

Allergens in bee venom

II. Two new high molecular weight allergenic specificities

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Two new allergenic specificities were detected in honeybee venom and the two corresponding protein substances isolated by gel filtration, immunoadsorption, and ion exchange chromatography. The first of these, allergen B, has a molecular weight ranging from 49,000 to more than 200,000 d and can be recognized by rabbit and guinea pig antisera as well as by human reagenic sera using the radioallergosorbent test (RAST). Allergen B gives a single line in immunodiffusion distinct from hyaluronidase, phospholipase A, melittin, and the other high molecular weight substances described and gives a single band at 49,000 d in sodium dodecyl sulfate (SDS) polyacrylamide gel. The second substance, allergen C, has a molecular weight of 105,000 d and was separated from allergen B by immunoadsorption with insoluble antibody. Allergen C was shown to be distinct from the other substances in bee venom by immunodiffusion with animal antisera. One human reagenic serum was monospecific for allergen C. Two other minor components of 86,000 and 71,000 d are present in bee venom; their allergenic activities are unknown. The two specificities, B and C, comprise most of the reactivity of the previously described Sephadex G-75 fraction 1 and clearly are important allergens, reacting with 98% of sera from bee venom-allergic individuals.

Three major allergen complexes have been demonstrated in honeybee venom^{1, 2}: hyaluronidase,¹⁻⁵ phospholipase A,^{6, 7} and the high molecular weight substances (fraction 1).^{1, 2} In our previously reported study,² sera from 40% of patients having systemic and large local reactions following bee stings were more reactive by RAST with the high molecular weight materials, fraction 1 from Sephadex G-75, than with the other allergens. Later studies of 68 additional sera yielded results comparable to those reported previously.

Since these high molecular weight materials appear to be important allergens, we have begun investigations of their nature and composition. In this paper we describe the isolation and characterization of two distinct allergenic specificities from this fraction.

MATERIALS AND METHODS

Bee venom

Bee venom collected by electrical stimulation was obtained from Champlain Valley Apiaries, Middlebury, Vt. It made little difference whether whole liquid venom (1974

crop) or air-dried venom (1975 or 1976 crop) was used. The venom must not be lyophilized or fraction 1 aggregates and becomes insoluble.

Human sera

Sera were obtained from patients who exhibited systemic or large local reactions within one hour following a bee sting. The criteria for the patient group is explained in greater detail by Hoffman and Shipman.² Sera were obtained before hymenoptera immunotherapy was started.

Antisera

Antisera to various fractions were produced by immunizing adult male New Zealand white rabbits or DHC/BA inbred guinea pigs with 0.5 to 1 mg of antigen emulsified in Freund's complete adjuvant. Immunizations were given in multiple intradermal sites and repeated at four- to six-week intervals until a satisfactory response was obtained. All sera were tested by double diffusion in gel against the various venom fractions.

Enzyme assays

The methods of assaying for hyaluronidase and phospholipase A are presented in detail by Hoffman and Shipman.² The hyaluronidase assay is based on that of Tolksdorf and co-workers⁸ and the phospholipase A assay on that of Magee and Thompson.⁹

Polyacrylamide gel electrophoresis (PAGE)

PAGE was performed in 4% and 7.5% acrylamide gels in 0.188 M glycine-acetic acid buffer, pH 3.6. Proteins were

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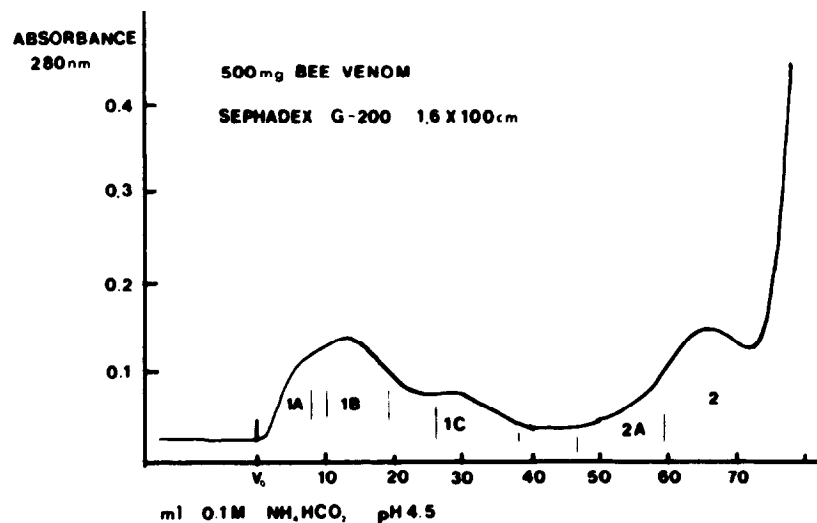


FIG. 1. Elution profile of 500 mg bee venom applied in 1.5 ml on a Sephadex G-200 column.

visualized by 1% amido black in 7% acetic acid or 0.5% Coomassie brilliant blue R-250 in 7% acetic acid.

Sodium dodecylsulfate PAGE

Samples were denatured overnight in 0.04 M dithiothreitol, 0.001 M ethylenediaminetetraacetic acid (EDTA), and 1% SDS in 0.04 M Tris acetate buffer, pH 6.6, at 40°. Electrophoresis of denatured samples was done in 4%, 7.5%, and 12% acrylamide gels in 0.205 M Tris acetate 0.1% SDS at pH 6.6 using a bromophenol blue tracking dye. Samples were fixed and SDS removed by soaking in four 20-ml changes of a mixture of 40% isopropyl alcohol, 10% acetic acid, and 50% water. Protein bands were visualized by staining with 0.1% Coomassie brilliant blue R-250 in 7% acetic acid. Destaining was by diffusion in 7% acetic acid. Molecular weights were calculated by constructing a standard curve on semilogarithmic paper using IgG, bovine serum albumin (BSA), H and L chains as reference proteins. Mobilities of reference proteins were determined in each run. Molecular weights are the average of at least four determinations.

Gel filtration

Bee venom (1 ml or 500 mg dry weight) was applied to a 1.6 × 100 cm column of Sephadex G-200 and eluted with 0.1 M ammonium formate, pH 4.5, at 7.5 ml/hr. Hyaluronidase, phospholipase A, and melittin were prepared from 500-mg batches of venom using a 1.6 × 100 cm Sephadex G-75 column. Fraction 1 from the G-75 columns was concentrated by ultrafiltration and separated on a G-200 column as above.

Isoelectric focusing in polyacrylamide gel

Bee venom fractions were focused in polyacrylamide gel rods by the method of Hoffman, Grossberg, and Pressman.¹⁰ Ampholyte pH 3-10 was obtained from Bio-Rad Laboratories, Richmond, Calif. The higher molecular weight allergens were focused in 4 M urea to prevent aggre-

gation and precipitation. Urea was not necessary for focusing hyaluronidase or phospholipase. Proteins were visualized by staining each gel in 20 ml of a mixture of 100 ml 5% trichloroacetic acid–5% sulfosalicylic acid, 20 ml of methanol, and 1 ml of 1% Coomassie blue R-250.

Purification of hyaluronidase

Fraction 2 (10 mg) was applied to a 1.5 × 24 cm column of carboxymethyl Sephadex C-25 equilibrated with 0.01 M ammonium formate pH 4.5. Elution was with a 500-ml linear gradient formed from equal volumes of starting buffer and 1.0 M ammonium formate, pH 4.5, containing 0.1 M NaCl. Hyaluronidase eluted in a broad peak centered at about 0.03 M NaCl.

Immunoabsorption

Immunoglobulins were isolated from antisera by precipitation with 33% saturated ammonium sulfate; after dialysis the globulin from 7 ml of serum was coupled to 10 ml of Sepharose 6B activated with cyanogen bromide.¹¹ After blocking with ethanolamine and extensive washings, the adsorbent was used to remove unwanted substances.

Amino acid analysis

Proteins were hydrolyzed for 22 hr using 6 N HCl in evacuated tubes. Amino acid compositions were determined on a Beckman model 120B analyzer.¹²

Radioallergosorbent test (RAST)

The method used in our laboratories for specific IgE antibody determinations has been described previously, and the evidence for its specificity has been presented.^{13, 14} Paper discs of 6 mm diameter punched from Schleicher and Schuell No. 595 filter paper were activated with cyanogen bromide.¹¹ Bee venom was coupled at a ratio of 10 mg dry weight per 200 activated discs in borate buffer, pH 8.0. The various fractions were coupled at a ratio of 1 mg per 200 discs; increasing the amount of antigen to 10 mg did not

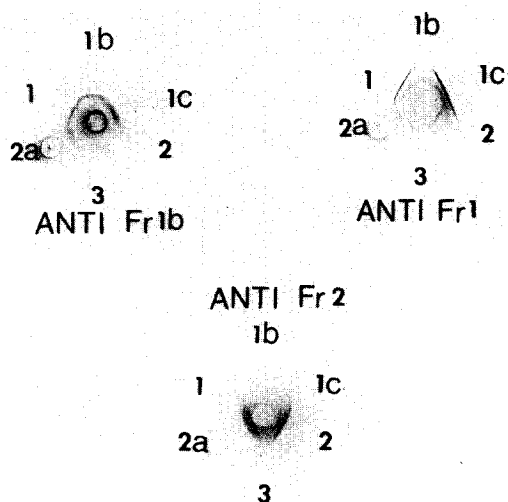


FIG. 2. Immunodiffusion analysis of bee venom fractions against rabbit and guinea pig antisera.

significantly increase specific IgE binding. Remaining reactive sites were blocked with ethanolamine, and the discs were extensively washed.

The Fc-specific anti-IgE was of horse origin raised against PS myeloma and specifically purified using ND myeloma.¹³ The anti-IgE was labeled with ¹²⁵I using 0.2 mCi per 50 μ g. Seven nanograms of radioactive anti-IgE was used for each assay tube.

The RAST was run in quadruplicate for each sample using one disc, 10 μ L of serum, and 100 μ L of buffer in each 12 $\times$ 75 mm plastic tube. After 18 hr, the discs were washed three times with 3 ml of 1% Tween 20 in saline. Radioactive anti-IgE was then added in 100 μ L of RAST buffer containing 6% normal horse serum. After 18 hr incubation, the discs were washed four times with Tween-saline and counted. Positive and negative controls were run for each antigen and batch of discs included in a run. Newborn sera and negative sera controls were comparable. Background binding for each antigen was calculated for each run by averaging the negative controls. The background was subtracted from the total binding for each sample. Background binding was a function of antigen and batch as well as of radioactive antibody. Since the controls bound an average of $1.1 \pm 0.1\%$ (SD) of counts added to bee venom discs, positive was defined as greater than 2.0% binding or 1.9 times background binding for this system. A further group of 91 controls bound $0.8 \pm 0.2\%$ (range) using a second batch of bee venom discs.

RESULTS

The elution profile of 500 mg of bee venom on a Sephadex G-200 column is shown in Fig. 1. Five fractions were collected before the large peak containing phospholipase A, melittin, and the other lower molecular weight substances. Fractions 1A, 1B, and 1C correspond to fraction 1, the G-75 exclusion

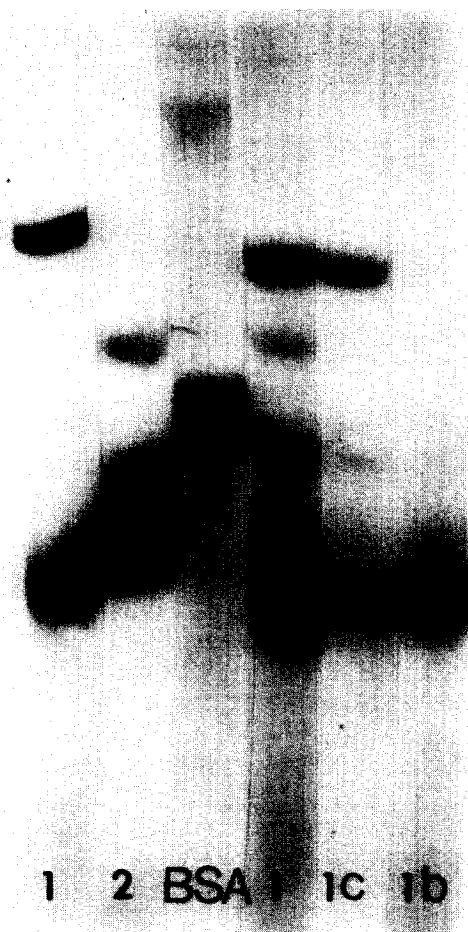


FIG. 3. Sodium dodecylsulfate polyacrylamide gel electrophoresis in 7.5% gels of bee venom fractions and a BSA standard; fraction 1 is shown at two different concentrations. The anode is at the bottom.

peak, fraction 2 consists primarily of the enzyme hyaluronidase, and fraction 2A is a mixture of hyaluronidase and two other minor components. If either whole venom or fraction 1 is freeze-dried, peak 1A becomes substantially larger as the result of aggregation. When separated from venom, fraction 1 tends to form insoluble aggregates, but can be dissolved in 8 M urea and deaggregated (although not completely) with dithiothreitol.

Analyses of the various fractions for both hyaluronidase activity and phospholipase activity were performed. Fraction 1 and its subfractions did not contain measurable activity of either of these enzymes (less than 0.1%). Fraction 2 contained hyaluronidase activity at a high level and contained about 0.5% phospholipase A. As has been reported previously,² fraction 3 from Sephadex G-75 is phospholipase A and fraction 4 is melittin.

Fraction 1 was isolated by Sephadex G-75 gel filtration from 2.2 gm of bee venom, concentrated by

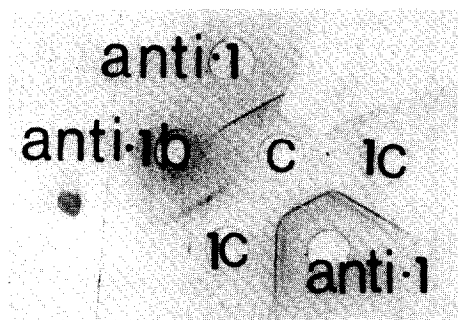


FIG. 4. Immunodiffusion analysis to follow the separation of fraction 1C by immunoadsorption; C in the center well is fraction 1C twice adsorbed with insoluble anti-1B.

ultrafiltration, and separated on Sephadex G-200 into fractions 1B, 1C, a small amount of hyaluronidase, and some small molecular weight materials identified as consisting mostly of melittin by amino acid analysis.

Analysis of these fractions by immunodiffusion with animal antisera raised against various bee venom fractions is shown in Fig. 2. Antiserum against fraction 1B gave identical single, somewhat diffuse, lines with fractions 1, 1B, 1C, and 2a. Antiserum against fraction 2 gave double lines with fractions 2a and 2 and very weak lines with fractions 1 and 1C; no reaction was observed with fractions 1B or 3. Antiserum against fraction 1 gave up to 4 lines of precipitate: one corresponding to the substance found in fraction 1B, a line with fraction 2 shown to be antihyaluronidase, and a strong line found in fractions 1 and 1C with a trace of related substance in fraction 2a. Fractions 1, 1B, 1C, 2a, and 2 did not react at all with antiphospholipase A. In immunodiffusion experiments, the three major lines anti-1B, antihyaluronidase, and anti-1C all crossed without fusion or spurring.

These same fractions were further analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results are shown in Fig. 3. Fraction 1 gave 4 bands, two strong bands at $105,000 \pm 10,000$ d and $49,000 \pm 5,000$ d and two weak bands at 71,000 d and 87,000 d. Fraction 2 gave two bands, the major band at $53,000 \pm 5,000$ d identified as the enzyme hyaluronidase, the minor at 87,000 d. Fraction 1B consists of only a single band at 49,000 d and fraction 1C a strong band at 105,000 d with a weaker band at 49,000 d and a trace at 71,000 d.

Absorption of fraction 1C with a column of insolubilized anti-fraction 1B and gel filtration on Sephadex G-150 completely removed immune reactivity with anti-1B as is shown in Fig. 4 and also removed the 49,000 d and 71,000 d bands seen in SDS-PAGE.

Isoelectric focusing in gel (IEF) shown in Fig. 5



FIG. 5. Isoelectric focusing of bee venom fractions in polyacrylamide gel, run in a gradient of pH 3.5 (bottom) to pH 10 (top).

gave a single band with two satellites for phospholipase A (fraction 3) and a three-line pattern for hyaluronidase (fraction 2) both in the high pH region. Although the patterns are complex, fraction 1B is clearly distinct from fraction 1C and whole fraction 1, and its components do not resemble fraction 2 or 3 at all. Amino acid analyses of fractions 1, 1B, hyaluronidase, phospholipase A, and melittin are presented in Table I. Substantial composition differences are apparent between 1B, hyaluronidase, and phospholipase A.

Allergenic activities of the venom fractions were tested by the radioallergosorbent test with sera from patients clinically allergic to bee venom. The results with 12 selected sera are presented in Figs. 6 and 7. The sera selected were chosen to represent the various patterns of binding observed and to have more than 4% binding above background with at least one fraction of bee venom. Sera 662, 1042, P2221, P2560, P2820, P2976, and P3305 were all primarily specific for fraction 1 and/or its subfractions. Most bee-allergic sera give patterns like those of 1143 or P2359.² Serum P2221 is virtually monospecific for a substance contained in fraction 1C. As this fraction

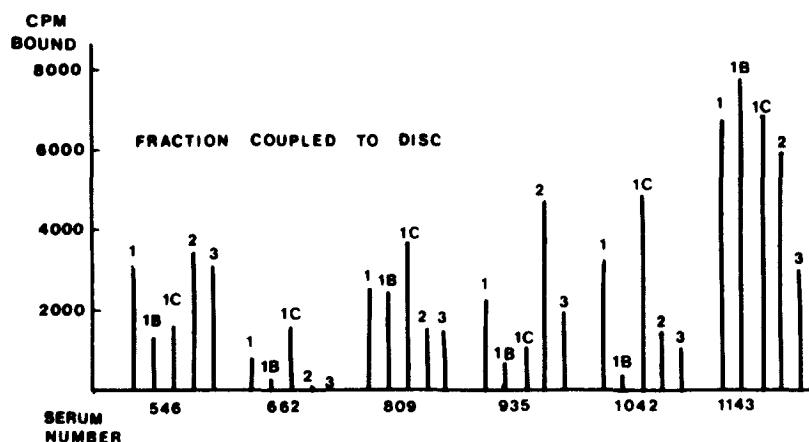


FIG. 6. Radioallergosorbent tests of sera from human subjects from California clinically sensitive to bee venom with G-75 fraction 1, fraction 2, hyaluronidase, fraction 3, phospholipase A, and G-200 fractions 1B, 1C, and 2A; 40,000 cpm of ^{125}I antibody were added. Nonallergic binding was 340 ± 60 cpm.

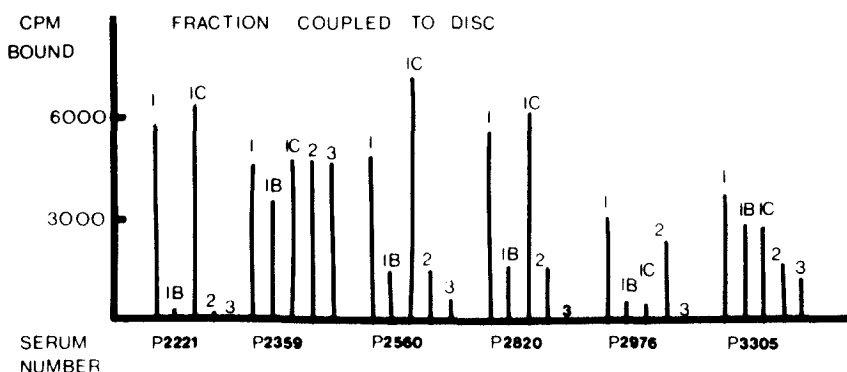


FIG. 7. Radioallergosorbent tests of sera from human subjects from the East and Midwest clinically sensitive to bee venom with G-75 fraction 1, fraction 2, hyaluronidase, fraction 3, phospholipase A, and G-200 fractions 1B, 1C, and 2A; 38,000 cpm of ^{125}I antibody were added. Nonallergic binding was 320 ± 60 cpm.

was purified, the IgE-binding activity followed the 105,000 d band in SDS-PAGE and also the corresponding immunodiffusion line. For clarity we will call this activity and associated substance allergen C. The allergenic and antigenic activity associated with fraction 1B and the 49,000 d SDS-PAGE band will be called allergen B. None of over 140 bee venom-allergic sera studied was monospecific for this specificity, allergen B. As has been shown previously,^{1, 2} fraction 1 and its subfractions are very potent allergens and virtually all (98%) bee allergic sera react with them.

The experiment shown in Fig. 6 was performed with sera from southern California and all tests shown were done at one time; Fig. 7 shows sera from the eastern United States tested with the same batches of discs in a second experiment. Negative control sera bound 340 ± 60 cpm out of 40,000 cpm added.

DISCUSSION

Bee venom is shown to contain four substances that elute from Sephadex in G-75 fraction 1 before

hyaluronidase by using immunodiffusion analysis and SDS-PAGE. In SDS-PAGE these substances gave molecular weights of 105,000, 87,000, 71,000, and 49,000 d. Fraction 1A and 1B were identical in immunodiffusion (not shown), giving a single precipitate line and in SDS-PAGE giving a single band at 49,000 d. This substance was also found in fraction 1C and in small amounts in fraction 2a. Because of its disperse molecular weight on Sephadex and elution position ranging from G-200 exclusion to about 50,000 d, it appears to exist in native form as a series of polymers up to at least tetramers. On storage of either fraction 1 or 1B insoluble aggregates form; these can be dissolved in 8 M urea but lose much of their antigenicity and allergenicity. Reduction with dithiothreitol in 8 M urea causes complete loss of the antigenic determinants of 1B. This aggregation appears to be primarily noncovalent but may involve some disulfide bonding. From the results of gel filtration using both Sephadex G-200 and G-150, it appears that this substance is found in native venom as at least tetramers, dimers, and a small amount of

TABLE I. Amino acid analyses of bee venom fractions

	Fraction 1	Fraction 1B	Hyaluron- idase	Phospholipase A		Melittin	
				Experi- mental	Literature (15)	Experi- mental	Literature (16)
Asp	129.5	129.0	70.3	15.2	16	tr	0
Thr	61.3	69.0	25.5	11.7	10	1.82	2
Ser	61.1	67.6	26.4	8.8	10	1.00	1
Glu	108.3	110.0	57.5	6.6	6	2.11	2
Pro	43.3	41.8	22.0	5.3	5	0.98	1
Gly	60.0	59.0	31.5	10.8	11	3.16	3
Ala	46.1	44.4	29.3	4.4	4	2.02	2
Cys	11.6	10.2	6.6	7.0	8	0	0
Val	54.6	55.4	30.7	5.0	5	2.06	2
Met	17.5	18.7	9.6	2.6	3	0	0
Ileu	61.8	61.1	24.6	3.9	4	3.02	3
Leu	94.1	88.1	42.1	9	9	4	4
Tyr	52.2	42.6	23.4	7.0	8	0	0
Phe	41.4	39.4	20.7	4.5	5	0	0
Lys	59.5	58.2	33.7	11.3	11	3.16	3
His	20.0	19.4	8.8	6.0	6	0	0
Arg	40.4	38.5	33.3	5.4	6	2.04	2
Trp	ND*	ND	ND	ND	2	0.6	1
Molecular weight (SDS-PAGE)		49,000 ± 5,000	53,000 ± 5,000	15,000 ± 2,000			
Residues		846	500			129	26

*Not determined.

monomer, but other polymers may be present. This is consistent with the diffuse precipitation line observed in immunodiffusion. This substance has substantial allergenic activity as measured by RAST, and this activity is distinct from hyaluronidase, phospholipase A, and melittin. It has been named allergen B.

An additional specificity defined by a monospecific human serum, P2221, has been identified in fraction 1C. This substance had been purified by immunoadsorption, and the allergenic activity was shown to be associated with a protein giving a molecular weight of 105,000 d on SDS-PAGE and with a sharp precipitation line in immunodiffusion. This substance is named allergen C. Allergen C is noncross-reactive with allergens B, hyaluronidase, phospholipase A, and melittin as well as with the other minor components in immunodiffusion. Allergens B and C are also shown to be unrelated to hyaluronidase, phospholipase A, and the basic peptides in venom by amino acid compositions, enzyme and biologic activity tests, and isoelectric focusing.

It has not yet been possible to determine if any allergenic activity is associated with the minor substances that give SDS-PAGE bands of 87,000 and 71,000 d. The 87,000 d band is found primarily in fraction 2a and is readily separable from hyaluronidase by carboxymethyl-Sephadex chromatography.

This report brings the number of allergens isolated from bee venom to five: phospholipase A,^{6, 7} hyaluronidase,¹⁻⁵ the weakly allergenic melittin,^{1, 2, 5} and allergens B and C. The variety of these allergens is surprising—they include both proteins and glycoproteins; some are basic and some neutral to slightly acidic, molecular weights range from 2,600 to over 200,000 d, and even chain size from 2,600 up to 105,000 d. Melittin has no disulfide bands and an isoelectric point above pH 10. These data indicate that theories of the chemical basis of allergenicity based on size, charge, and carbohydrate content are premature at the present time and that more data on the structure and properties of various allergenic substances are needed.

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