

Wasp Venom Proteins: Phospholipase A₁ and B¹

TE PIAO KING,² LOUCIA KOCHOUMIAN, AND ALISON JOSLYN

The Rockefeller University, New York, New York 10021

Received June 20, 1983, and in revised form November 28, 1983

Three major venom proteins from different species of wasps have been isolated and characterized. They are hyaluronidase, phospholipase, and antigen 5 of as yet unknown biochemical function. These three proteins are allergens in wasp venom-sensitive persons. The species of wasps studied, of the genus *Polistes*, were *annularis*, *carolina*, *exclamans*, *fuscatus*, and *instabilis*. Antigen 5 and phospholipase from wasp venoms were shown to be antigenically distinct from homologous proteins of yellowjacket venoms. The venom phospholipase from wasp, as well as that from yellowjacket (*Vespula germanica*), appears to have dual enzymatic specificities of the A₁ and B types. That is, hydrolysis takes place at the 1-acyl residue of phosphatidylcholine and at the 1- or 2-acyl residue of lysophosphatidylcholine.

Most allergies to insect venoms are caused by bees and by vespids, which include hornets, yellowjackets and wasps. Allergic persons who have been sensitized by insect stings have venom-specific antibodies of the IgE³ and IgG classes as well as of other Ig classes (1). We have previously identified three homologous proteins from hornet and yellowjacket venoms as important allergens in humans (2, 3). These proteins are hyaluronidase, phospholipase, and antigen 5 of as yet unidentified biochemical function. Antigen 5 may be a pre-synaptic toxin which is active at the neuromuscular junction of crustaceans, because its physicochemical properties are similar to those reported for a neurotoxin from an Asian species of hornet, *Vespa mandarinia* (4). We have now isolated and immunochemically characterized the aforementioned three homologous venom proteins from three species of wasps, *Pol-*

istes annularis, *P. exclamans*, and *P. fuscatus*. We have also compared the antigenic relationship of antigen 5's or phospholipases from these three species of wasps and from *P. carolina* and *P. instabilis*.

MATERIALS AND METHODS

Lyophilized venom sac extracts of *P. annularis* (Lot No. 01198106 PW), *P. exclamans* (Lot No. 12198026 PO), and *P. fuscatus* (Lot No. 02258107 PW) were prepared by Vespa Laboratories, Spring Mills, Pennsylvania, and they were obtained in the same way as for yellowjacket venom sac extracts (3). Venoms of *P. carolina* and *P. instabilis* in milligram amounts were collected by Dr. James E. Gillaspay, Texas A & I University, Kingsville, Texas. 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°C in sealed and evacuated tubes (<0.1 Torr). The venom weights, as determined by amino acid analysis (5), were approximately 66% 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 performic acid-oxidized derivatives. About 1 nmol of protein was treated with 50 μl of 3% H₂O₂ in 90% formic acid for 3 h at 25°C. The reagent was blown off under nitrogen at 50°C. After drying *in vacuo*, the sample was hydrolyzed as usual.

¹ Supported in part by Grant AI 17021 from the National Institutes of Health.

² To whom correspondence should be addressed.

³ Abbreviations used: Ig, immunoglobulin; PC, 1-(11-carboxyundecyl)-2-hexadecyl-vac-glycero-3-phosphorylcholine; SDS, sodium dodecyl sulfate.

The absorbent for affinity chromatography of phospholipase (6) was prepared by a carbodiimide-promoted coupling of 240 mg of 1-(11-carboxyundecyl)-2-hexadecyl-rac-glycero-3-phosphorylcholine (PC) to 6 g of AH-Sepharose 4B (Pharmacia); the phospholipid analog was obtained from R. Berchtold Chem. Labor, Bern, Switzerland. Heparin-Sepharose, the absorbent for affinity chromatography of hyaluronidase (7), was also from Pharmacia. Honey bee and yellowjacket (*V. germanica*) venom phospholipases and yellowjacket (*V. vulgaris*) venom antigen 5 were isolated in this laboratory (3, 8).

Protein solutions were concentrated by ultrafiltration through Amicon YM-2 membrane. Dialysis was carried out in Visking 8/32 tubing. Sodium dodecyl sulfate slab gel electrophoresis (9) was carried out using the published procedure. The protein bands in the gels were stained with 0.02% Coomassie blue R-250 in 2-propanol-acetic acid-water (25:10:65).

Hyaluronidase activity was measured turbidimetrically (10). One unit of activity is defined as the amount of enzyme required to hydrolyze 1 μ g of hyaluronic acid (U. S. Biochemical) per minute at 25°C in 200 μ l of a 200 μ g/ml solution of hyaluronic acid at pH 5.3.

Phospholipase activity was determined routinely by a titrimetric method with 10% egg yolk as the substrate in 0.2 N NaCl containing 0.5% Triton X-100 (11). 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 1.5 ml of substrate solution.

Phospholipase activity was also measured titrimetrically with several other natural or synthetic phospholipids as substrates; they were obtained from Sigma Chemical Company. Fine suspensions of phospholipids were prepared by sonication in 1 mM Tris-HCl buffer (pH 8) containing 100 mM NaCl, 20 mM KCl, 10 mM CaCl₂, and 0.5% Triton X-100 in an ultrasonic cleaner bath (Cole-Palmer). For the purpose of studying the products formed on phospholipase digestion, the same buffer as described above was used, but the concentration of Tris-HCl buffer was increased to 50 mM. The digests were examined by thin-layer chromatography on silica gel plates (Whatman LK6DF); 20 μ l of digest (2 mg/ml of phospholipid) was applied to the preadsorbent zone, and the plate was developed in a system of chloroform-methanol-0.1 N HCl (70:30:5). The spots were visualized after exposure to iodine vapor; the R_f values of phosphatidylcholine, lysophosphatidylcholine, and glycerophosphorylcholine were 0.20, 0.14, and 0.01, respectively. The digests were also examined directly for the presence of saturated and unsaturated fatty acids by chromatography on freshly prepared silica gel plates which had been dipped in 5% AgNO₃, and then dried at 110°C for 1 h. After developing in a system of hexane-diethyl ether-acetic acid (70:30:1),

the spots were visualized under uv after spraying with 0.2% dichlorofluorescein in ethanol; the R_f values for saturated and unsaturated fatty acids were 0.36 and 0.28, respectively.

Rabbit antisera specific for the venom sac extracts were prepared in three New Zealand white rabbits (2). Mouse ascitic fluid specific for purified venom proteins was prepared according to the procedure of Tung *et al.* (12). Groups of three to five mice of CAF₁ strain (20–25 g weight) were given repeated intraperitoneal immunizations with about 2 μ g of purified protein in 0.2 ml of an emulsion containing 9 parts of complete Freund's adjuvant (CalBiochem) and one part of 0.02 M ammonium acetate buffer (pH 4.7). Mouse antibodies specific for purified venom proteins were isolated by affinity chromatography (3). The required immunosorbent was prepared by coupling 1 mg of protein to 0.25 g of CNBr-activated Sepharose 4B (Pharmacia). Human sera with high levels of IgE specific for wasp or yellowjacket venom were gifts from Dr. Donald R. Hoffman (13).

Enzyme immunoassays were used to measure the binding of human or mouse antibodies to solid-phase antigen. The published procedure (14) was modified by using 20- μ l volumes of all reagents; the absorbance change in each well (50 μ l of substrate solution) was read directly with an EIA reader (Bio-Tek Instruments). All the reagents used have been described previously (3).

Immunodiffusion and rocket or crossed immunoelectrophoresis were carried out in 1% agarose gel and the precipitates were visualized after dye staining of dried and washed gel (15).

RESULTS

Isolation and Characterization of Wasp Venom Proteins

Stability studies with a mixture of venom sac extracts from *P. annularis* and *P. exclamans* indicated little or no loss of phospholipase activity after a 9-day storage in ammonium acetate buffer of pH 4.75 at room temperature. However, there was a loss of more than 80% in phospholipase activity after similar storage in Tris-HCl buffer, pH 7.95. This loss is probably associated with the presence of proteases since it has been demonstrated to take place with yellowjacket venom sac extracts (3).

To minimize possible protease actions, wasp venom sac extract was treated with the known inhibitor phenylmethylsulfonyl fluoride, and then separated by gel filtration on Sephadex G-100 (Fig. 1A). The re-

gion containing proteins of 20,000–80,000 Da was next separated by affinity chromatography on an adsorbent containing a substrate analog of phospholipase (Fig. 1B). The bound fraction, which was eluted with acetic acid, contained phospholipase;

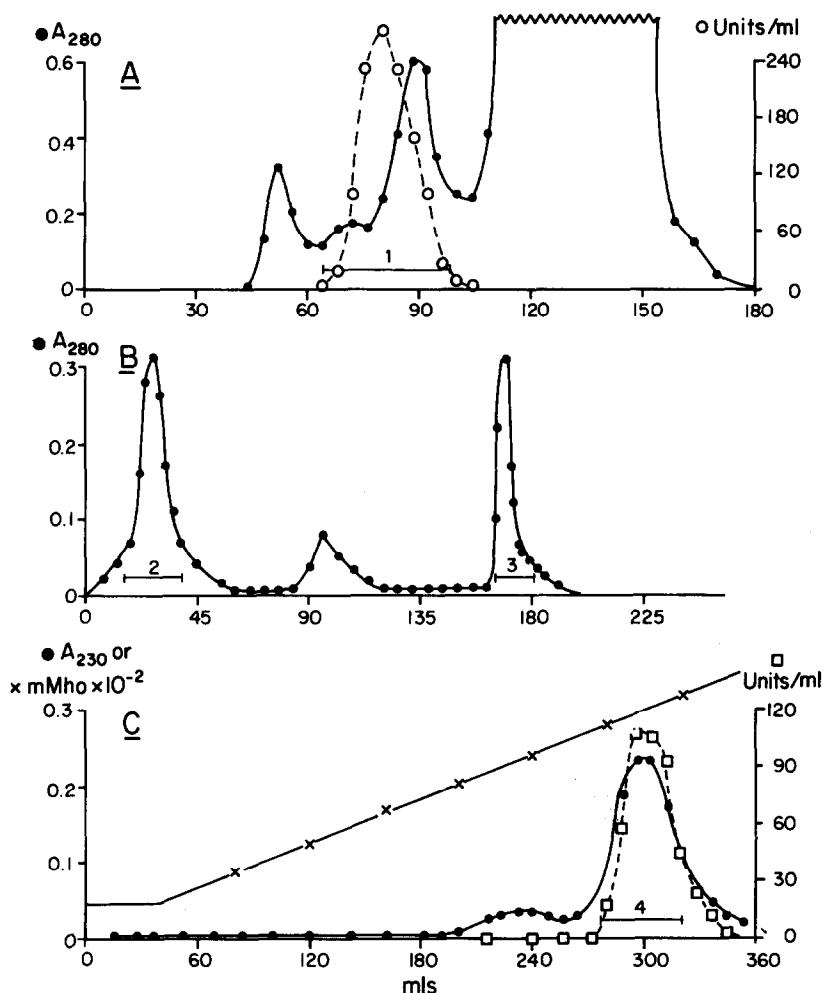


FIG. 1. (A) Gel filtration of venom sac extract of *P. exclamans* on a 200 × 0.9-cm column of Sephadex G-100. About 55 mg was dissolved in 8 ml of 0.05 M ammonium acetate buffer (pH 4.75) containing 2 mM phenylmethylsulfonyl fluoride. After 3 h at 25°C, the mixture was concentrated to <4 ml, and then applied. The column was eluted with 0.05 M ammonium acetate buffer (pH 4.75) at 12 ml/h. The indicated fraction 1 was concentrated and dialyzed against the starting buffer of (B). Open circles represent hyaluronidase activity. (B) Affinity chromatography of fraction 1 on an 8 × 0.9-cm column of Sepharose with a bound substrate analog of phosphatidyleholine. The column was eluted at 90 ml/h, first with 0.05 M Tris-HCl buffer (pH 7.95) containing 0.01 M CaCl₂ (60 ml), then with 0.05 M Tris-HCl buffer (pH 7.95) containing 0.5 M NaCl (60 ml), and finally with 0.05 M acetic acid (120 ml). Fraction 3 was collected in tubes containing ammonium acetate in an equimolar amount to that of acetic acid. The indicated fractions 2 and 3 were concentrated and dialyzed against the starting buffer of (C). (C) Ion-exchange chromatography of fraction 3 on a 20 × 0.9-cm column of CM-32 cellulose. The column was eluted at 60 ml/h with a linear NaCl gradient from 0 to 0.5 M in 500 ml of 0.05 M ammonium acetate buffer (pH 4.75). The indicated fraction 4 containing phospholipase was concentrated and dialyzed against the starting buffer. Open squares represent phospholipase activity.

it was further purified by ion-exchange chromatography on CM-32 cellulose (Fig. 1C). The breakthrough fraction from Fig. 1B was chromatographed successively on columns of CM-32 cellulose and heparin-Sepharose to yield antigen 5 and hyaluronidase (Fig. 2A to 2C). A better separation of antigen 5 and hyaluronidase was

obtained on heparin-Sepharose than on CM-32 cellulose; it is likely that heparin-Sepharose functions both as an ion exchanger and as a specific adsorbent for hyaluronidase (7).

The results given in the figures are for the venom sac extract from *P. exclamans*. Similar findings were obtained with venom

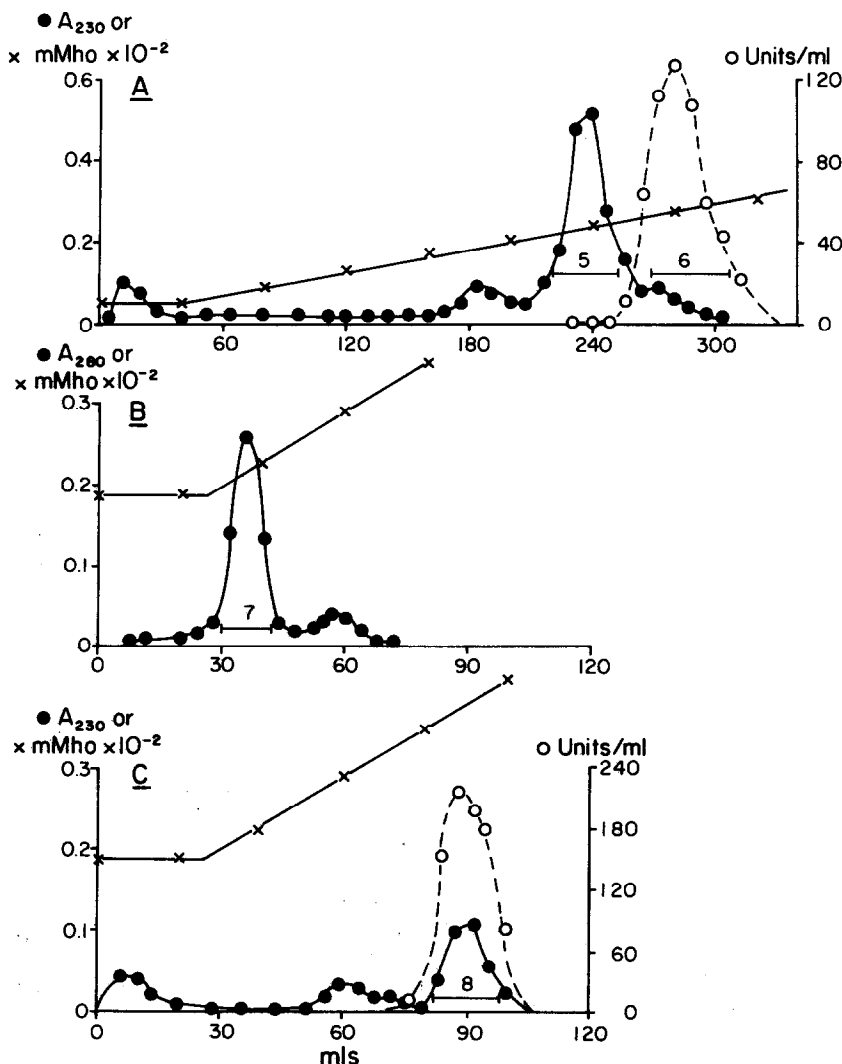


FIG. 2. (A) Ion-exchange chromatography of fraction 2 on a 20 × 0.9-cm column of CM-32 cellulose. Conditions were the same as those in Fig. 1C. The indicated fractions 5 and 6 were concentrated and dialyzed against the starting buffer. (B) and (C) Ion-exchange chromatographies of fractions 5 and 6 on a 5 × 0.9-cm column of heparin-Sepharose, respectively. The fractions applied were obtained from 135 mg of venom. The column was eluted with a linear NaCl gradient from 0.15 to 1.0 M in 200 ml of 0.05 M ammonium acetate buffer (pH 4.75). The indicated fractions 7 and 8 containing antigen 5 and hyaluronidase, respectively, were concentrated and dialyzed against the starting buffer.

sac extracts from *P. annularis* and *P. fuscatus*. The hyaluronidases from the three species of wasps were eluted at nearly the same salt concentration of ion-exchange chromatography on CM-cellulose, but the antigen 5's, as well as the phospholipases, differed in their elution positions. These chromatographic differences, as well as the yields and other relevant data on these proteins from the three species of wasps, are listed in Table I.

In Table II are summarized the amino acid compositions of the three proteins from *P. annularis* venom. The compositions of homologous proteins from *P. exclamans* and *P. fuscatus* are very similar to those from *P. annularis* (less than 10% difference); these are not shown. The compositions of homologous proteins from yellowjacket (*V. germanica*) venom are included for comparison. The overall compositions of the homologous proteins from wasp and yellowjacket venoms are quite similar, although they do differ significantly in some amino acid residues.

The purified venom proteins from *P. exclamans* were examined on SDS-gel electrophoresis (Fig. 3A). Antigen 5 and hyaluronidase each gave a single band before and after mercaptoethanol reduction (lanes 4, 6, 7, and 9 of Fig. 3A).

The mobility of antigen 5 decreased on reduction while that of hyaluronidase did not. The molecular weights of antigen 5 for native and reduced samples, respectively, were estimated to be about 2.2×10^4 and 2.7×10^4 , while that of hyaluronidase was estimated to be about 4.4×10^4 . Native phospholipase gave two equally intense bands of about 3.6×10^4 and 3.8×10^4 Da (lane 5), but the reduced sample gave one intense band of about 3.7×10^4 Da together with other fainter bands of about 2×10^4 Da or less (lane 8). These minor bands cannot be removed on repeated affinity or ion-exchange chromatography.

The purified venom proteins from *P. annularis* and *P. fuscatus* gave SDS-gel patterns that were nearly identical to those shown in Fig. 3A; these results are not

TABLE I
SOME PROPERTIES OF WASP VENOM PROTEINS

Protein	Estimated venom composition ^a (%)	Yield on isolation (%)	NaCl concentration for elution from CM-32 (M)	A _{280 nm} for 1 mg/ml	Enzymatic specific activity ^c
Antigen 5					
<i>P. annularis</i>	4.0	14	0.15	1.94	
<i>P. exclamans</i>	5.2	9	0.20	2.24	
<i>P. fuscatus</i>	2.8	12	0.25		
Hyaluronidase					
<i>P. annularis</i>	0.4	25	0.23	2.41	4.5×10^4
<i>P. exclamans</i>	0.7	22	0.24		4.2×10^4
<i>P. fuscatus</i>	0.8	16	0.28		3.5×10^4
Phospholipase ^b					
<i>P. annularis</i>	1.2	21	0.32	1.73	5.2×10^3
<i>P. exclamans</i>	3.5	62	0.26	1.69	6.4×10^3
<i>P. fuscatus</i>	2.0	22	0.29		3.2×10^3

^a Venom composition for antigen 5 was estimated by rocket immunoelectrophoresis in gels containing specific antibodies, while those for hyaluronidase and phospholipase were estimated by enzyme activity.

^b In addition to the phospholipase component indicated, the venom of *P. annularis* contained another minor component eluted at 0.27 M NaCl, and that of *P. fuscatus* contained two minor components eluted at 0.25 and 0.33 M NaCl.

^c Enzymatic specific activities in units per milligram are averages of two or more experiments; the values for different experiments varied by about 30%.

TABLE II
AMINO ACID COMPOSITIONS OF VENOM PROTEINS FROM *P. annularis*

Amino acid	Antigen 5		Phospholipase		Hyaluronidase	
	Average	Nearest integer	Average	Nearest integer	Average	Nearest integer
Lys	20.6	21 (24)	30.4	30 (24)	40.3	40
His	6.7	7 (6)	9.7	10 (9)	9.4	9
Arg	5.5	6 (5)	9.7	10 (12)	22.2	22
Asp	24.4	24 (26)	34.9	35 (27)	45.6	46
Thr	10.9	11 (9)	17.0	17 (24)	22.5	23
Ser	11.6	12 (8)	21.1	21 (26)	27.2	27
Glu	24.7	25 (26)	21.2	21 (32)	47.6	28
Pro	8.1	8 (7)	9.5	10 (13)	16.6	17
Gly	19.7	20 (16)	25.5	25 (23)	31.6	32
Ala	14.3	14 (13)	25.9	26 (21)	20.5	21
Cys/2	8.9	9 (7)	12.7	13 (12)	8.2	8
Val	12.3	12 (13)	22.9	23 (18)	22.1	22
Met	3.4	3 (2)	2.0	2 (4)	5.7	6
Ile	11.2	12 (7)	17.0	17 (22)	23.2	23
Leu	11.6	12 (12)	24.4	24 (22)	24.6	25
Tyr	10.2	10 (11)	14.7	15 (13)	15.8	16
Phe	4.4	4 (5)	15.0	15 (14)	16.7	17
Trp	n.d.		n.d.		n.d.	
		210 (209)		314 (316)		402

Note. Calculated on the assumption that the numbers of Asp, Glu, Gly, Ala, and Phe residues are, respectively, 24, 25, 20, 14 and 4 for antigen 5; 35, 21, 25, 26 and 15 for phospholipase; and 46, 48, 32, 21 and 17 for hyaluronidase. Values given are averages of three or more determinations with average standard deviations of 5% or less. Values given in parentheses are those of the homologous protein from yellowjacket, *V. germanica* (3).

shown, except for phospholipase from *P. annularis*, which is shown in lane 3 of Fig. 3A. In addition to the main bands, the native sample gave other faint bands of about 2×10^4 Da and less. These bands of lower molecular weight are also present in the phospholipase from *P. exclamans*, but in much lower concentration. The distribution of low-molecular-weight bands in wasp venom phospholipase is very similar to those seen with yellowjacket venom phospholipase following partial degradation with trypsin (3); for comparison, the pattern for a sample of yellowjacket phospholipase which had been stored in pH 4.75 buffer at 4°C for about 1 year is shown in lane 2 of Fig. 3A. These findings together suggest that the low-molecular-weight components present in phospholipase from *P. annularis* venom represent protease-di-

gested fragments. In accord with this suggestion is the lower specific activity of phospholipase samples from *P. annularis*, which is about 20% less than that of phospholipase from *P. exclamans* (Table I).

In Fig. 3B are shown, for comparison, the electrophoretic patterns of venom sac extract from *P. annularis*, *P. exclamans*, and *P. fuscatus*. The electrophoretic properties of antigen 5 and phospholipase in the venom sac extract, before and after reduction, are just like those of the purified components in Fig. 3A.

Antigen 5 and phospholipase from *P. exclamans* were examined by agarose gel electrophoresis at pH 8.6 and by crossed immunoelectrophoresis with their respective specific mouse antisera, as shown in Fig. 4. Both samples migrated as a single basic protein toward the cathode. Antigen

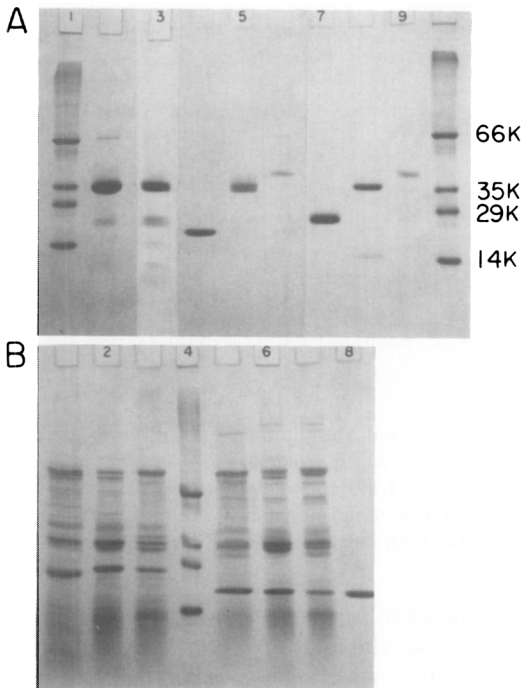


FIG. 3. Sodium dodecyl sulfate-gel electrophoresis; (A) Protein standards (lanes 1 and 10); *V. germanica* phospholipase (lane 2); *P. annularis* phospholipase (lane 3); antigen 5, phospholipase, and hyaluronidase from *P. exclamans* (lanes 4, 5, 6); the same three preceding samples after 1% mercaptoethanol reduction at 100°C for 2 min (lanes 7, 8, 9). (B) Venom sac extracts of *P. fuscatus*, *P. exclamans*, and *P. annularis* after mercaptoethanol reduction (lanes 1, 2, 3); protein standards (lane 4); the same three venom sac extracts without reduction (lanes 5, 6, 7); antigen 5 from *P. annularis* (lane 8). About 4 μ g of purified protein and 40 μ g of venom sac extract were used.

5 showed one immune precipitate band, while phospholipase showed one main band together with a small band of similar mobility. An exactly identical finding was made with phospholipase from *P. annularis* (result not shown).

Specificity of Wasp and Yellowjacket Phospholipases

The activity of purified phospholipase from *P. exclamans* was measured at 23–25°C with five different substrates, each at several concentrations by titrimetric assay as given under Materials and Methods (Fig. 5). When compared under conditions of

substrate saturation, the specific activity was highest with egg yolk as the substrate, about 6500 units/mg of enzyme. The specific activity was 400–800 units/mg of enzyme with egg lysophosphatidylcholine, egg phosphatidylcholine, or 1,2-dioleoyl-3-*sn*-glycerophosphorylcholine substrates. However, it was only about 100 units/mg of enzyme with 1,2-distearoyl-3-*sn*-glycerophosphorylcholine as substrate.

The assay data in Fig. 5 were obtained in solutions containing 10 mM Ca²⁺ ion. When assays with egg phosphatidylcholine were repeated in the absence of Ca²⁺ ion but in the presence of 1 mM EDTA, the specific activity of wasp phospholipase decreased about 50%. A control experiment with bee venom phospholipase indicated complete inactivation in the presence of EDTA. Bee venom phospholipase is known to have the A₂ type of specificity and its activity requires the presence of Ca²⁺ (16).

The specificity of purified phospholipases (3 μ g/ml) from *P. annularis* and *P. exclamans* was examined by thin-layer chromatography of their digests of different phospholipids (2 mg/ml) at pH 8 and 23–25°C. Under these conditions, complete conversion of egg phosphatidylcholine, 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphorylcholine, and 1-oleoyl-2-stearoyl-3-*sn*-glycerophosphorylcholine to their respective monoacyl phospholipids occurred in about 10 min, followed by their conversion to glycerophosphorylcholine in <6 h. Similarly, egg lysophosphatidylcholine was converted to glycerophosphorylcholine in <6 h.

Qualitative estimation of thin-layer chromatograms showed that, after a 1-min digestion with wasp venom phospholipase, stearic acid, together with oleic acid in smaller amounts, was released from 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphorylcholine, but only oleic acid was released from 1-oleoyl-2-stearoyl-3-*sn*-glycerophosphorylcholine. After a 6-h digestion, oleic and stearic acids were released from both synthetic phospholipids. These studies, as well as those in the preceding paragraph, were carried out with purified venom phospholipase from yellowjacket (*V. germanica*) (3); exactly the same results

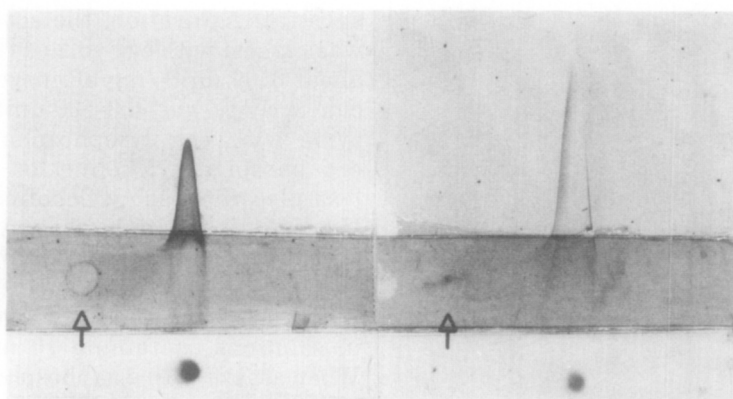


FIG. 4. Crossed immunoelectrophoresis of antigen 5 (1 μ g) and phospholipase (2 μ g) from *P. exclamans* (right and left pattern, respectively). Electrophoresis was carried out in 1% agarose gel containing 0.087 M sodium barbital buffer (pH 8.6) at 100 V for 1 h; immunoelectrophoresis in the second direction was in the same agarose gel containing 2–10% of specific mouse antisera at 200 V for 2–4 h. Cathode is on the right side and at the top for both patterns.

were obtained. As control experiments, bee venom phospholipase A_2 digestion of these synthetic phospholipids was also examined,

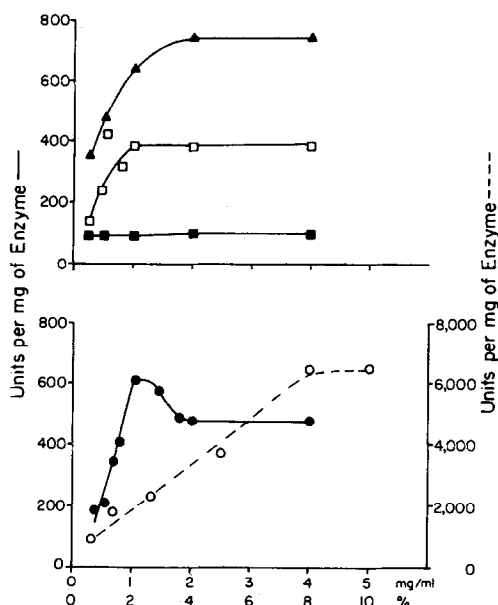


FIG. 5. Activity of *P. exclamans* phospholipase as measured with different substrates at pH 8. The concentration of egg yolk (○) is given in weight percent while those of other substrates (egg lecithin, ●; egg lysophosphatidylcholine, ▲; 1,2-dioleoyl-3-*sn*-glycerophosphocholine, □; and 1,2-distearoyl-3-*sn*-glycerophosphocholine, ■) are in milligrams per milliliter.

and only products corresponding to the hydrolysis of the acyl group at the 2 position were obtained. Since the specificity of bee venom phospholipase A_2 is well established (16), the results of these control experiments also serve as structural proof of the synthetic substrates that we used.

The above results indicate that the initial site of hydrolysis catalyzed by the wasp or yellowjacket venom phospholipase is the 1 position of phosphatidylcholine and that the second slower site is the 2 position of 2-acyl-3-*sn*-glycerophosphorylcholine. The second slower site may also be the 1-position of 1-acyl-3-*sn*-glycerophosphorylcholine which can be formed on acyl migration of the 2-isomer (17). The apparent simultaneous release of fatty acids from the 1 and 2 positions of 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphorylcholine is likely to be a consequence of the fact that the rate of hydrolysis of the second site can be as fast as or faster than that at the first site because of an enzymatic preference for unsaturated fatty acid residues. This is supported by the data in Fig. 5, showing that the dioleoyl derivative was hydrolyzed at least five times faster than the distearoyl derivative. Therefore, the phospholipase from wasp and yellowjacket venoms has both A_1 and B types of activities. Since the enzyme samples used are of high purity, as indicated by the chromatographic and

electrophoretic data of Figs. 1C, 3 and 4, the results suggest that both activities reside in a single protein.

Immunological Studies with Wasp-Specific Animal and Human Sera

Immunodiffusion analysis showed that the phospholipase samples isolated from *P. annularis*, *P. exclamans*, and *P. fuscatus* were indistinguishable antigenically since they showed a single precipitin line of identity with *P. exclamans* phospholipase-specific mouse sera or with *P. fuscatus* venom-specific rabbit sera. Immunodiffusion also showed antigenic identity of the phospholipase samples from *P. annularis* and *P. exclamans* with those present in *P. carolina* and *P. instabilis* venoms. Antigen 5 samples from *P. annularis* and *P. exclamans* were also antigenically indistinguishable since they showed a single precipitin line of identity with *P. annularis* antigen 5-specific mouse sera. The precipitin lines of these two antigen 5 samples showed a faint spur with that of antigen 5 from *P. fuscatus*, indicating partial iden-

tity, but they showed a single precipitin line of identity with that of antigen 5 in *P. carolina* or in *P. instabilis* venoms.

Phospholipase samples from *P. annularis* and *P. exclamans* venom did not form a precipitin line on immunodiffusion against yellowjacket phospholipase-specific mouse sera. Nor did antigen 5 samples from these two species of wasps react with yellowjacket antigen 5-specific mouse sera. The immunodiffusion studies referred to in this and the above paragraph are summarized in Table III, but their patterns are not shown.

Solid-phase enzyme immunoassays were used to establish the presence of antibodies specific for wasp venom proteins in sera of wasp venom-allergic persons. The solid-phase immunosorbents were wells of polyvinylchloride plates which had been coated with purified wasp venom protein (3 μ g/ml) or whole venom (40 μ g/ml). The specific antibodies bound to the solid-phase immunosorbents were detected with rabbit antibodies specific for the Fc region of human IgE or IgG, followed with peroxidase-labeled sheep antibodies specific for rabbit

TABLE III

IMMUNODIFFUSION OF WASP VENOM PROTEINS AGAINST DIFFERENT ANIMAL SERA OR ASCITES^a

	<i>P. fuscatus</i> venom-specific rabbit sera	<i>P. exclamans</i> PLA-specific mouse ascites	<i>P. annularis</i> Ag5-specific mouse ascites	<i>V. germanica</i> PLA-specific mouse ascites ^b	<i>V. vulgaris</i> Ag5-specific mouse ascites
Phospholipase					
<i>P. annularis</i>	+	+	—	—	—
<i>P. exclamans</i>	+	+	—	—	—
<i>P. fuscatus</i>	+	+	—	n.d.	n.d.
Antigen 5					
<i>P. annularis</i>	+	—	+	—	—
<i>P. exclamans</i>	+	—	+	—	—
<i>P. fuscatus</i>	+	—	+	n.d.	n.d.
Venom					
<i>P. carolina</i>	n.d.	+	+	n.d.	n.d.
<i>P. instabilis</i>	n.d.	+	+	n.d.	n.d.

^a + and — refer, respectively, to the presence or absence of a precipitin line, and n.d. denotes not done. Precipitin lines of identity were observed for homologous proteins of all wasp species, with the single exception of antigen 5 from *P. fuscatus*; its precipitin line showed a faint spur with that of antigen 5 from *P. annularis* or from *P. exclamans*.

^b *V. germanica* phospholipase-specific ascites and *V. vulgaris* antigen 5-specific ascites each formed a precipitin line with their respective immunogen.

IgG. In Fig. 6 are shown the data for specific IgG antibodies in sera of two allergic persons and one normal one, as well as those for specific mouse IgG antibodies. The mouse antibodies were detected by using rabbit antibodies specific for the Fc region of mouse IgG. The concentrations of mouse antibodies are known since they were isolated by affinity chromatography on specific immunosorbents. The concentrations of human antibodies are not known and they are expressed as serum dilutions in Fig. 6.

The binding curves of the human and the mouse antibodies for solid-phase wasp proteins are similar in shape. On the assumption that mouse and human antibodies have the same affinity for solid-phase

wasp proteins, one can use the binding curve of mouse antibodies for estimating the concentration of human antibodies. The binding curve of mouse antibodies specific for phospholipase was arbitrarily chosen as the standard for estimation of human antibodies specific for all wasp venom proteins. This procedure was used to estimate the concentrations of IgG antibodies, specific for different wasp proteins, present in the human sera as shown in Fig. 6. The estimated specific IgG levels of these three sera, as well as those for nine other human sera, together with their specific IgE levels, are given in Table IV. The results for specific IgE are given qualitatively because there was competition by specific IgG antibodies, which are usually present in higher concentration than those of IgE, for the solid-phase immunosorbents.

The data in Table IV show that, compared to the normal serum samples, sera from wasp venom-sensitive patients contained elevated levels of IgE and IgG specific for *P. annularis* or *P. exclaimans* venom, while sera from yellowjacket venom-sensitive patients did not. The wasp venom-specific human IgGs consist mainly of phospholipase-specific IgG since in the sera tested they are present in concentrations at least several times greater than those of antigen 5- or hyaluronidase-specific IgGs.

DISCUSSION

The presence of phospholipase A and B activities in hornet, wasp, and yellowjacket venoms has been reported earlier by others (cf. (18)). Our present results establish that the phospholipase activity of yellowjacket and wasp venoms is of the A₁ type. Furthermore, our results suggest that the A₁ and B activities of yellowjacket and wasp venoms reside in a single protein. Our unpublished results indicate that the phospholipase in hornet venoms has the same enzymatic specificity as that shown for phospholipase from wasp or yellowjacket venoms. The presence of these two activities in one single enzyme has its precedence in the phospholipase A₁ from bovine pancreas or other microbial sources (19,

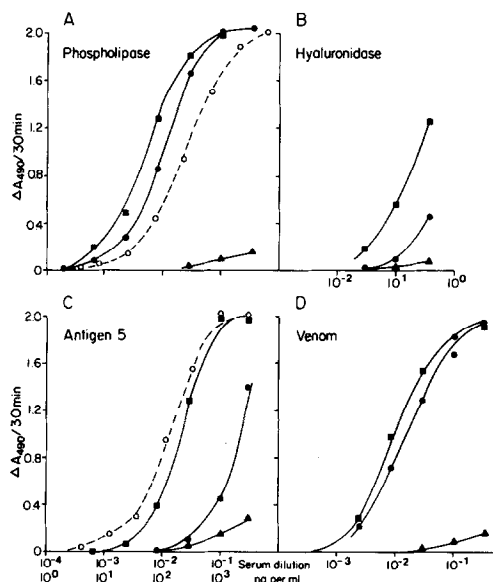


FIG. 6. Solid-phase enzyme immunoassay of wasp-specific human or mouse IgGs. The solid-phase immunosorbents in patterns A and C were, respectively, phospholipase and antigen 5 from *P. exclaimans*, and those in patterns B and D were hyaluronidase and venom from *P. annularis*. Solid circles, squares, and triangles are for human sera Nos. 3747, 5755, and one normal control; their concentrations are given in serum dilutions. Open circles are for affinity-purified specific mouse antibodies; their concentrations are in nanograms per milliliter. The maximal absorbance change for mouse antibodies was about 1.9; the data were normalized to a maximal absorbance change of 2.0 in this figure.

TABLE IV
WASP-SPECIFIC IgG AND IgEs IN INSECT VENOM ALLERGIC PATIENTS

Patient no. ^a	Specific IgG (units/ml) ^{b,c}				Specific IgE ^d	
	Venom	PLA	Ag5	HYA	Venom	PLA
3747	55 (19)	27 (19)	0.8 (0.3)	(0.22)	++	+++
3801	82	89 (60)	0.8 (0.3)	(0.59)	+++	+++
5339	6	9 (4)	3.5 (1.2)	(0.20)		+++
5755	25 (23)	45 (59)	9 (5.4)	(1.2)	++	++
5938	16	21	2.2	(0.13)	++	+
6074	15 (16)	27	3.5	(0.09)	++	+++
7562	3	4	0.5	(0.02)	—	++
8801	(0.4)		0.8	(0.04)	—	—
8431	(0.3)	1 (0.1)	0.7 (0.1)	(0.07)	—	—
8633	(0.2)	1	0.7	(0.04)	—	—
1	(0.4)	0.1 (0.1)	0.2 (0.1)	(0.06)	—	—
2	(0.4)	0.3	0.5	(0.03)	—	—

^a Patients numbers 3747 to 7562 and 8801 to 8633 were shown by RAST to be wasp and yellowjacket venom sensitive, respectively (13), while patients numbers 1 and 2 were normal controls.

^b The venom and the purified proteins used were mainly from *P. exclamans*, except for those for the values given in parentheses, which were obtained with samples from *P. annularis*.

^c Concentrations of specific IgGs are given in arbitrary units per milliliter, which were determined using the lower half of the binding curve of PLA-specific mouse antibodies as a standard; one unit per milliliter is equivalent to 1 μ g/ml of mouse antibodies.

^d Concentrations of specific IgEs are given qualitatively; the plus signs refer to the highest serum dilution tested (1/2.9, 1/10 and 1/35) which gave $\Delta A_{490}/30$ min values of at least twice those of controls.

20). All other known phospholipases (cf. (20)) from venoms of snakes, honey bees, and an Asian species of hornet (*V. orientalis*) are reported to be of the A₂ type. The Asian hornet venom is reported to contain one phospholipase with both A₂ and B types of activities (21) as well as another phospholipase with only the B type of activity (22).

The five species of wasps studied in this report are widely distributed in this country (13, 23). The homologous phospholipases from these five species of wasps show apparent immunological identity. This is also the case for homologous antigen 5's, with the single exception of that for *P. fuscatus*. The partial identity of antigen 5 from *P. fuscatus* with those of other species may reflect that *P. fuscatus* and the other species of wasps studied belong to different subgenera (24). Our findings on the antigenic identity of homologous venom proteins from different species of wasps are in general accord with other studies (13,

23) where whole venoms were compared with animal or human antibodies.

Our results show that antigen 5, hyaluronidase, and phospholipase from wasp venom are allergens in man, as are the homologous proteins from yellowjacket venom. Phospholipase is the most immunogenic one of the three proteins since all seven wasp venomsensitive patients tested showed levels of phospholipase-specific antibodies as high as, or higher than, those of antibodies specific for venom, antigen 5, or hyaluronidase.

It is of interest to note that phospholipases from wasp and yellowjacket venoms show closely similar molecular sizes, amino acid compositions, and enzymatic specificities, yet they do not show any apparent antigenic identity when tested with the animal or human sera. This is the case also for antigen 5's from these two venoms. The results would suggest that the structurally common regions of these homologous proteins from wasp and yellowjacket venoms

are antigenically inactive, possibly because these common regions are in the inaccessible portion of the molecule.

We are currently working on the characterization of these three homologous proteins from different hornets. These sets of homologous proteins from different vespids will be useful diagnostic reagents for our better understanding of the common observation of patients' multiple sensitivity to venoms from different insects. This multiple sensitivity can be due to multiple exposures or to cross-reactivity of different insect venoms (cf. (1)).

ACKNOWLEDGMENTS

We would like to thank Dr. Allen W. Benton of Vespa Laboratories, Inc. for generous gifts of wasp venom sac extracts; Dr. James E. Gillaspay of Texas A & I University for gifts of wasp venoms; and Dr. Donald R. Hoffman of East Carolina University for gifts of sera from insect venom-allergic patients.

REFERENCES

1. LICHTENSTEIN, L. M., VALENTINE, M. D., AND SOBOTKA, A. K. (1979). *J. Allerg. Clin. Immunol.* **64**, 5-12.
2. KING, T. P., SOBOTKA, A., ALAGON, A., KOCHOUMIAN, L., AND LICHTENSTEIN, L. M. (1978) *Biochemistry* **17**, 5165-5174.
3. KING, T. P., ALAGON, A. C., KUAN, J., SOBOTKA, A. K., AND LICHTENSTEIN, L. M. (1983) *Mol. Immunol.* **20**, 297-308.
4. ABE, T., KAWAI, N., AND NIWA, A. (1982) *Biochemistry* **21**, 1693-1697.
5. LIAO, T. H., ROBINSON, G. W., AND SALNIKOW, K. (1973) *Anal. Chem.* **45**, 2286-2288.
6. ROCK, C. O., AND SNYDER, F. (1975) *J. Biol. Chem.* **250**, 6564-6566.
7. SRIVASTAVA, P. N., AND FAROOQUI, A. A. (1979) *Biochem. J.* **183**, 531-537.
8. KING, T. P., SOBOTKA, A. K., KOCHOUMIAN, L., AND LICHTENSTEIN, L. M. (1976) *Arch. Biochem. Biophys.* **172**, 661-671.
9. LAEMMLI, U. K. (1970) *Nature (London)* **227**, 680-685.
10. TOLKSDORF, S., MCCREADY, M. H., MCCULLAGH, D. R., AND SCHWENK, E. (1949) *J. Lab. Clin. Med.* **34**, 74-89.
11. SHILOAH, J., KLIBANSKY, C., DE VRIES, A., AND BERGER, A. (1973) *J. Lipid Res.* **14**, 267-278.
12. TUNG, A. S., JU, S. T., SATO, S., AND NISONOFF, A. (1976) *J. Immunol.* **116**, 676-681.
13. HOFFMAN, D. R., AND McDONALD, C. A. (1982) *Ann. Aller.* **48**, 82-86.
14. GIALONGO, A., KOCHOUMIAN, L., AND KING, T. P. (1982) *J. Immunol. Methods* **52**, 379-393.
15. AXELSEN, N. H., KRÖLL, J., AND WEEKE, B. (1973) *Scand. J. Immunol.* **2**(Suppl. 1), 15-90.
16. SHIPOLINI, R. A., CALLEWAERT, G. L., COTTRELL, R. C., DOONAN, S., VERNON, C. A., AND BANKS, B. E. G. (1971) *Eur. J. Biochem.* **20**, 459-468.
17. PLÜCKTHUN, A., AND DENIS, E. A. (1982) *Biochemistry* **21**, 1743-1750.
18. FLETCHER, J. E., ELLIOTT, W. G., ISHAY, J., AND ROSENBERG, P. (1979) *Toxicon* **17**, 591-599.
19. VAN DEN BOSCH, H., AARSMAAN, A. J., AND VAN DEENEN, L. L. M. (1974) *Biochim. Biophys. Acta* **348**, 197-209.
20. VAN DEN BOSCH, H. (1980) *Biochim. Biophys. Acta* **604**, 191-246.
21. TUICHIBAEV, M. U., MUKSIMOV, F. A., BABAIEV, M., RAKHIMOV, M. M., AND TASHMUKHAMEDOV, B. A. (1981) *Biokhimiya* **46**, 1215-1220.
22. MIROSHNIKOV, A. I., GRITSUK, V. I., MESHSHERYAKOV, E. A., OKHANOV, V. V., TUICHIBAEV, M. U., AND TASHMUKHAMEDOV, B. A. (1981) *Bioorg. Chem.* **7**, 494-501.
23. REISMAN, R. E., WYPYCH, J. I., MUELLER, U. R., AND GRANT, J. A. (1982) *J. Allerg. Clin. Immunol.* **70**, 281-287.
24. RICHARDS, O. W. (1973) *Rev. Brasil. Entomol.* **17**, 85-103.