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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 172, 661-671 (1976)

Allergens of Honey Bee Venom¹TE PIAO KING, ANNE K. SOBOTKA, LUCIA KOCHOUMIAN,
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The allergenic activities of four purified components of honeybee venom were studied by using histamine release from leukocytes of bee sting-allergic patients. The components studied were hyaluronidase, phospholipase A₂, melittin and apamin with molecular weights, respectively, of about 50,000, 15,800, 2840 and 2038 d. In six of the seven patients studied, hyaluronidase and phospholipase were, respectively, on the average about two and eight times more active by weight than the venom. The situation was reversed in one patient in that hyaluronidase and phospholipase A₂ were, respectively, 90 and 0.5 times more active than the venom. With this single exception, hyaluronidase and phospholipase were about equally active on a molar basis as allergens. Melittin was on the average about one-tenth as active as the venom, and apamin was inactive as an allergen.

Chemical modifications of phospholipase A₂ were carried out. Succinylation of eight of its eleven amino groups yielded a derivative that retained 4% of the enzymic activity of the native enzyme. Reduction and carboxymethylation of its four disulfide bonds or cyanogen bromide cleavage of its three methionyl bonds yielded enzymatically inactive derivatives. These derivatives showed varying decreases of allergenic activities when compared to the native enzyme. The results indicate that the antigenic determinants of phospholipase depend on the charge, the amino acid sequence and the conformation of the molecule.

One of the most common types of insect sting allergy is that caused by the honeybee (1). As bee sting allergy is caused by the venom injected on stinging (2, 3), several laboratories have made studies to characterize the bee venom allergens by various immunochemical methods (4-7). In this paper our studies on bee venom allergens will be reported.

Haberman and his colleagues (8) have shown that about 70% of the dry weight of honeybee venom is accounted for as polypeptides and proteins. Several of the com-

ponents have been isolated and characterized. Two peptides known as melittin and apamin and two proteins having phospholipase A₂ and hyaluronidase activities represent, respectively, about 50, 2, 12 and 2% of the venom weight. Melittin and apamin contain 26 and 18 amino acid residues, respectively, and their sequences are known. Phospholipase A₂ is a glycoprotein with a molecular weight of 15,800, and its covalent structure has been reported recently (9). Only limited chemical data are available for the hyaluronidase. We have purified these four known components of bee venom and we have studied their relative allergenic activities in sensitive individuals. As phospholipase A₂ was found to be an important allergen, chemical modifications of this protein were carried out and the allergenic and enzymatic activities were studied.

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MATERIALS AND METHODS

Honeybee venom, grades I and IV, were obtained from Sigma Chemical Co. Two different lots of grade I venom (122C-2170 and 640-0096) and a single lot of grade IV venom (1220-2410) were used. Hyaluronic acid was from Nutritional Biochemicals Corp. Sephadex G-50 and G-100 and carboxymethyl cellulose CM-32 were from Pharmacia and Whatman Biochemical, respectively.

Phospholipase assays were made by measuring the clearing of egg yolk suspension in agarose gel (10) or by a titrimetric procedure with egg yolk as the substrate (11). One unit of phospholipase activity is defined as that amount of enzyme releasing 1 μ mol of acid per minute at pH 8 and 25°C in 3.0 ml of a 10% (w/v) solution of egg yolk in 0.1 M NaCl. Hyaluronidase activity was measured by a turbidimetric method (12), with the modification that the assays were carried out at 25°C. One unit of activity is defined as that amount of enzyme required to hydrolyze 1 μ g of hyaluronic acid per minute at 25°C in 200 μ l of a 200 μ g/ml solution of hyaluronic acid in pH 5.3 buffer.

Ninhydrin analysis of samples was carried out by using the Technicon Autoanalyzer system. Concentration of samples by ultrafiltration was done in Visking 8/32 tubing (13). Hydrolysis of samples for amino acid analysis was carried out in 6 N HCl or in 6 N HCl containing 0.1% (w/v) phenol at 110°C for 22 h in sealed and evacuated tubes (less than 0.1 mm Hg pressure). Amino acid analyses were made on a Beckman-Spinco Model 120B analyzer; the analyzer was modified for use with narrow-bore columns so that nanomole amounts of amino acids were measured (14). Circular dichroic spectra were taken in a Cary spectropolarimeter. Discontinuous (disc) electrophoresis was carried out in polyacrylamide gels containing pH 4.1 acetate buffer in 6 M urea (15), and the gels were stained with Coomassie blue (16). Sodium dodecyl sulfate (SDS)² gel electrophoresis was carried out in gels containing 12% acrylamide, 0.6% methylene bisacrylamide and 0.2% SDS with or without 6 M urea (17). Electrophoresis in agarose gels containing pH 8.6 barbital buffer was carried out as described in the literature (18). The number of free amino groups in samples was determined colorimetrically by use of the reagent 2,4,6-trinitrobenzene sulfonic acid (19).

The allergenic activities of the fractions were studied by the histamine release method from leukocytes of bee sting-allergic patients (20) or from leukocytes of normal persons passively sensitized with allergic patients' sera (21).

Precipitin analyses of bee venom phospholipase or its derivative with rabbit anti-bee venom sera

were carried out in the manner described previously (22). The antisera were prepared by immunization of rabbits with bee venom in complete Freund's adjuvant (7).

RESULTS

There are two different grades of commercially available honeybee venom, I and IV. Examination of two separate lots of Grade I venom showed that they contained, within experimental error, identical hyaluronidase and phospholipase activities, 370 ± 70 and 62 ± 6 enzyme units/mg, respectively. Examination of a single lot of Grade IV venom showed that its specific phospholipase activity was the same as that of Grade I venom, but it contained negligible hyaluronidase activity. Other investigators have also reported the absence of hyaluronidase in certain commercial samples of bee venom (23). The fractionation experiments described below were carried out with the Grade I bee venom, and the procedures used are similar but not identical to those employed by others workers (4, 8, 23, 24). These procedures are described fully, as they are pertinent to the characterization of the isolated components.

Fractionation of Bee Venom

The venom was chromatographed on a column of Sephadex G-50 to yield fractions of different molecular sizes (Fig. 1). Fractions 1 and 2 were found to contain hyaluronidase and phospholipase activities, respectively, by using enzyme assays. Amino acid analysis of fractions 3 and 4 showed them to contain melittin and apamin, respectively. These four fractions were further purified by ion-exchange chromatography. The yields of the purified components are given in Table I.

Fraction 4 was chromatographed on CM-cellulose by using a linear gradient of increasing concentration of pyridine-acetate buffer (pH 4.65). Two fractions, 4A and 4B, were obtained (Fig. 2A), and both were found to have amino acid compositions identical to that reported for apamin. Only data for cut 4A are given in Table II. Fraction 3 was also chromatographed on CM-cellulose, but a linear gradient of increasing concentration of ammonium acetate

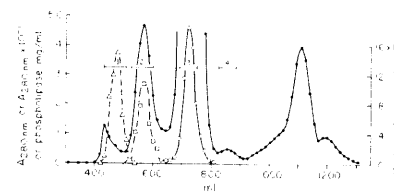


Fig. 1. Separation of bee venom components. The bee venom (2 g) was dissolved in 15 ml of 0.05 M ammonium acetate and 0.05 M acetic acid buffer (pH 4.75) and, after clarification by centrifugation, the solution was applied to a column (2.6 $\times$ 198 cm) of Sephadex G-50. The column was eluted with the same ammonium acetate buffer at a flow rate of 56 ml/h, and fractions of 15-ml volume were collected. Cuts 1 and 2 were concentrated by ultrafiltration, while cuts 3 and 4 were recovered on lyophilization. (●) and (○), A_{280} and $A_{280}/10$, respectively; (△), hyaluronidase activity; (□), phospholipase activity.

TABLE I
YIELDS OF THE PURIFIED COMPONENTS FROM 2 g OF BEE VENOM^a

Protein	Fraction	Amount (mg)	Specific activity (units/mg)
Hyaluronidase ^b	1A	0.43	7.8×10^4
Phospholipase	2A	73	5.5×10^2
	2B	24	5.5×10^2
Melittin	3A	320	
	3B	320	
	3C	212	
Apamin	4A	30	
	4B	7	

^a The sample of bee venom used was found to contain 370 ± 60 and 62 ± 6 units of hyaluronidase and phospholipase activities per milligram of venom, respectively.

^b The weight of hyaluronidase was estimated by amino acid analysis. The value may be an underestimate, as it does not take into account the possible presence of other neutral carbohydrates.

buffer (pH 4.75) was used. A broad peak, shown in Fig. 2B, was observed and three fractions, 3A, 3B and 3C, were taken as indicated. Amino acid analyses showed these fractions to have indistinguishable compositions within experimental error and their compositions to be the same as that reported for melittin. Only data for fraction 3A are given in Table II. Rechromatography of fraction 3A under the same

initial conditions but with the change that the sample size was reduced to one-fifth of that used originally showed that the peak position was delayed to that of 3C and that the peak broadness was reduced markedly as shown by the dashed curve in Fig. 2B. This finding indicates that the broadness of the melittin peak in Fig. 2B is related to its concentration-dependent aggregation reaction (8) and that it does not represent the separation of melittin into separate components differing in their electrical charges. A portion of the melittin in the venom is known to be formylated at its amino terminus. (8).

Fraction 2 was purified by chromatography on CM-cellulose by using a linear gradient of increasing concentration of ammonium acetate buffer (pH 4.75). Two fractions, 2A and 2B, containing phospholipase activity were obtained (Fig. 3A). These two fractions together accounted for 80% of the absorbance units at 280 nm applied to the column. On rechromatography, fraction 2A was eluted at the same position as in Fig. 3A (result not shown). Fractions 2A and 2B showed identical specific enzymatic activities (Table I) and identical amino acid compositions (only data for fraction 2A are given in Table II). The fractions had identical mobilities on disc electrophoresis in polyacrylamide gel containing acetate buffer in 6 M urea, but fraction 2B showed other bands in addition to the main band (Fig. 4A). On SDS-polyacrylamide-gel electrophoresis, fraction 2A showed only one band and its mobility indicated a molecular weight of $18,000 \pm 3000$, in accord with its known value (9); but fraction 2B gave two bands with mobilities indicating molecular weights of 18,000 and 15,000 (Fig. 4B). The nature of chemical differences between these two phospholipase fractions is not known.

Fraction 1 was also purified by chromatography on CM-cellulose, and a linear gradient of increasing concentration of sodium chloride in ammonium acetate buffer (pH 4.75) was used. Two peaks having hyaluronidase activity were obtained (Fig. 3B), accounting for 90% of the enzyme units applied to the column. Only the main peak fraction, 1A, was recovered, and rechromatography did not increase its

² Abbreviations used: CM-, carboxymethyl; IgE, immunoglobulin E; SDS, sodium dodecyl sulfate.

TABLE II
AMINO ACID COMPOSITIONS OF PURIFIED BEE VENOM COMPONENTS

Amino Acid ^a	Hyaluronidase			Phospholipase		Melittin		Apamin	
	Found ^b	Found ^b	Reported	Found ^b	Reported	Found ^b	Reported	Found ^b	Reported
Lysine	36.0	10.8	11	2.96	3	1.01	1		
Histidine	11.0	6.1	6	0		1.07	1		
Arginine	31.0	6.0	6	2.02	2	2.01	1		
Tryptophan	n.d. ^c	n.d.		n.d.	1		0		
Aspartic Acid	63.0	15.3	16	0.09		1.07	1		
Threonine	27.2	9.9	10	1.95	2	1.07	1		
Serine	34.3	9.0	10	1.03	1	0			
Glutamic Acid	47.3	6.3	6	1.98	2	3.00	3		
Proline	21.0	5.2	5	1.11	1	1.00	1		
Glycine	32.6	11.1	11	3.31	3	0			
Alanine	28.0	3.9	4	1.92	2	2.96	3		
Half-cystine	7.5	9.1	8	0		4.14	4		
Valine	24.2	4.9	5	1.55	2	0			
Methionine	7.8	2.6	3	0		0			
Isoleucine	16.0	2.9	4	2.82	3	0			
Leucine	39.7	8.4	9	3.80	4	1.01	1		
Tyrosine	25.9	7.8	8	0		0			
Phenylalanine	19.9	5.0	5	0		0			
Glucosamine	6.0	0	0	0		0			
Weight recovery	n.d.	91 ± 3% ^d		97 ± 3%		100 ± 3%			
A ₂₈₀ for 1 mg/ml solution	2.12 ^e	1.30 ^f		1.60 ^g		Negligible ^h			
	±0.20	±0.05		±0.05					

^a Only samples of 20-h hydrolysates were analyzed.

^b Calculated by taking the number of residues per mole of lysine, arginine, aspartic acid, glutamic acid, glycine and alanine to be respectively 36, 31, 63, 48, 32 and 28 for hyaluronidase; 11, 6, 16, 6, 11 and 4 for phospholipase (9); 3, 2, 0, 2, 3 and 2 for melittin (8); 1, 2, 1, 3, 0 and 3 for apamin (8).

^c n.d., Not determined.

^d The expected weight recovery for phospholipase is 92% as it is a glycoprotein.

^e In 0.1 M ammonium acetate buffer (pH 4.7); the weight of sample was estimated by amino acid analysis.

^f In 0.1 M ammonium bicarbonate.

^g In 0.2 M acetic acid.

specific activity (chromatogram not shown). The homogeneity of fraction 1A is indicated by disc electrophoresis in acetate-urea gel (Fig. 4A) and by electrophoresis in SDS gel (Fig. 4B). Its mobility on SDS-gel electrophoresis indicated its molecular weight to be $50,000 \pm 10,000$. The weight recovery of fraction 1A given in Table I was estimated from its amino acid composition given in Table II. This value may be an underestimate, as bee venom hyaluronidase is likely to be a glycoprotein and the composition of neutral sugars, if present, is not known.

It was found best not to concentrate the purified hyaluronidase fraction by lyophilization, as there was a significant loss of enzymic activity on such treatment. However, there was no detectable loss of hyaluronidase activity on lyophilization of bee venom solutions in ammonium acetate buffer (pH 4.75) or in ammonium bicarbonate (pH 8.4). Purified or crude phospholipase was stable to lyophilization from ammonium acetate or bicarbonate buffers.

Chemical Modifications of Bee Venom Phospholipase.

In the following studies, only the major form of phospholipase (fraction 2A) was used.

Succinylation of phospholipase (8.5 mg,

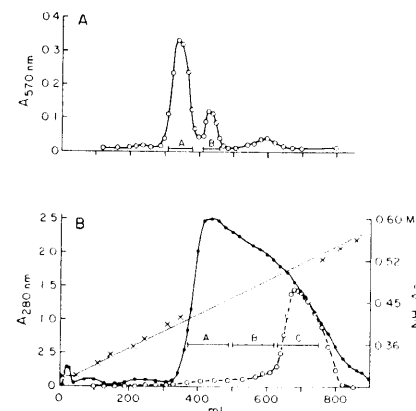


FIG. 2. (A), Purification of apamin. The entire sample of cut 4 of Fig. 1 (186 mg) in 10 ml of 0.1 M pyridine-acetate buffer was applied to a column (0.9 × 20 cm) of CM-cellulose, CM-32. The column was eluted with a linear gradient containing 500 ml each of 0.1 and 0.6 M pyridine-acetate buffer (pH 4.65). The flow rate was 60 ml/h. Ninhydrin analyses were made on 50-μl aliquots of the fractions (10-ml volume) to detect the peptide. (B), Purification of melittin. One-half of the sample of cut 3 of Fig. 1 (610 mg) in 10 ml of 0.2 M ammonium acetate buffer (pH 4.75) was applied to a column (0.9 × 20 cm) of CM-cellulose. The column was eluted with a linear gradient of 500 ml each of 0.3 and 0.7 M ammonium acetate buffer (pH 4.75) at a flow rate of 56 ml/h. The fractions (10-ml volume) were analyzed by absorbance at 280 nm. The dashed curve was obtained on rechromatography of fraction A (122 mg dissolved in 5 ml of starting buffer) under the same initial conditions.

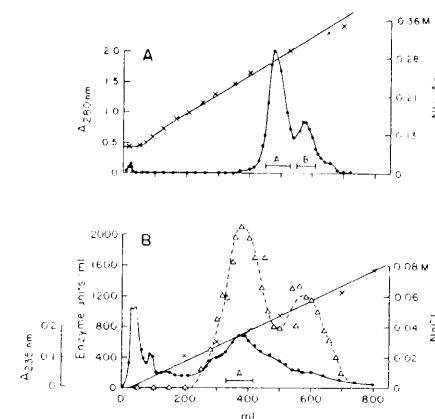


FIG. 3. (A), Purification of phospholipase A₂. The entire sample of cut 2 of Fig. 1 in 10 ml of 0.1 M ammonium acetate buffer (pH 4.75) was applied to a column (0.9 × 20 cm) of CM-cellulose. The column was eluted with a linear gradient of 500 ml each of 0.1 and 0.4 M ammonium acetate buffer (pH 4.75) at 56 ml/h, and fractions of 10-ml volume were collected. (B), Purification of hyaluronidase. The entire concentrate (9 ml) of cut 1 of Fig. 1 was used. The column (0.9 × 20 cm) of CM-cellulose was eluted with a linear sodium chloride gradient in 0.2 M ammonium acetate buffer (pH 4.75), increasing from 0 to 0.1 M in a total volume of 1 liter. The flow rate was 60 ml/h, and fractions of 10-ml volume were collected. (○), Absorbance at 280 nm; and (△), hyaluronidase activity.

5.4 μmol) was carried out at 25°C in 2 ml of 0.05 M Tris-HCl buffer (pH 7.95) by two successive additions of succinic anhydride (27 mg, 230 μmol) at 30-min intervals. The pH of the reaction was kept constant by the addition of 2 M Tris. The product was isolated by chromatography on Sephadex G-100 with 0.1 M ammonium bicarbonate as the eluant, and its elution position was the same as that of the native enzyme. The product migrated rapidly as an anion on agarose-gel electrophoresis in pH 8.6 barbital buffer, while the native enzyme migrated slowly as a cation; the presence of as little as 1% of native enzyme in the product could be easily detected. Using the trinitrobenzene sulfonic acid method (19), the native and the succinylated enzymes

were found to contain 11.2 and 3.5 amino groups per mole, respectively. The native enzyme has one α-amino and eleven ε-amino groups (9); therefore about eight succinyl groups were introduced per molecule of enzyme. The native and the succinylated enzymes had similar dichroic spectra (Fig. 5), indicating that no major conformational change of the molecule occurred on modification. By the titrimetric assay, the specific activities of the native and the modified enzymes were found to be, respectively, 550 and 22 units/mg. Lineweaver-Burk plots for the native and the modified enzymes are given in Fig. 6. The data show that the decrease in specific activity of the modified enzyme is mainly a consequence of a reduction of its catalytic efficiency as the K_m 's for the two enzymes differ only by about twofold.

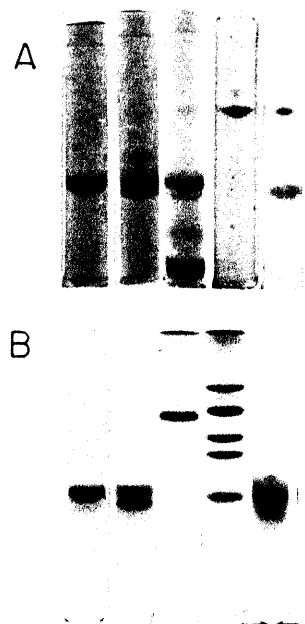


Fig. 4. (A), Acetate-urea-gel electrophoresis: from left to right, fractions 2A, 2B, bee venom, 1A and admixture of 2A and 1A. About 5 μ g of each protein was used per gel with the following exceptions: for the admixture of 2A and 1A, about 1 μ g of each protein was used; for the venom, 48 μ g were used. (B) SDS-polyacrylamide-gel electrophoresis: from left to right, fractions 2A, 2B, 1A, an admixture of 1A and standard proteins and, on the far right, bee venom. About 3 μ g of each protein were used per gel, and the amount of venom used was 25 μ g. The standard proteins from top to bottom are: bovine plasma albumin (M.W. 66,000), pepsin fragments A and B of bovine albumin (M.W. 35,000 and 29,000) (25) and ribonuclease (M.W. 13,600).

The four disulfide bonds of phospholipase (4.1 mg) were reduced with dithiothreitol (10 mg) in 2 ml of 6 M guanidine hydrochloride containing 0.1 M Tris-HCl buffer (pH 8.6). After 18 h at 25°C, the reduced protein was alkylated with iodoacetic acid (24 mg) and the pH was kept at 8.0 by the addition of 2 M Tris. After 30 min, the excess reagents were removed by dialysis against 0.1 M ammonium bicarbonate. The mixture was then fractionated on a column (200 $\times$ 0.9 cm) of Sephadex G-100.

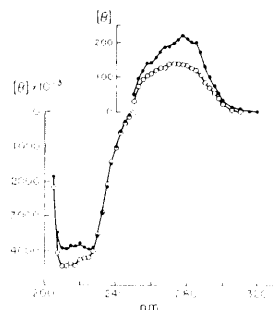


Fig. 5. Circular dichroic spectra. (●), Phospholipase; and (○), succinylated phospholipase. The protein concentration was in the range of 0.9–1.0 mg/ml in 0.05 M sodium phosphate buffer (pH 7.2).

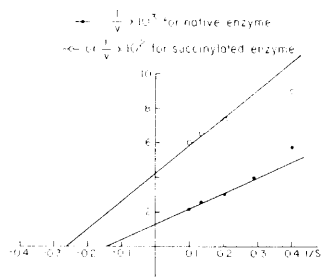


Fig. 6. Lineweaver-Burk plots for phospholipase (●) and its succinylated derivative (○). Velocity (v) is the rate of release of acid in micromoles per minute per milligram of enzyme, and s is the percent concentration of egg yolk.

Two peaks of about equal amounts of proteins were obtained; one eluted at the void volume and the other at the same position as that of the native enzyme (chromatogram not shown). Both peaks were found to contain fully reduced and carboxymethylated phospholipases as they showed identical amino acid compositions. Their carboxymethyl cystine content was 9.4 ± 0.3 residues/mol, while that expected is 8.0 residues (9).

Cleavage of the three methionyl bonds of phospholipase (10 mg) with cyanogen bromide (100 mg) was carried out at 25°C in 75% aqueous formic acid (1.4 ml). After a reaction time of 2 h, the product was recovered by lyophilization after dilution with water. On SDS-gel electrophoresis,

the product gave only one band having a mobility identical to that of the native enzyme, indicating no change in molecular weight on cleavage. After reduction of the cleaved product with mercaptoethanol, two bands were seen on SDS gels. Their mobilities indicated molecular weights of about 4000 and 7000 (results not shown). These findings are in agreement with the known covalent structure and the disulfide bond distribution of phospholipase (9). The cleaved product was enzymatically inactive. A reduced and carboxymethylated derivative of the cyanogen bromide-cleaved phospholipase was also prepared by following a procedure identical to that given above for the native enzyme except that the product was purified on a Sephadex G-25 column.

Immunological Studies

The purified bee venom components were assayed for their allergenic activities by measuring their ability to release histamine from the leukocytes of honeybee-allergic patients (20) or from the cells of normal donors passively sensitized with sera from allergic patients (21). Figure 7 shows representative data from one patient. From such data the concentration of allergens required for 50% release of cellular histamine is extrapolated. The extrapolation error is estimated to be about 30%. Both unfractionated venom and hyaluronidase caused 50% histamine release from this patient's cells at about 5×10^{-4} μ g/ml, whereas one-fifth of that concentration

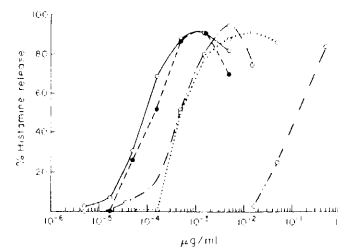


Fig. 7. Histamine release from leukocytes of a bee sting-allergic patient (R.W.) challenged with bee venom or its purified components: bee venom (.....); hyaluronidase fraction 1A (-----); phospholipase fraction 2A (— · — · —); melittin fractions 3A (————).

was required for phospholipase. Melittin was weakly allergenic in this patient, releasing 50% histamine at 1×10^{-1} μ g/ml. Since melittin is known to cause nonspecific lysis, concentrations up to 10 μ g/ml were tested on the cells of normal donors; nonspecific histamine release did not occur. Apamin had been tested twice previously on this patient's leukocytes and found nonallergenic at concentrations up to 1 μ g/ml. Consequently, it was not included in this experiment. Experimental data on seven patients studied are summarized in Table III. The specificity of the assay is indicated by the fact that both normal cells sensitized with ragweed-allergic serum and nonsensitized leukocytes from nonallergic donors released very little or no histamine when challenged with bee venom or its purified components at concentrations which were 100 times greater than those indicated in Table III (data not shown).

The relative allergenic activities of the components are indicated by the ratio of their concentration required for 50% histamine release to that required when using whole venom. These values are given in parentheses in Table III. In six of the seven patients studied, phospholipase was more allergenic than the venom, its relative activity is in the range of 3–12, and the average value is about 8. In these same six patients, the relative activity of hyaluronidase is in the range of 1–5 and the average value is about 2. In only one of the seven patients (J.G. in Table III) was phospholipase less allergenic than the venom, and this is also the only case in which hyaluronidase was more active than phospholipase.

The relative allergenic activity of melittin also covers a wide range (0.01–0.30, Table III); for the majority of patients the value is about 0.1. This amount of activity could not be the result of trace contamination with the most active phospholipase, as all the melittin samples (fractions 3A, 3B and 3C) were found to contain less than 0.01% phospholipase by the titrimetric assay. Further confirmation of the allergenicity of melittin was obtained from a series of passive sensitization experiments with use of the sera from several bee-allergic

TABLE III

ALLERGENIC ACTIVITY OF THE PURIFIED BEE VENOM COMPONENTS AS MEASURED BY HISTAMINE RELEASE FROM LEUKOCYTES OF ALLERGIC PATIENTS OR FROM LEUKOCYTES OF A NORMAL DONOR PASSIVELY SENSITIZED WITH ALLERGIC SERA

Patient ^a	Allergen required for 50% histamine release ($\mu\text{g/ml}$) ^b				
	Honey bee venom	Hyaluronidase Fraction 1A	Phospholipase ^c Fraction 2A	Melittin Fraction 3A	Apamin Fraction 4A
R.W.	5×10^{-4} (1)	5×10^{-4} (1)	1×10^{-4} (5)	1×10^{-1} (0.005)	— ^d
D.P.	4×10^{-3} (1)	1×10^{-3} (4)	7×10^{-4} (6)	1×10^{-1} (0.04)	n.d. ^e
S.M.	1×10^{-5} (1)	$<10^{-5}$ (>1)	4×10^{-6} (3)	n.d.	— ^d
J.G.	9×10^{-3} (1)	1×10^{-4} (90)	2×10^{-2} (0.5)	1×10^{-1} (0.09)	n.d.
P.P.	6×10^{-4} (1)	1×10^{-3} (0.6)	3×10^{-4} (12)	1×10^{-1} (0.006)	n.d.
J.V.	1×10^{-2} (1)	1×10^{-2} (1)	8×10^{-4} (12)	1×10^{-1} (0.1)	n.d.
K.B.	1×10^{-2} (1)	2×10^{-3} (5)	1×10^{-3} (10)	3×10^{-2} (0.33)	n.d.

^a Values for the first four patients were obtained by using their own leukocytes while those for the remaining three patients were obtained by using normal cells passively sensitized with their sera.

^b Values in parentheses are activities relative to that of bee venom which is taken to be 1.

^c The activity of phospholipase fraction 2B was found to be within the experimental error, the same as that of fraction 2A.

^d No histamine release at a test allergen concentration of $1 \mu\text{g/ml}$.

^e n.d., Not determined.

patients. An example of one such experiment is shown in Fig. 8. The sera from three bee-allergic patients contained specific IgE antibodies capable of transferring melittin sensitivity to normal cells while the same cells, sensitized with a serum from a ragweed-allergic patient, released no histamine when challenged with melittin. Apamin is apparently inactive as an allergen since, at a test concentration of $1 \mu\text{g/ml}$, no histamine was released from any patient's cells.

The allergenic activities of the chemically modified phospholipases were determined by using passively sensitized leukocytes (Table IV). The relative activity of the succinylated enzyme compared to that of the native enzyme covers a range of 0.003–0.7 in the five patients studied. The relative activity of the cyanogen bromide-cleaved enzyme covers as wide a range as does the succinylated enzyme. The widely differing decreases in activity of the succinylated or the cyanogen bromide-cleaved phospholipase in the five patients tested may reflect that the sera of these patients are specific for different antigenic determinants of phospholipase used for sensitization. The relative activities of the reduced and carboxymethylated enzyme and of the

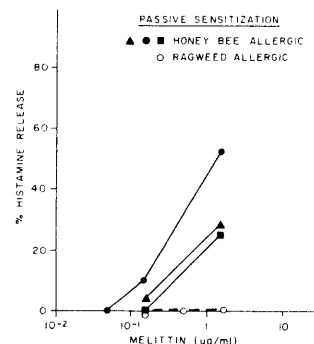


FIG. 8. Passive sensitization of normal leukocytes with three sera from honeybee-allergic patients (●) and a serum from a ragweed-allergic patient (○) followed by challenge with melittin.

cyanogen bromide-cleaved enzyme followed by reduction and carboxymethylation are, respectively, about 10^{-4} and 10^{-5} of that of the native enzyme.

Precipitin analyses in liquid medium of phospholipase and its succinylated derivative were carried out with rabbit anti-bee venom sera (Fig. 9). The amount of immune precipitate obtained with the succinylated enzyme at maximal precipitation

TABLE IV

ALLERGENIC ACTIVITIES OF NATIVE AND MODIFIED PHOSPHOLIPASES

Patient ^a	Allergen required for 50% histamine release ($\mu\text{g/ml}$) ^b				
	Native	Succinylated	Reduced and carboxymethylated ^c	Cyanogen bromide cleaved	Cyanogen bromide cleaved then reduced and carboxymethylated
S.M.	1×10^{-5} (1)	3×10^{-3} (0.003)	7×10^{-2} (0.0001)	4×10^{-3} (0.003)	1 ^d ($<10^{-5}$)
R.W.	5×10^{-4} (1)	7×10^{-4} (0.7)	2 (0.0003)	3×10^{-3} (0.2)	1 ^d ($<10^{-5}$)
J.V.	4×10^{-5} (1)	2×10^{-4} (0.2)	2×10^{-2} (0.0002)	1×10^{-4} (0.4)	1 ^d ($<10^{-5}$)
J.G.	3×10^{-3} (1)	6×10^{-1} (0.005)	n.d. ^e	1 (0.003)	n.d.
K.B.	2×10^{-4} (1)	5×10^{-3} (0.04)	n.d.	2×10^{-2} (0.01)	n.d.

^a Normal cells passively sensitized with patients' sera were used.

^b Values in parentheses are activities relative to that of the native enzyme.

^c Two chromatographic forms of reduced and carboxymethylated phospholipase were obtained (see text), and they showed identical allergenic activities.

^d Incomplete or no release of histamine at $1 \mu\text{g/ml}$ of allergen.

^e n.d., Not determined.

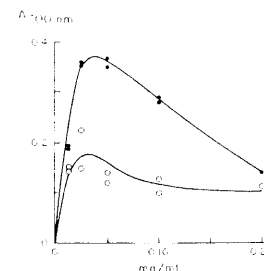


FIG. 9. Precipitin analyses of phospholipase and its succinylated derivative with a rabbit anti-bee venom serum. The volumes of antigen and antiserum solutions are respectively 25 and 50 μl .

is about one-half that obtained with native enzyme, indicating that most of its antigenic determinants are retained on succinylation.

DISCUSSION

Hyaluronidase, phospholipase A₂, melittin and apamin were isolated from honeybee venom. These four components were characterized by their chromatographic properties and/or electrophoretic mobilities, amino acid compositions and, where applicable, their enzymatic activities. Their allergenic activities were examined by following their capacities to induce histamine release from leukocytes of allergic patients (Table III).

For the majority of patients tested (six out of seven) hyaluronidase and phospholipase were respectively on the average two and eight times more active by weight than the bee venom. This indicates that the relative allergenic activities of these two proteins are about equal on a molar basis, as the molecular weights of these two proteins differ by a factor of about 3. Only in one of seven patients studied is hyaluronidase more active than phospholipase on a weight or molar basis. Several reports (5–7) have appeared indicating phospholipase to be the major allergen of bee venom. One report (5) indicated that the purified phospholipase was no more active, on a weight basis, than the bee venom in causing histamine release from sensitive leukocytes. Another report (6) indicated that, in addition to phospholipase, hyaluronidase and another high molecular weight fraction of bee venom were important allergens, as shown by the radioallergosorbent test. The present findings extend these preliminary reports in the following way: Both hyaluronidase and phospholipase are important allergens, but the relative responses of allergic individuals to these two allergens vary. The varying responses of individuals to these two bee venom allergens find precedence in the literature on the allergens of ragweed pollen. Allergens Ra3 and Ra5 are highly active only in a selected population of ragweed-

sensitive individuals while the major allergen antigen E is highly active in the overwhelming majority of patients (26).

The results presented in Fig. 8 and in Table III indicate that melittin is a weak allergen. As noted earlier, the weak activity of melittin is not the result of cross contamination. Other investigators (6) have also reported that melittin might be a weak allergen in bee sting patients. Melittin has also been reported to be immunogenic in mice, stimulating the formation of skin-sensitizing antibodies (27). Both apamin and melittin are peptides of about the same size, containing about 20 amino acid residues. However, we found apamin to be nonallergenic in all patients studied. In addition to its much greater abundance in bee venom, melittin is known to be strongly associated in solution as well as to possess surfactant properties (8). These two characteristics may be of importance as to why melittin is an allergen while apamin is not. The small size of melittin indicates that it can be another useful model allergen for studying genetic aspects of immune response (cf. Ref. (26)).

The allergenicity of phospholipase was decreased on succinylation of its accessible amino groups, cyanogen bromide cleavage of its methionyl bonds or reduction and carboxymethylation of its disulfide bonds (Table IV). Succinylation of phospholipase changed the net charge of the protein from a basic one to an acidic one but it produced only a limited conformational change of the molecule, as indicated by the similarity of its circular dichroic spectrum to that of the native enzyme and by its retention of enzymatic activity though of reduced catalytic efficiency. The primary structure of phospholipase was altered on cyanogen bromide cleavage, and the product was enzymatically inactive. Complete reduction of the cross-linking disulfide bonds of phospholipase produced a large conformational change, as indicated by its complete loss of enzymatic activity and its altered chromatographic behavior on Sephadex G-100. The greatest decrease in allergenic activity (greater than 10^5 -fold) was obtained when the enzyme was first cleaved with cyanogen bromide, then reduced and car-

boxymethylated. The above results taken together show that the allergenic (or antigenic) determinants of phospholipase depend on the charge, the conformation and the amino acid sequence of the molecule, just as has been found for the other globular protein allergens from ragweed pollen (22) and rye grass pollen (26).

The amino acid sequences of phospholipases A_2 from porcine pancreas (28), snake venom (*Naja melanoleuca*) (29) and honeybee venom (9) are known, and they all consist of about 130 amino acid residues. Alignment of the sequences of porcine pancreatic and snake venom enzymes shows that about half of the amino acid residues occupies identical positions. Similar alignment of the sequences of porcine pancreatic and bee venom enzymes shows that less than 20% of the residues occupies identical positions. Also, bee venom phospholipase differs significantly from the other two phospholipases in the number as well as the pairing of half-cystine residues. These structural differences between the bee venom and the mammalian phospholipases probably play an important role in the high immunogenicity of bee venom in sensitive people, as it is well known that the immunogenicity of a protein antigen depends on its degree of foreignness to the host being immunized (30).

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