

Allergens in Hymenoptera venom XXVII: Bumblebee venom allergy and allergens

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Background: Allergic reactions to bumblebee stings are much less common than allergic reactions to honeybee stings. Preliminary studies suggest that there may be a high degree of cross-reactivity between honeybee and bumblebee venoms.

Objective: This study was done to determine the immunochemical and structural relationships between bumblebee and honeybee venom allergens.

Methods: Allergens were purified from bumblebee venom and compared immunochemically with sera from patients with allergy and hyperimmunized rabbits. The purified proteins were characterized, enzyme activities were measured, and the complete amino acid sequences of two proteins were determined.

Results: The venoms were highly cross-reactive, consistent with the degree of structural similarity in the phospholipases. Hyaluronidases and acid phosphatases were also similar. Bumblebee venom contained several proteins not found in honeybee venom, including an interesting tryptic amidase related to clotting enzymes and acrosin.

Conclusion: Skin testing with honeybee venom will detect almost all cases of bumblebee venom allergy, however; RAST with bumblebee venom can detect some additional cases. (*J ALLERGY CLIN IMMUNOL* 1996;97:812-21.)

Key words: Cross-reactivity, bee, phospholipase, venom, bumblebee, protease, Hymenoptera

Bumblebee venom allergy is uncommon relative to honeybee venom allergy. Our laboratory previously reported five cases of allergic reactions to bumblebee stings.¹ In addition, one case of a fatal reaction to a bumblebee sting has been reported,² a near fatal case was reported in New Zealand,³ and seven cases of occupational allergy to bumblebee venom have been reported.⁴ Bumblebees are members of the genus *Bombus* of the family Apidae. There are approximately 44 species in the United States and Canada and about 160 additional species in Europe, Asia, North Africa, and South and Central America. The classification into subgenera and species groups is controversial,⁵⁻⁷

Abbreviation used

SDS-PAGE: Sodium dodecylsulfate-polyacrylamide gel electrophoresis

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and identification of many species requires significant taxonomic expertise. In Europe some bumblebees, especially *B. terrestris* (L.), have been domesticated and used to pollinate plants grown in greenhouses,⁸⁻¹⁰ especially tomatoes. In general, bumblebees are more docile than either honeybees or vespid wasps, so stings are much less common.¹¹ Most species are ground-dwelling, live in relatively small, inconspicuous nests, and have little contact with people. Three North American and one European species present a greater threat, because their nests are commonly found in areas that conflict with human use. *Bombus pennsylvanicus* (Degeer) and *B. fervidus* (F.) commonly build nests in woodpiles and birdhouses; *B. impatiens* Cresson commonly nests in yards and is more irritable than other species; and the European species *B. terrestris* has been domesticated and introduced into greenhouses and fields.¹⁰ Bumblebees will generally only sting to protect their nests or if captured

and held. Bumblebees will also sting when they are trapped between skin and clothing.

Kochuyt et al.⁴ confirmed the observations of Hoffman¹ that bumblebee venom appears to be highly cross-reactive with honeybee venom and that honeybee venom can be used for testing both in vivo and in vitro. In this study we present additional cases of patients with allergic reactions to bumblebee stings, further investigate the immunologic cross-reactivity between honeybee and bumblebee venoms, and report on the isolation and characterization of bumblebee venom allergens.

METHODS

Venoms

B. fervidus venom sac extract was obtained from Vespa Laboratories, Inc. (Spring Mills, Pa.). *B. pennsylvanicus* and *B. impatiens* venoms were obtained by hand milking of live insects captured in Greenville, N.C.¹² Honeybee venom was purchased from Champlain Valley Apiaries (Middlebury Vt.).

Patient sera

Sera were obtained from patients with recent histories of systemic reactions to either honeybee or bumblebee stings. All sera for this study were screened by RAST, and only positive sera were used. Sera were stored at -20°C until use.

RAST

RAST was performed by the method previously described by our laboratory.¹³ Disks prepared from both commercial *B. fervidus* and hand-collected *B. pennsylvanicus* venoms were used for bumblebee testing. Purified allergens were coupled at 0.2 microgram per disk. RAST inhibition testing was performed as previously described.¹⁴

Separation and purification of proteins

Honeybee venom allergens were purified as previously described.^{15, 16} Bumblebee venom was first separated on a 1.6×160 cm column of Sephadex G75 superfine (Pharmacia, Piscataway, N.J.).¹⁷ Pooled fractions were further separated by cation exchange chromatography on a Mono S FPLC column from Pharmacia Fine Chemicals (Piscataway N.J.) with a gradient of 0 to 1 mmol/L NaCl in 0.05 mmol/L sodium acetate, pH 5.2.¹⁷ Final fractions were desalted on Sephadex G75. Fractionation was monitored by use of enzyme assays for acid phosphatase, hyaluronidase, and phospholipase and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver stain (Bio-Rad, Hercules, Calif.).¹⁵⁻¹⁸ Enzyme screening was carried out with Api-Zym test strips purchased from Analytab Products

(Chamblee, Ga.).¹⁸ Protease testing was also performed with the casein protease test kit from Bio-Rad.

Rabbit antisera and immunodiffusion testing

Laboratory animals were kept in compliance with the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 86-23). New Zealand white rabbits were immunized with purified allergens as described for the production of antisera to yellow jacket venom allergens.¹⁸ Immunodiffusion experiments were performed in 1% ME agarose, (FMC Bioproducts, Rockland, Maine) in Tris-barbital buffer.¹⁸ Gels were stained with Serva violet 49 (Serva Fine Biochemicals, Garden City, N.Y.).

Enzyme digestion, peptide isolation, and amino acid sequencing

Proteins were reduced with dithiothreitol and alkylated with iodoacetic acid as previously described.¹⁹ Digestions were performed with sequencing grade staphylococcal V8 protease, endoproteinase Asp-N, endoproteinase Lys-C, and cyanogen bromide according to previously described methods.¹⁹ Peptides were isolated on an 0.46×25 cm Vydac 218TP54 reverse-phase column (The Separations Group, Hesperia, Calif.) with a gradient of 0.07% trifluoroacetic acid in acetonitrile in 0.1% trifluoroacetic acid in water.¹⁹ Peptides were collected in siliconized tubes to prevent loss. Proteins were coupled to 1,4-phenylene diisothiocyanate functionalized disks and reduced and alkylated in situ,¹⁹ and peptides were coupled to arylamine functionalized disks with carbodiimide¹⁹ (Millipore Corp., Bedford, Mass.). Sequencing was performed on a Milligen Biosearch 6625 automated protein sequencer (Milligen Biosearch, Framingham, Mass.). Database comparisons were made by using the Prosis program (Hitachi Software Engineering America, Ltd., Brisbane, Calif.) with data downloaded from Entrez (National Center for Biotechnology Information, Bethesda, Md.).²⁰

RESULTS

Serologic studies of sera from patients allergic to bumblebee and honeybee

Sera from 94 patients allergic to honeybee venom and 13 patients allergic to bumblebee stings, as determined by history, were studied. The patients allergic to bumblebee included the five previously reported.¹ Only two sera from the patients allergic to bumblebee did not react with honeybee venom as determined by RAST, but both showed significantly positive responses to bumblebee venoms (*B. fervidus* and *B. pennsylvanicus*). The sera from patients allergic to honeybee were generally less reactive with bumblebee venom, with 73.4% demonstrating positive RAST results. Correlation studies were performed with 56 sera compared in a single pair of RAST experiments; the correlation coefficient was 0.59 with a

slope of 0.43, which was statistically significant but indicated that the allergens are not identical.

Because many of the honeybee-allergic sera had been previously characterized to determine profiles of allergen reactivity,^{15, 21} the RAST data were analyzed to determine whether there was any correlation with specific honeybee venom allergen reactivity. Two sera that were specific for honeybee venom allergen C, P2221 and P4962,^{15, 21} were not reactive with bumblebee venom. There was no correlation with other allergen reactivity, including reactivity to acid phosphatase, hyaluronidase, and phospholipase A. These findings were confirmed by RAST inhibition studies. None of the honeybee-allergic sera were positive for melittin alone, but there is no melittin-like peptide in bumblebee venoms.²²

RAST disks were prepared from high molecular weight protein, acid phosphatase, hyaluronidase, fraction 4 proteins, protease, and phospholipase purified from *B. pennsylvanicus* venom. RAST was performed with a panel of six sera from patients allergic to bumblebee and 12 sera from patients allergic to honeybee. IgE binding to each of the fractions with from 14 to 17 of the sera was observed. The sera from patients allergic to bumblebee were most strongly reactive with hyaluronidase in four cases, acid phosphatase in one case, and phospholipase in one case. Very high correlation was observed among reactivities to high molecular weight protein, fraction 4, and protease, suggesting that there may be cross-contamination by proteolytic fragments of the larger molecules. Correlations of reactivities among other allergen pairs were similar to those seen among other series of allergens,¹⁸ consistent with independent sensitization.

Preliminary characterization of bumblebee venoms

Pure hand-collected venom from *B. pennsylvanicus* was tested for enzyme activities. It contained large amounts of hyaluronidase, phospholipase A, acid phosphatase, and protease activities. The bumblebee venom did not hemolyze sheep red blood cells like honeybee venom did, a property of the amphiphilic peptide melittin. The proteolytic activity was further characterized with several substrates; casein and intact proteins were hydrolyzed in the presence of calcium, tosyl-L-arginine methyl ester was not hydrolyzed, but N-benzoyl-DL-2-arginine-2-naphthylamide was. A variety of chymotryptic substrates were not hydrolyzed. The protease has the specificity of a tryptic amidase. Because protease activity had not been previously

detected in either honeybee^{15, 21} or vespid^{17, 18} venoms, some additional species of bees were studied. Venom was hand collected from the bumblebees *B. impatiens*, *B. fraternus*, and *B. bimaculatus*, and the large carpenter bee *Xylocopa virginica*. Venoms from all four species contained significant amounts of tryptic activity, although the amounts were significantly lower in venoms from *B. impatiens* and *B. fraternus*. All of the bee venoms contained large amounts of acid phosphatase activity.

Separation and purification of *B. pennsylvanicus* allergens

Pure venom from *B. pennsylvanicus* was fractionated by gel filtration on a Sephadex G75 column as shown in Fig. 1. The separation was followed by absorbance at 280 nm and by monitoring enzyme activities, as well as SDS-PAGE with silver staining. SDS-PAGE of the partially purified fractions is shown in Fig. 2. A high molecular weight material of about 95,000 kd under reducing conditions was found in the earlier fractions. The acid phosphatase monomers had a molecular weight of 39,000 kd; hyaluronidase, 38,000 kd; protease, 28,000 kd; and phospholipase, about 14,200 kd. Additional proteins of molecular weights 36,000, 33,000, 29,000, and 22,000 kd were also seen, as well as some smaller peptides. Pooled fractions from the G75 column were further separated by high-performance cation exchange chromatography on a Mono S column. The high molecular weight material, acid phosphatase, hyaluronidase, protease, and phospholipase A were all prepared in highly purified form. The separation on a Mono S cation exchange column of a G75 fraction containing hyaluronidase, protease, and the uncharacterized proteins with molecular weight of 22 to 36,000 kd is shown in Fig. 3. Purities of final fractions were evaluated with SDS-PAGE and enzyme activity measurements to rule out cross-contamination. Both phospholipase and protease were greater than 97% pure as determined by N-terminal amino acid analysis. The hyaluronidase had a ragged N-terminal and could not be assayed by sequencing.

Immunodiffusion studies

Antisera were prepared by immunizing rabbits with purified bumblebee venom allergens. Strong cross-reactivity was observed between honeybee and bumblebee phospholipases and hyaluronidases, and moderate cross-reactivity was observed

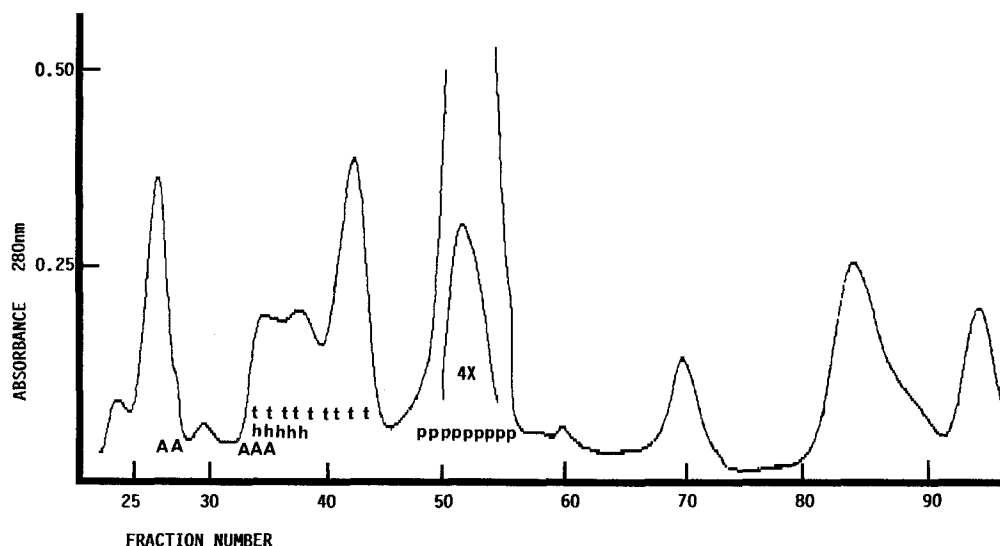


FIG. 1. Elution profile of *B. pennsylvanicus* venom on a 1.6×160 cm column of Sephadex G75 superfine. The letters under the tracing indicate the presence of enzyme activities A, Acid phosphatase; h, hyaluronidase; t, tryptic protease; p, phospholipase A.



FIG. 2. SDS-PAGE with silver stain of fractions from the Sephadex G75 separation shown in Fig. 1 and from Mono S ion-exchange chromatography. Fractions are: 1, exclusion peak; A, acid phosphatase-rich peak; H, hyaluronidase-rich fractions; T, tryptic protease; 4, other proteins (see Fig. 3); P, phospholipase. V indicates whole venom. Molecular weight standards shown are: B, bovine serum albumin at 66,000 kd; O, ovalbumin at 45,000 kd; CT, chymotrypsinogen at 25,000 kd; Cy, cytochrome c at 12,800 kd; Ph, phosphorylase at 97,400 kd; G, β -galactosidase at 116,000 kd.

between acid phosphatases. The antiserum against bumblebee venom protease was unreactive with honeybee venom and its fractions.

Amino acid sequences of *B. pennsylvanicus* venom phospholipase and protease

The complete amino acid sequence of *B. pennsylvanicus* venom phospholipase A was determined

from the sequencing of the N-terminus, and peptides isolated from staphylococcal V8 protease and endoproteinase-Asp-N digestions of the reduced and alkylated molecule, as shown in Fig. 4. The complete molecule contained 136 amino acids having a molecular weight of 15,488 kd. An asparagine with N-linked carbohydrate was found at position 16 by the direct observation of aspartic

COMPARISON OF BUMBLEBEE AND HONEYBEE PHOSPHOLIPASE A₂'s

53.7% identity in 136 aa overlap

	10	20	30	40	50	60
BOMPPLA	IIYPGTLWCGNGNIA	XGTNELGLWKETD	ACCRTTHDMCPDIE	AHGSKHGLTNPADY	TRLN	
						
BPLADNA	IIYPGTLWCGHGNKSS	GPNELGRFKHTD	ACCRTTHDMCPD	VMSAGESKHGLTNT	ASHTRLS	
	30	40	50	60	70	80
	70	80	90	100	110	120
BOMPPLA	CECDEEFRHCLHNSG	DAVSAAFVGR	TYFTILGTQCFRLD	YPIVKCKVKSTILRE	CKEYEF	
						
BPLADNA	CDCDDKFYDCLKNSA	DTISSYFVGKMYFN	LIDTKCYKLEHPVT	GCGERTEG--RCLHY	TV	
	90	100	110	120	130	140
	130					
BOMPPLA	DTNAPQKYQWFDVLSY					
						
BPLADNA	DKSKPKVYQWFDLRKY					
	150	160				

FIG. 5. Comparison of the amino acid sequence of *B. pennsylvanicus* venom phospholipase with the cDNA-derived sequence²³ of phospholipase from honeybee venom. Double dots indicate identical residues in each sequence, and single dots indicate residues differing by a single base substitution.

has two more residues than the honeybee enzyme. The cysteine residues and the functional residues are all conserved.

The complete amino acid sequence of the venom protease was determined from N-terminal sequencing and sequencing of peptides isolated from staphylococcal V8 protease, endoproteinase-Lys-C, tryptic, and cyanogen bromide digests as shown in Fig. 6. There were 243 amino acid residues in the complete molecule, having a molecular weight of 27,249 kd. There were two positions at which heterogeneity was observed: glutamic acid and an atypical residue at position 202 and glutamic acid and glutamine at position 31. No carbohydrate was detected. All eight of the required catalytic and accessory residues for a tryptic protease were present and are shown in Fig. 6.²⁴ The sequence of the protease was related to the sequences of a large series of serine proteases found in the Protein Identification Resource database. Among the most notable was the 41.1% identity with a large region of a horseshoe crab proclotting factor,²⁵ shown in Fig. 7, and a 37% identity with a large area of mouse acrosin,²⁶ the sperm head protease, also shown in Fig. 7. The venom protease was also closely related to kallikreins²⁷ with 36.9% identity over 241 residues and clotting factors, such as factor XIa,²⁸ with 33.6% identity over 241 amino acid residues.

DISCUSSION

Bumblebee venom appears to be chemically and antigenically related to honeybee venom. About 75% of sera from patients allergic to honeybee react in RAST with bumblebee venom, and 85% of sera from patients with histories of allergy to bumblebee stings demonstrate positive RAST responses to honeybee venom. The hyaluronidases and acid phosphatases appear to be closely related as determined by both RAST and immunodiffusion with rabbit antisera. The phospholipases appear to exhibit cross-reactivity with many patient sera in RAST and rabbit antisera in immunodiffusion. The primary amino acid sequence of bumblebee venom phospholipase shows 53.7% identity with that from honeybee venom with 33% additional conservative changes, a level of structural similarity consistent with immunologic cross-reactivity.^{19, 20} Honeybee venom contains tetramers of the peptide melittin, which is allergenic in some persons. No similar peptide is found in bumblebee venom. Two sera from patients allergic only to honeybee venom allergen C, probably α -D-glucosidase, were unreactive with bumblebee venom.

The sera from 13 patients known by history to be allergic to bumblebee stings were studied, and 11 demonstrated positive RAST responses to honeybee venom also. Kochuyt et al.⁴ reported on seven cases of occupational allergy to bumblebee venom and

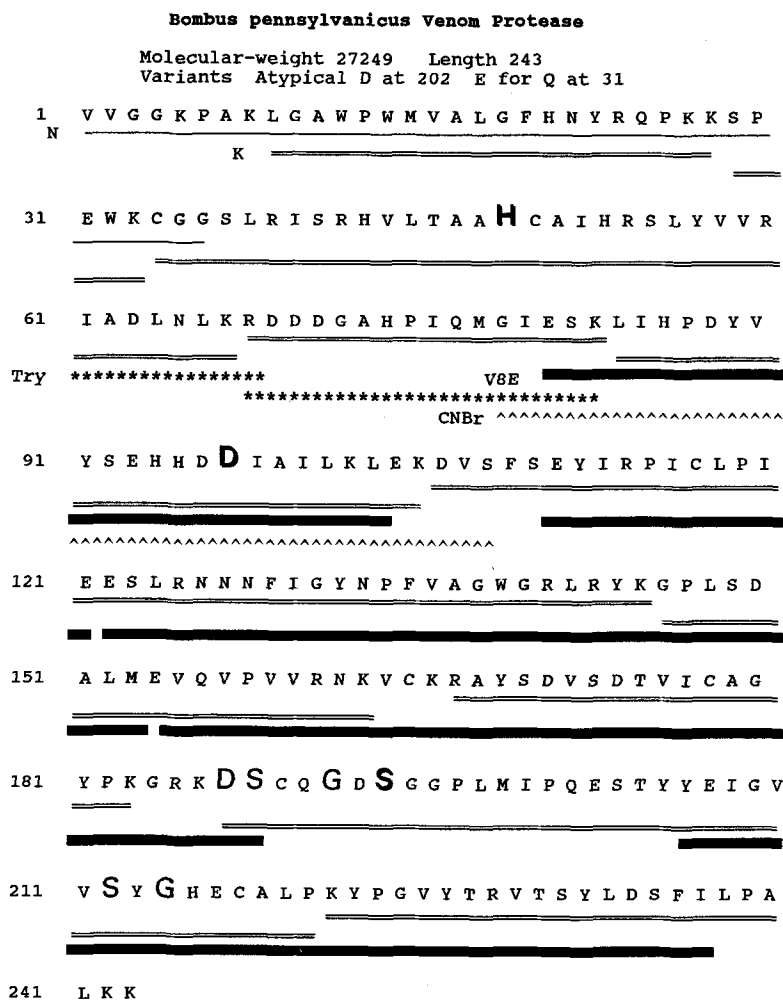


FIG. 6. The complete amino acid sequence of *B. pennsylvanicus* venom protease. The N-terminal sequence is indicated by N and a single thin line; K and double thin lines indicate fragments generated by endoproteinase-Lys-C; V8E and a heavy line indicate fragments generated by staphylococcal V8 protease; Try and asterisks indicate tryptic fragments; and CNBr and carets (^) indicate a cyanogen bromide-derived fragment. Heterogeneity was detected at positions 31 and 202. The catalytic residues are shown in large boldface type (H-49, D-97, and S-193); and the specificity-determining residues are shown in large type.^{24, 34}

found that six of the seven patients had positive RAST responses to honeybee venom. They performed intradermal skin tests on five patients, and all results were positive, although two were only positive at the 0.05 μ g level. In addition to the significant differences in the C-terminal part of the venom phospholipases, there are a number of other differences between the venoms of honeybees and bumblebees. Bumblebee venom contains a protease of tryptic amidase specificity, whereas honeybee venom does not contain any detectable protease. Allergen C from honeybee venom has a molecular weight of 105,000 kd,²¹ whereas the largest protein in bumblebee venom appears to be

95,000 kd and is not reactive with human IgE against allergen C. Bumblebee venom contains a number of uncharacterized components, called fraction 4, with molecular weights of 36,000, 33,000, 29,000, and 22,000 kd, which could be proteolytic fragments of other venom proteins or distinct components. The presence of the venom protease has been verified in three additional species of bumblebees and large carpenter bees.

Because commercial bumblebee venom preparations were not available for immunotherapy, Kochuyt et al.⁴ treated all seven of the occupationally exposed patients with honeybee venom immunotherapy. This therapy appeared to be highly

		10	20	30	40	50	
BPENPROTEASE	1	VVGKPAKLG	AWPVMVALGF	HNYRQPKKSP	EWKCGGSLRI	SRHVLTAABC	50
A23689	128	II**RE*PI*	*****T*V--	--*IKQGGIR	SVQ***A*VT	N***I**S**	177
JX0172	43	I*S*QS*Q**	*****S*--	QIFTSHNSRR	YHA*****LN	*HW*****	92
		60	70	80	90	100	
BPENPROTEASE	51	-----AI	HRSLYVVRIA	DLNLKRDDDG	AHPIQMGIES	KLIHPDYVYS	100
A23689	178	VVNSAGTDVM	PADVFS**LG	EH**YST**D	SN**DFAVT*	VKH*EHF*LA	227
JX0172	93	--FDNKKKVY	DWR*VFQAQE	IEYGRNKPVK	EPQQERYVQK	IV**EK*NVV	142
		110	120	130	140	150	
BPENPROTEASE	101	EHDDIAILK	LEKDVFSFSEY	IRPICLPIEE	SLRNNNFIGY	NPFVAGWGRL	150
A23689	228	TYLN*****T	*NDT*T*TD*	*****-YR	K**YDDLAMR	K**IT***TT	277
JX0172	143	TEGN***L**	ITPP*TCGNF	*G*C***--H	FKAGPPQ*PH	TCY*T***YI	192
		160	170	180	190	200	
BPENPROTEASE	151	RYKGPL-SDA	LMEVQVPVVR	NKVCKRAY--	-SDVSDTVIC	AGYPKGRKDS	200
A23689	278	AFN**S-*AV	*R***L*IWE	HEA*RQ**EK	DLNITNVYM*	**FAD*G**A	327
JX0172	193	KE*A*RP*PV	***AR*DLID	LDL*NSTQWY	NGR*TS*NV*	*****E*KI*T	242
		210	220	230	240	250	
BPENPROTEASE	201	CQGDGGGGLM	IP--QESTYY	EIGVVSYGHE	CALPKYPGVY	TRVTSYLDSE	250
A23689	328	*****M*	L*VKTGEF*L	--*I**F*KK	****GF****	*K**EF**WI	377
JX0172	243	*****M*	CRDNVD*PFV	VV*IT*W*VG	**RA*R****	*ATWD***WI	292
		251	260	270	280	290	
BPENPROTEASE	251	ILPALKK					
A23689	378	AEHNV					
JX0172	293	ASKIGPN					

FIG. 7. Comparison of amino acid sequences of bumblebee venom protease and horseshoe crab proclotting factor, A23689,²⁵ and mouse sperm acrosin, JX0172.²⁶ Asterisks indicate residues identical to those in the bumblebee venom protease; dashes are gaps inserted to maximize the alignment.

efficacious, with no systemic and only two large local reactions occurring after over 36 natural stings in six patients. Of particular significance is the patient who had a negative RAST response to honeybee venom; she had more than 10 stings while receiving immunotherapy and had no reactions. This study suggests that enough cross-reactivity exists between honeybee and bumblebee venoms to provide protection, even when cross-reactive antibody cannot be demonstrated before immunotherapy.

The reported sequence of *B. pennsylvanicus* venom phospholipase A₂ is the second complete sequence of phospholipases in this group. Bee venom phospholipase has only a limited amount of sequence similarity with vertebrate phospholipases²⁹ but is functionally and three-dimensionally similar,³⁰⁻³² and it has nearly identical catalytic mechanism with segmental similarity of sequence and conservation of substructures. The bumblebee venom phospholipase is also a class II family member of the same subfamily as honeybee venom phospholipase.

The bumblebee venom serine protease is a particularly interesting molecule in several re-

spects. No similar molecule has been found in either honeybee venom, vespid venoms, or fire ant venom.^{15, 18, 33} The bumblebee tryptic amidase contains all of the active site-associated residues of serine proteases^{34, 35} but does not have the histidines or tryptophan residues characteristic of tryptases.³⁵ Comparisons of the sequence of the bumblebee enzyme with the Protein Identification Resource database show the most similarity to clotting factors of the kallikrein family^{25, 27, 28} and to acrosins, the mammalian sperm head proteases.²⁶ The relationship to sperm proteases is particularly intriguing, because other Hymenoptera venom proteins have been shown to be related to proteins of the male reproductive tract. Antigen 5s from vespid venoms have been shown to be related to an epididymal protein,³⁶ hyaluronidases from both bee and vespid venoms are closely related to sperm hyaluronidase,³⁷⁻³⁹ and bee venom acid phosphatase has recently been shown to be a prostatic type enzyme, not a lysosomal type enzyme.⁴⁰ Type II phospholipase A₂ molecules are important constituents of seminal plasma, sperm, and male reproductive organs.^{41, 42} Taken

together, these observations suggest that the venom proteins may be derived from male proteins, not from female proteins, as entomologists had assumed from morphologic studies. Because most hymenopterans are arrhenotokous, where male insects are haploid and female insects diploid, the females carry the complete genetic information for the male reproductive system.⁴³ In honeybees, sex determination is based on a series of alleles at a single locus, where the males are hemizygous and the females heterozygous.⁴⁴ Both workers and queens have fully developed ovipositors in addition to sting apparatuses, so it is improbable that the sting apparatus has developed from the ovipositor. The observation that many venom proteins are derived from male antigens suggests that the venom apparatus is derived from the male genes, rather than the female genes.

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