leukocytes of patients sensitive to yellowjacket venom (Lichtenstein & Osler, 1964; Siriganian, 1974).

Isolation procedure of yellowjacket venom proteins

Lyophilized venom sac extracts (15–45 mg) were dissolved in about 7 ml of 50 mM Tris-HCl buffer at pH 7.95 and dialyzed against the same buffer overnight at 4°C. The dialyzed solution, after adjusting to 10 mM CaCl₂, was applied to a PC column (22 $\times$ 60 mm). The breakthrough peak contained antigen 5 and hyaluronidase. The affinity column was washed with 50 mM Tris-HCl buffer of pH 7.95 containing 10 mM CaCl₂ and 0.5 M NaCl, then the bound phospholipase was eluted with 0.1 M acetic acid at 90 ml per hr. Fractions of 3 ml volume were collected in tubes containing 0.4 ml of 1 M Tris-HCl of pH 7.95. In some instances the breakthrough peak had to be re-chromatographed on the same column because of incomplete absorption of phospholipase.

The breakthrough peak from the PC column was concentrated and used for gel filtration on Sephadex G-100 (200 $\times$ 0.9 cm). A buffer of 50 mM ammonium acetate (pH 4.75) was used at a flow rate of 10 ml/hr. The fractions containing hyaluronidase or antigen 5 were concentrated and re-chromatographed on Sephadex G-100. They were then separated by ion-exchange chromatography on CM-32 cellulose. The column (20 $\times$ 0.9 cm) was eluted at 60 ml/hr with a linear NaCl gradient of 1.25 mM per ml in 20 mM ammonium acetate buffer of pH 4.75. Alternatively, a 10 $\times$ 0.9 cm column was used with a linear NaCl gradient of 2.08 mM per ml in 50 mM sodium phosphate buffer of pH 7.02. The phospholipase peak from PC column was also purified on CM-32 cellulose under these conditions.

RESULTS

In our previous study of yellowjacket venom proteins, venom sac extracts had been collected from a mixture of three species of yellowjackets (*Vespa maculifrons*, *V. germanica*, and *V. vulgaris*). In the present work, venom sac extracts from each of these three species of yellowjackets were collected and examined separately. Small amounts of venom which were obtained on milking the extirpated venom sacs of *V. maculifrons* and *V. germanica* were studied also.

In Table 1 are listed the hyaluronidase, phospholipase, peptidase, and proteinase activities present in the venoms or venom sac extracts of three species of yellowjackets. Similar data for the venom sac extract of a mixture of these three species, reported previously, are included for comparison. The venom sac extracts from the three species of yellowjackets differed in their contents of hyaluronidase and phospholipase activities. The sac extract of *V. germanica* contained the highest phospholipase activity while that of *V. vulgaris* contained the lowest phospholipase activity but the highest hyaluronidase activity. The venom of *V. maculifrons* contained nearly twice the phospholipase activity as did its venom sac extract, and that level of activity was nearly the same as that in the venom or the venom sac extract of *V. germanica*. The hyaluronidase activities in the venoms and the venom sac extract were the same for these two species.

The venom and venom sac extracts from *V. germanica* or *V. maculifrons* differed in their contents of peptidase and proteinase activities (Table 1). The large difference in proteinase activities of the venom and the venom sac extracts suggests that this activity is probably a tissue contaminant in the venom. For convenience, we designate the activity detected with the substrate, D-valyl-L-leucyl-L-arginyl *p*-nitroanilide, as a peptidase although this substrate has been used for assaying glandular kallikrein (Claeson *et al.*, 1978). The venom sac extracts were also tested with other substrates; these were *N*, α -benzoyl-L-arginine ethyl ester and its *p*-nitroanilide, and *N*, α -benzoyl-L-tyrosine ethyl ester. The results were negative.

The peptidase and the proteinase have about the same molecular size as the phospholipase and hyaluronidase. Gel filtration of *V. maculifrons* venom sac extract on Sephadex G-100 showed that these four activities were eluted in approximately the same region (Fig. 1). The

Table 1. Comparative biochemical properties of yellowjacket venoms

	<i>V. germanica</i>			<i>V. maculifrons</i>			<i>V. vulgaris</i> sac extract	Yellowjacket ^a sac extract
	sac extract	venom	venom	sac extract	venom	venom		
Hyaluronidase (units/mg of venom)	190 ± 28	193 ± 17	171 ± 6	174 ± 28	171 ± 6	171 ± 6	464 ± 65	470
Phospholipase (units/mg of venom)	940 ± 110	810 ± 90	780 ± 90	360 ± 90	780 ± 90	780 ± 90	170 ± 40	210
Peptidase (m units/mg of venom)	154 ± 9	11.2 ± 0.5	25.9 ± 1.0	34.9 ± 1.3	25.9 ± 1.0	25.9 ± 1.0	86.1	—
Proteinase (units/mg of venom)	0.48 ± 0.20	0.07 ± 0.20	0.34 ± 0.08	6.31 ± 0.99	0.34 ± 0.08	0.34 ± 0.08	2.5	—
% of venom as proteins ^b	34.4 ± 0.2			43.3 ± 2.6			38.8 ± 2.3	30 ± 3
% of venom as: ^c								
hyaluronidase	0.62			0.5			1.5	1.5
phospholipase	6.9			6.9			3.3	0.69
antigen 5	1.4			8.7			8.1	4.5

^aPreviously reported data for a mixture of the indicated three species of yellowjackets. Phospholipase activity, previously determined with a 10% egg yolk solution, was 4.3 times lower than the present one in which a 10% egg yolk solution containing 0.5% Triton X-100 was used.

^bVenom and venom protein weights were determined by amino acid analysis following acid hydrolysis of venom samples before and after dialysis.

^cThe amounts of antigen 5 in venoms were estimated from isolation experiments after correction for a 50% loss in workup. The amounts of hyaluronidase and phospholipase in venoms were estimated from the specific activities of purified enzymes. The specific activities of the purified phospholipases from *V. germanica*, *V. maculifrons*, and *V. vulgaris* were, respectively, taken to be 1.2×10^4 , 4.3×10^5 , and 4.3×10^5 units per mg, while those of hyaluronidases were taken to be 3.1×10^4 units per mg.

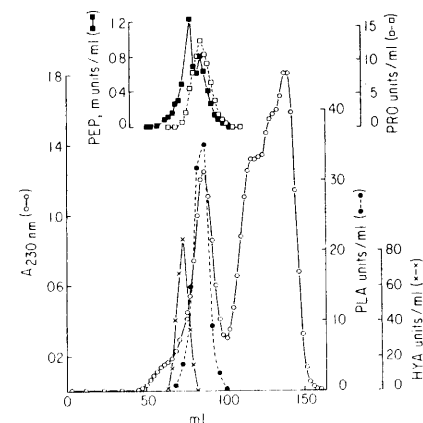


Fig. 1. Separation of venom sac extract of *Vespula maculifrons* on a 200 × 0.9 cm column of Sephadex G-100. About 7.6 mg of venom proteins were applied and the column was eluted with 20 mM ammonium acetate (pH 4.75). Fractions were analyzed for hyaluronidase (HYA), phospholipase (PLA), peptidase (PEP), and proteinase (PRO) activities.

large peak at about 85 ml contained antigen 5 in addition to the indicated phospholipase and proteinase, and the results below indicate that the proteinase is a trace component in that peak.

The stability data of phospholipase activity in yellowjacket venoms, venom sac extracts, and preparations partially purified by affinity chromatography given in Table 2 are described below. Good stability of phospholipase in the venom of *V. maculifrons* or *V. germanica* was observed at pH 4.7 or 8. However, phospholipase in the venom sac extract of *V. maculi-*

frons showed very poor stability, while good stability was found in the venom sac extracts of *V. germanica* and *V. vulgaris*. The stability of phospholipase from venom sac extract of *V. maculifrons* was markedly improved following partial purification by affinity chromatography. The stability of hyaluronidase in the venom and in the venom sac extract of *V. maculifrons* and *V. germanica* was also compared under the conditions given in Table 2, and no significant differences were observed between the venom and the venom sac extract.

The yellowjacket venom proteins were best fractionated according to the following scheme: the venom was separated by affinity chromatography on an adsorbent containing a substrate analog for phospholipase, namely an alkyl ether derivative of phosphatidyl choline (Rock & Snyder, 1975). The bound fraction yielded purified phospholipase after ion exchange chromatography (Fig. 2A). The unbound fraction, containing the proteases as well as other venom proteins, was separated by gel chromatography to give antigen 5 and hyaluronidase rich fractions. Re-chromatography of these fractions on Sephadex G-100, followed by ion exchange chromatography, yielded antigen 5 and hyaluronidase of high purity (Fig. 2B and 2C). The results of gel chromatography are not given since they are similar to that shown in Fig. 1 for whole venom sac extract.

In the chromatograms of Fig. 2 the proteins were isolated from the venom sac extract of *V. germanica*. The NaDodSO₄ gel electrophoretic patterns of these purified proteins and of the

Table 2. Stability of phospholipase in various preparations of yellowjacket venoms under different conditions

Preparation	$t_{1/2}$ (days) at pH 4.7		$t_{1/2}$ (days) at pH 7.5-8.0	
	4°C	25°C	4°C	25°C
<i>V. maculifrons</i>				
venom		> 25		> 25
venom sac extract	16	1	10	4
partially purified sample		17		28
<i>V. germanica</i>				
venom		> 25		
venom sac extract	> 25	16	> 25	8
partially purified sample			23	
<i>V. vulgaris</i>				
venom sac extract	> 25	16	> 25	3

Phospholipase activities of venom or venom sac extract were followed for 4 days while those of partially purified samples, which were obtained by affinity chromatography of venom sac extracts, were followed for about one month. The $t_{1/2}$ values were extrapolated from plots of log % activity remaining vs time.

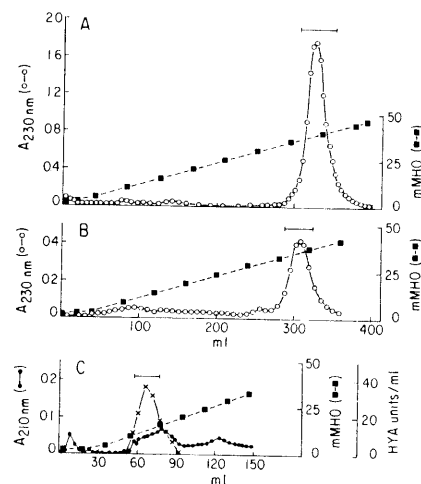


Fig. 2. Separation of venom proteins from *V. germanica* on CM-32 following affinity chromatography on an absorbent specific for phospholipase. Pattern A was obtained with the affinity column enriched sample of phospholipase. Patterns B and C were obtained, respectively, with antigen 5- and hyaluronidase-enriched fractions that had been obtained from a phospholipase-depleted venom protein mixture by gel filtration on G-100. In patterns A and B, a 20×0.9 cm column was used with a linear NaCl gradient in 20 mM ammonium acetate buffer (pH 4.75); in pattern C, a 10×0.9 cm column was used with a linear NaCl gradient in 50 mM sodium phosphate (pH 7.05).

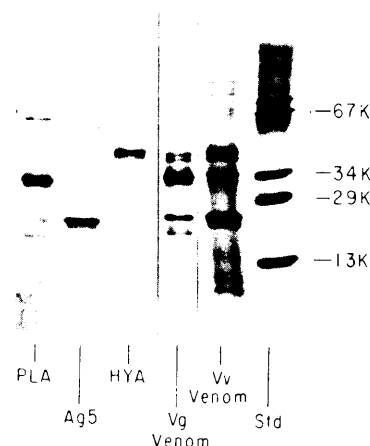


Fig. 3. Sodium dodecyl sulfate gel electrophoresis of venom proteins. From left to right: phospholipase, antigen 5, and hyaluronidase from *V. germanica*, venom sac extracts of *V. germanica*, and *V. vulgaris*, followed by a mixture of standard proteins. The gel contained 12.5% polyacrylamide. About $0.2 \mu\text{g}$ of protein sample was used in each instance, and the bands were detected by silver stain. A separate experiment was also done with about $5 \mu\text{g}$ of each protein sample and the gel was stained with Coomassie Blue. Results similar to those shown were obtained.

venom sac extracts of *V. germanica* and *V. vulgaris* are given in Fig. 3. The results show that the three purified proteins are the major venom components and that the mol. wts of phospholipase, antigen 5 and hyaluronidase are about 37,000, 23,000 and 42,000, respectively. The amino acid compositions of antigen 5 and phospholipase from *V. germanica* are given in Table 3.

The homologous proteins from venom sac extracts of *V. maculifrons* and *V. vulgaris* showed similar chromatograms as those in Fig. 2 for *V. germanica*. They showed similar electrophoretic patterns as those in Fig. 3. Their amino acid compositions were the same within experimental error as those in Table 3. Therefore these results are not given. The absorbance values at 280 nm for phospholipase, antigen 5 or hyaluronidase from these three species of yellowjackets were the same within experimental error. The values were, respectively, 0.885 ± 0.031 , 1.93 ± 0.01 , and 1.86 ± 0.07 for 1 mg per ml solution with a light path of 1 cm; the sample weights were determined by amino acid analysis.

The gel electrophoretic pattern of phospholipase in Fig. 3 showed, in addition to the main band of 37,000 mol. wt, faint bands of 74,000 and 20,000–40,000 mol. wt. The band of 74,000 is apparently a dimer band since it disappeared following 1% mercaptoethanol reduction. The other faint bands are probably degradation products, since the intensity of these bands increased with the decreased specific activity of the sample. The hyaluronidase sample in Fig. 3 showed one band of 42,000 mol. wt. When a higher concentration was used, a weaker band of 45,000 mol. wt was present also. Both bands are present in the venom sac extract. These bands probably differed in their carbohydrate contents, as described below.

The purified phospholipase samples from the venom sac extracts of *V. germanica*, *V. maculifrons* and *V. vulgaris* showed av. sp. act. of 1.2×10^4 , 4.3×10^3 and 4.3×10^3 units per mg, respectively. These differences do not necessarily indicate species differences, as is suggested by the following findings. The yields (30–80%) and specific activities of phospholipase varied in different experiments with the same lot of venom sac extract, in particular that from *V. maculifrons*, the loss invariably occurring during ion exchange chromatography. In one single experiment, phospholipase isolated from the milked venom of *V.*

Table 3. Amino acid compositions of phospholipase and antigen 5 from *V. germanica* venom^a

Amino acid	Phospholipase		Antigen 5	
	Average ^b	Nearest integer	Average ^b	Nearest integer
Lys	24.06	24	24.37	24
His	8.87	9	5.69	6
Arg	12.18	12	4.59	5
Asp	26.99	27	25.98	26
Thr ^c	24.31	24	9.33	9
Ser ^c	26.35	26	7.51	8
Glu	32.15	32	25.85	26
Pro	12.66	13	6.98	7
Gly	23.04	23	15.52	16
Ala	20.52	21	13.09	13
1/2-Cys	11.80	12	6.70	7
Val	17.78	18	12.83	13
Met	4.11	4	2.14	2
Ile	22.05	22	7.28	7
Leu	22.35	22	12.08	12
Tyr	13.44	13	10.78	11
Phe	13.55	14	4.85	5
Trp	n.d. ^d	n.d. ^d	n.d. ^d	n.d. ^d

^aCalculated on the assumption that the numbers of Asp, Glu, Ala, and Leu are, respectively, 27, 32, 21, and 22 for phospholipase; 26, 26, 13, and 12 for antigen 5.

^bAverages of three analyses of two different preparations. Average standard deviations were, respectively ± 2.77 , and $\pm 1.34\%$, for phospholipase and antigen 5.

^cCorrected values, assuming 5 and 10% hydrolytic loss for Thr and Ser, respectively.

^dn.d. = not determined.

maculifrons had a specific activity of 7.6×10^3 units per mg, nearly double that of the sample from venom sac extracts. The hyaluronidase samples from the three *Vespa* species showed nearly the same sp. act. of 3.1×10^4 units per mg ($\pm 30\%$), and they were isolated in yields of 30–70%.

Hyaluronidase and phospholipase from these three species of yellowjackets are probably glycoproteins, as indicated by their binding to concanavalin-Sepharose 4B in 0.1 M phosphate of pH 7.0. The bound phospholipase could be eluted with the same buffer containing 0.5 M NaCl. The bound hyaluronidase was partly eluted with the buffer containing 0.5 M NaCl and partly with the buffer containing 0.5 M NaCl and 0.5 M methyl mannoside. On NaDodSO₄ gel electrophoresis, the hyaluronidase eluted with mannoside was mainly of 45,000 mol. wt. These two molecular forms of hyaluronidase were also partially separable on CM-32 chromatography, the form of 45,000 mol. wt being eluted slightly behind that of 42,000 mol. wt. Antigen 5 from *V. germanica* venom bound to concanavalin-Sepharose, that from *V. maculifrons* showed variable binding, and that from *V. vulgaris* did not bind.

The purified phospholipases were readily susceptible to proteolysis by bovine pancreatic trypsin, and less readily susceptible to proteolysis by chymotrypsin or *S. aureus* protease. After 20 hr digestion of phospholipase ($40 \mu\text{g}$ per ml) with trypsin ($4 \mu\text{g}$ per ml) at pH 8 and 25°C containing 1.0 mM CaCl₂, less than 1% of the phospholipase activity remained. On NaDodSO₄ gel electrophoresis, the band of 37,000 mol. wt showed greatly decreased intensity while the bands of 23,000 showed strongly increased intensity together with other bands of less than 21,000 mol. wt. Only the results obtained with trypsin digestion of phospholipase from *V. germanica* are given in Fig. 4, but those with phospholipases from the other two species were identical; they are not shown. Antigen 5 was also susceptible to proteolysis; the results with trypsin digestion of antigen 5 from *V. vulgaris* are shown in Fig. 4. Reduction of the trypsin digest of phospholipase caused disappearance of the bands of about 20,000 mol. wt, together with the appearance of bands of 10,000–15,000 mol. wt. Reduction of antigen 5 did not change its size (these results not shown).

Enzyme immunoassays were used for

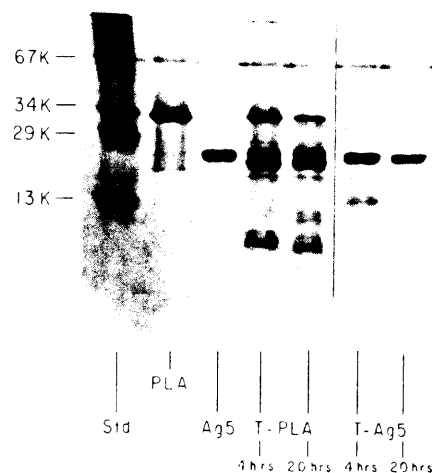


Fig. 4. Sodium dodecyl sulfate gel electrophoreses of phospholipase and antigen 5 before and after 4-hr and 24-hr digestions. The phospholipase sample was from *V. germanica* venom; the antigen 5 samples were from *V. vulgaris* venom. The gel used contained 17% polyacrylamide.

measuring antigen 5- or phospholipase-specific antibodies present in sera of rabbits that had been immunized with venom sac extract of *V. maculifrons*. The solid-phase immunosorbents were wells of vinyl plates which were coated with antigen 5 or phospholipase (5 μ g per ml) from each of the three species of yellowjackets studied. The amounts of specific rabbit antibodies bound to the solid-phase antigens were detected by their reaction with horseradish per-

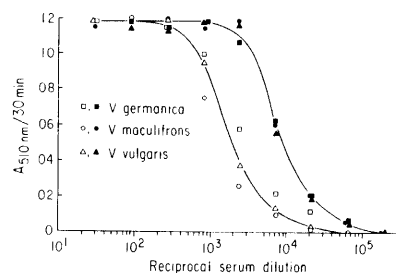


Fig. 5. Enzyme immunoassay of an antiserum sample from a rabbit immunized with venom proteins of *V. maculifrons*. Patterns with open and closed symbols were obtained with solid-phase immunosorbents containing antigen 5 and phospholipase, respectively. Symbols $\square$, $\circ$, and $\triangle$ represent, respectively, proteins isolated from *V. germanica*, *V. maculifrons*, and *V. vulgaris*. The serum sample was estimated to contain about 1.2 and 0.23 mg per ml of antibodies specific for phospholipase and antigen 5, respectively, by affinity chromatography on specific immunosorbents.

oxidase-labeled sheep antibodies specific for rabbit immunoglobulin. The results with one serum sample which had been collected at week 7 after immunization are shown in Fig. 5. Identical concentrations of phospholipase-specific antibodies were obtained with immunosorbents containing phospholipase from any of the three species of yellowjackets. This was also the case for antigen 5-specific antibodies. The concentration of phospholipase-specific antibodies is about 8 times that of antigen 5-specific antibodies. Similar results were also obtained with a serum sample collected at week 3 from the same rabbit from which the week 7 sample was taken (Fig. 5), as well as with serum samples from a second rabbit.

The above results indicate that antigen 5s as well as phospholipases from these three species of yellowjackets are antigenically indistinguishable. The findings were confirmed by immunodiffusion. Lines of identity were observed when antigen 5s, or phospholipases, from the three species of yellowjackets were tested with rabbit anti-serum specific for *V. maculifrons* venom (results not shown).

Inhibition of binding of venom-specific rabbit antibodies for solid-phase antigen 5 or phospholipase was studied (Fig. 6A and 6B).

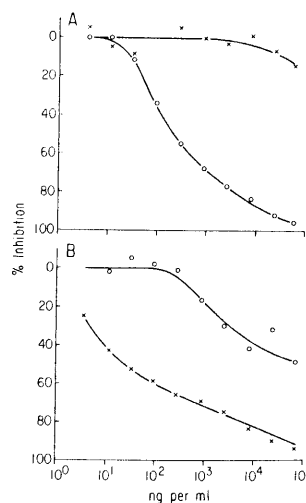


Fig. 6. (A) Inhibition of binding of venom-specific rabbit antibodies for solid-phase phospholipase by phospholipase (O) or by antigen 5 (x). (B) Inhibition of binding of venom-specific rabbit antibodies for solid-phase antigen 5 by antigen 5 (x) or by phospholipase (O). Phospholipase was from *V. germanica*, and antigen 5 was from *V. vulgaris*. Rabbit serum is the same sample as in Fig. 5; it was used at dilutions of 1/7500 and 1/1500, respectively, in A and B.

Table 4. Antigen 5- and phospholipase-specific IgG responses in mice

Group	IgG specificity	IgG titer at week			
		3	34	35	36
A	Ag5	1.4×10^3	2.3×10^3	3.5×10^3	1.1×10^5
	PLA	<10	<10	<10	<10
B	Ag5	1.9×10^3	6.0×10^3	4.0×10^3	5.2×10^3
	PLA	<10	<10	1.0×10^1	6.0×10^1

Group A mice were immunized with antigen 5 at weeks 0 and 34. Group B mice were immunized with antigen 5 at week 0 and with phospholipase at week 34. Specific IgG titers were measured by enzyme immunoassay similar to that for human IgG in Fig. 7; they are defined as the reciprocal of serum dilution required to give half maximal binding.

Antigen 5 inhibited very poorly, if at all, the binding of solid-phase phospholipase for its specific antibodies. Also, phospholipase inhibited poorly the binding of solid-phase antigen 5 for its specific antibodies, less than 4×10^{-3} -fold as effectively as antigen 5. Immunodiffusion tests with venom-specific rabbit antisera also suggested the lack of cross-reactivity between antigen 5 and phospholipase, since no partial fusion of their precipitin lines was observed.

Immunogenicity of antigen 5 and phospholipase was studied in mice (Table 4). Mice receiving primary and secondary immunization with antigen 5 gave a vigorous secondary IgG response which is specific for antigen 5. Mice receiving a primary immunization with antigen 5 and a secondary one with phospholipase showed no secondary response specific for antigen 5, nor were such mice primed for a rapid response specific for phospholipase. On immunodiffusion the serum sample from mice,

which received primary and secondary immunizations with antigen 5, formed a single precipitin line with antigen 5 but not with phospholipase. These results, as well as those from the inhibition experiment in Fig. 6, suggest that the two proteins lack common antigenic determinants.

Enzyme immunoassays were used to measure venom-, antigen 5- or phospholipase-specific IgE and IgG antibodies in sera of patients who had been treated with venom sac extracts of mixed species of yellowjackets. The solid-phase bound specific IgE or IgG was detected with rabbit antibodies specific for human IgE or IgG, followed with peroxidase labeled sheep antibodies specific for rabbit IgG.

In Fig. 7 are shown the assays for specific IgG in one human serum sample. The reciprocals of serum dilution required for 50% maximal binding for the samples shown in Fig. 7 as well as those for seven other samples are summarized in Table 5. The results for specific IgE assays of these samples are given qualitatively in Table 5 because there was significant competition by specific IgG which is present in the serum samples at concentrations that exceed the limited capacities of solid-phase antigens. These results show that patients have antigen 5- and phospholipase-specific IgE and IgG. The ratio of concentrations of phospholipase- and antigen 5-specific IgGs varied with individual patients in the range of 0.6–3.5. Also included in Table 5 for comparison are the yellowjacket venom-specific IgG contents of these serum samples. The data suggest that the sum of antigen 5- and phospholipase-specific IgGs can account for the bulk of venom-specific IgG in these serum samples, if one assumes that the different venom proteins bind to the plastic wells with equal affinity so as to give a

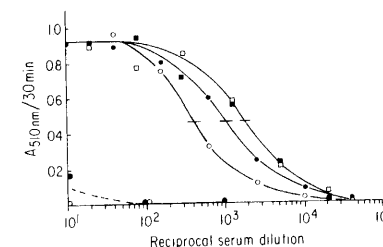


Fig. 7. Enzyme immunoassays of yellowjacket venom-specific human IgGs. Serum samples from one patient (No. 8971) and one normal control are represented, respectively, by solid and dashed lines. Four different solid-phase antigens were used: phospholipase (●) and dialyzed venom sac extract (■) from *V. germanica*, and antigen 5 (○) and dialyzed venom sac extract (□) from *V. maculifrons*. Solid-phase antigens were prepared by coating wells of vinyl plates with purified protein (5 μ g per ml) or venom (equivalent to 5 μ g per ml of phospholipase).

Table 5. Yellowjacket-specific IgE and IgG in sera of patients on venom therapy

Patient No.	Specific IgE		Specific IgG			
	PLA	Ag5	Venom	PLA	Ag5	PLA, Ag5
8119	0	+	990	460	860	0.5
8541	+	+		760	360	2.1
8553	+	+		450	420	1.1
8599	+	+		560	160	3.5
8971	+	+	1900	1100	420	2.6
9045	0	+	560	230	390	0.6
9051	0	+		90	169	0.6
9365	0	+	260	170	100	1.7

Specific IgG was measured as given in Fig. 7 and the concentrations are expressed as reciprocals of serum dilution to give half maximal binding. Specific IgE was measured similarly, but at a single reciprocal serum dilution of 10 and the results are expressed qualitatively (+ denoting an absorbance change of two-fold or greater than that of a normal serum). The contents of venom-specific IgG are averages of the values for *V. germanica* and *V. maculifrons* which differ by 20% or less.

solid-phase immunosorbent having the same antigenic composition as that in venom.

The presence of antigen 5- and phospholipase-specific IgE in patients sensitive to yellowjacket venom was also demonstrated by the histamine release assay with their leukocytes. Release of histamine and other pharmacologic mediators occurs when leukocyte-bound specific IgE binds with antigen. In Table 6 the concentrations of antigens required for 50% maximal release of histamine from leukocytes of four sensitive patients are summarized. The results in Table 6, like those in Table 5, show individual variation of patients in their IgE responses to antigen 5 and phospholipase.

DISCUSSION

Yellowjacket venom consists mainly of three proteins: antigen 5, hyaluronidase, and phospholipase. A main obstacle to its fractionation is instability of hyaluronidase and, in particular, that of phospholipase. The instability of phospholipase is due to proteolysis as evi-

denced by the sensitivity of the purified phospholipase to different proteases. Phospholipase can be isolated in good yield from the venom by the use of affinity chromatography because it is removed in a single step from the bulk of proteolytic activity. The phospholipases so isolated may still be contaminated with traces of proteases since there was variable loss of activity on storage of the samples following ion exchange chromatography.

As indicated in Results, yellowjacket venom sac extracts or venom collected by milking contained two kinds of proteolytic activities. One is a cowhite solubilizing activity and the other a kallikrein-like peptidase activity. The kallikrein-like peptidase is probably a true venom component since it is present in high concentrations in the venom sac extract and in the venom. Furthermore, it has been reported that yellowjacket venom contains a bradykinin analog (Yoshida *et al.*, 1976).

The improved isolation procedure makes it possible to show that both phospholipase and antigen 5 are major protein components of yellowjacket venom. This is in contrast to our

previous work suggesting that antigen 5 is the major protein component in yellowjacket or hornet venom (King *et al.*, 1978b). The concentrations of phospholipase (about 6%) and hyaluronidase (about 1%) found in this study for yellowjacket venoms (Table 1) nearly approached those reported for honey bee venom, which are 12 and 2%, respectively (Habermann, 1972). It is likely that the present findings with yellowjacket venoms are also applicable to hornet venoms.

Antigen 5 is present in yellowjacket venom sac extract or in venom collected by milking, as shown in this work; others have shown it to be present in venom collected by electrical stimulation (Mueller *et al.*, 1981). The content of antigen 5 and phospholipase in venom differed in the three species of yellowjackets studied, but their combined content was about the same in the three species. This finding suggests the possibility of antigen 5 being a fragment of phospholipase.

In a preliminary report (Alagon *et al.*, 1982) we indicated that absorption of rabbit anti-venom sera with a phospholipase-immunosorbent reduced its phospholipase-specific antibody titer about 400-fold while also reducing its antigen 5-specific titer 4-fold. A similar finding was made with an antigen 5-immunosorbent. These findings were taken to indicate the presence of common determinants in these two proteins and appeared to favor the hypothesis advanced above.

However, other data in the present work failed to support this initial finding. First, there was very poor inhibition of phospholipase binding for its specific rabbit antibodies by antigen 5, and of antigen 5 binding by phospholipase. Second, both antigen 5 and phospholipase stimulated antibody responses in mice which were specific only for the immunogen employed. Therefore, the findings indicate a lack of common determinants on these two proteins.

The venom phospholipases isolated from the three species of yellowjackets in this report were found to be immunochemically indistinguishable from each other using sera from rabbits immunized with venom from a single species of yellowjacket. Similar findings were obtained with antigen 5. We did not attempt to study the question of species specificity using sera from allergic humans, since these patients had been immunized with venom sac extracts from mixed species of yellowjackets. Further-

more, it would be difficult to ascertain whether an individual has been stung by only a single species of yellowjacket.

Sera from allergic patients immunized with venom sac extracts contain both IgEs and IgGs specific for antigen 5 and phospholipase. The results suggest that the bulk of venom protein-specific IgG in these sera is accounted for as antigen 5- and phospholipase-specific IgGs. Therefore, these two yellowjacket venom proteins are of importance in insect allergy. It is likely that the homologous proteins from hornets and wasps are of similar importance. The availability of these proteins from different species of hornets, wasps and yellowjackets will be useful in acquiring a better understanding of the antigenic relationship of these venoms. Such knowledge is of significance in the diagnosis and treatment of insect sting allergy in humans since it can help to define the number of venoms from different insects required for such purposes.

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Table 6. Histamine release assays of yellowjacket venom proteins with leukocytes from patients

Donor	Venom	Concentration in μg per ml required for 50% maximal release	
		Antigen 5	Phospholipase
1	1.1 $\pm$ 0	50 $\pm$ 0	2.5 $\pm$ 1.4
2	1.7 $\pm$ 0.6	4 $\pm$ 0	0.4 $\pm$ 0
3	0.17 $\pm$ 0.04	0.20 $\pm$ 0.06	2.1 $\pm$ 1.1
4	0.07 $\pm$ 0.05	1.8 $\pm$ 1.5	0.0009 $\pm$ 0.0006

Values given are averages of three samples from *V. germanica*, *V. maculifrons*, and *V. vulgaris*.

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