

Occupational allergy to bumblebees: Allergens of *Bombus terrestris*

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Background: With the increase in commercial vegetable production in greenhouses, occupational sensitization to bumblebee venom is becoming more common. Studies using sera from subjects thus sensitized allow evaluation of the allergenic specificity of bumblebee sensitization.

Objective: The purposes of this study were to determine the degree of species group specificity of bumblebee venom allergens in sera of allergic patients and to investigate the structural basis of this specificity.

Methods: Allergens were purified from bumblebee venom, studied serologically by direct binding and inhibition techniques, and characterized by enzyme analysis and amino acid sequencing. Three-dimensional models of the phospholipases were constructed and analyzed.

Results: *Bombus terrestris* venom contains phospholipase A₂, venom protease, hyaluronidase, and acid phosphatase allergens. The protease and phospholipase A₂ allergens contain IgE-reactive epitopes that are different from those seen in *Bombus pennsylvanicus*, a North American species. Bumblebee phospholipase A₂ is only 53% identical to honeybee phospholipase A₂. The results of 3-dimensional modeling are consistent with the immunologic observations.

Conclusions: Patients with primary bumblebee sensitization should be diagnosed and treated with venom from the appropriate species group of bumblebees. Bumblebee venom phospholipase A₂ and protease are antigenically distinct from honeybee venom proteins. There are significant species group-specific epitopes on bumblebee venom proteins. (J Allergy Clin Immunol 2001;108:855-60.)

Key words: Bumblebee, venom, phospholipase, protease, occupational allergy, epitope, honeybee

Bumblebees are commonly used in Europe and increasingly in North America to pollinate vegetable crops grown in greenhouses. This has led to an increased

Abbreviation used
PLA: Phospholipase A₂

incidence of bumblebee stings and occupationally related anaphylactic reactions to bumblebee venom.¹⁻³ Previous studies from our laboratory⁴ were carried out with sera from 13 patients sensitized by field stings. Eleven of these patients demonstrated significant cross-reactivity between honeybee and bumblebee venoms. Studies of patients with occupational sensitization from *Bombus terrestris* demonstrated a high degree of bumblebee venom specificity in most subjects^{3,5,6}—both nonreactivity with honeybee venom allergens and bumblebee species specificity. In this report we describe the purification and properties of *B terrestris* phospholipase (Bom t 1) and venom protease (Bom t 4). Sera from bumblebee venom-allergic patients are used to compare *B terrestris* and *Bombus pennsylvanicus* venom allergens.

METHODS

Patients and sera

Studies were approved by the Institutional Review Boards at East Carolina University and the University Hospital Rotterdam. Sera were collected from 6 Europeans with histories of occupational allergy to *B terrestris* venom reported previously,^{5,6} 1 American with occupational sensitization to *B terrestris* venom, and 1 American sensitized to a field sting by *B pennsylvanicus* or *Bombus fervidus*.⁴ Sera were collected before any allergen vaccine therapy and stored at -20°C until use.

Venoms

Venoms from workers of *B terrestris* and *B pennsylvanicus* were supplied by Vespa Laboratories, Inc (Spring Mills, Pa).

RAST and RAST inhibition

Paper-disk RAST with whole venoms and purified fractions was performed as described previously.⁷ Purified materials were coupled to CNBr-activated disks at 0.2 µg per disk. RAST inhibition followed the method that we have reported previously.⁸

Purification of venom components

Venom protease was separated from the commercial extracts by affinity chromatography with benzamidine agarose⁹ (Sigma Chemical, St Louis, Mo). Venom protease and phospholipase fractions were separated and purified by cation exchange chromatography on

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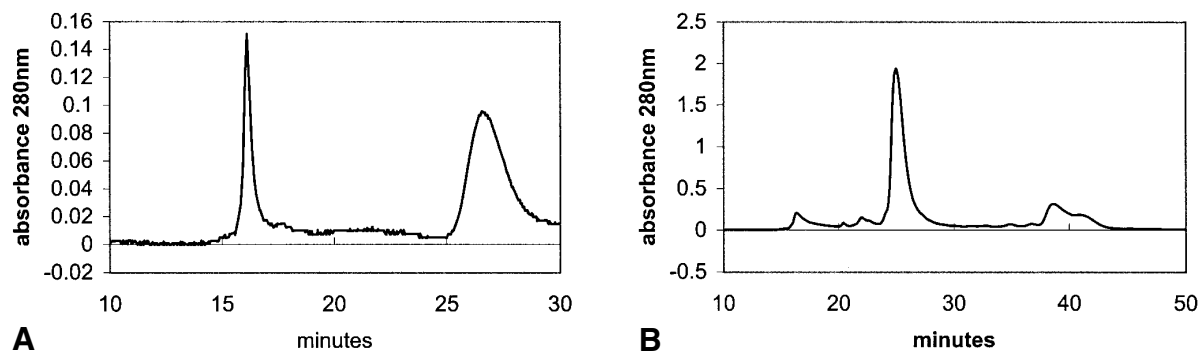


FIG 1. Cation exchange separations of *B terrestris* venom benzamidase eluate (A) and flow-through (B). The protease was found in the peak at 16.1 minutes in the eluate fraction. Phospholipase was found in the large peak centered at 25 minutes in the flow-through fraction, hyaluronidase activity eluted at 21-22 minutes, and acid phosphatase activity at 16-21 minutes in the flow-through fraction.

		10	20	30	40	50
Bom t PLA	1	IIFPGTLWCG	NGNLANGTNQ	LGSKWETDSC	CRTHDMCPDL	TEAHGSKHGL
Bom p PLA	1	IIFPGTLWCG	NGNIANGTNE	LGLWKETDAC	CRTHDMCPDI	TEAHGSKHGL
Api m PLA	1	IIFPGTLWCG	HGNKSSGPNE	LGRFKHTDAC	CRTHDMCPDV	MSAGESKHGL
		60	70	80	90	100
Bom t PLA	51	TNAADYTRLN	CECDEEFRC	LHNSGDTVSA	GFVGRITYFTV	LHTQCFRLDY
Bom p PLA	51	TNPADYTRLN	CECDEEFRC	LHNSGDAVSA	AFVGRITYFTI	LGTQCFRLDY
Api m PLA	51	TNTASHTRLN	CDCDDKFYDC	LKNSADTISS	YFVGKMYFNL	IDTKCYKLEH
		110	120	130		
Bom t PLA	101	PIVKCKVKST	ILHRSKCYDF	ETFAPKKYQW	FVDLQY	
Bom p PLA	101	PIVKCKVKST	ILRECKEYEF	DTNAPQKYQW	FVDLSY	
Api m PLA	101	PVTGCGERTE	--GRCLHYTV	DKSKPKYQW	PDLRKY	

FIG 2. Comparison of the amino acid sequences of phospholipases from *B terrestris*, *B pennsylvanicus*, and *Apis mellifera* venoms.

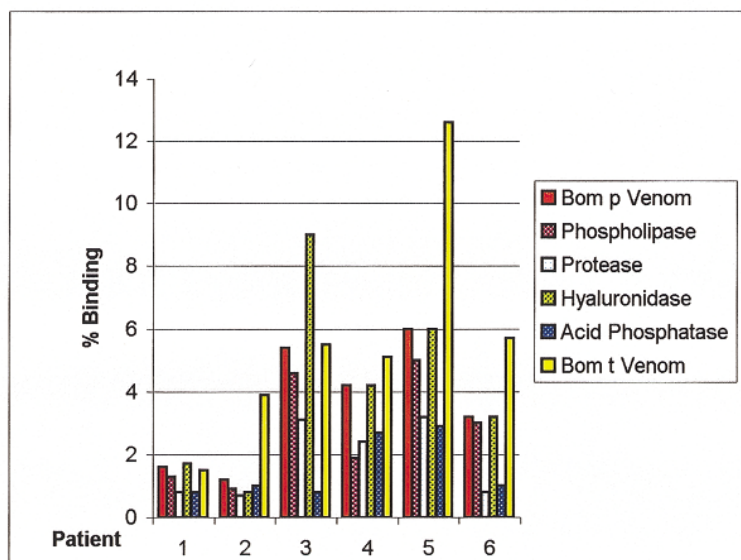


FIG 3. IgE antibody binding activity of sera from European *B terrestris* venom-sensitized patients to phospholipase, protease, hyaluronidase, and acid phosphatase from *B pennsylvanicus* venom and whole venoms from both species. Results are reported after subtraction of nonspecific binding.

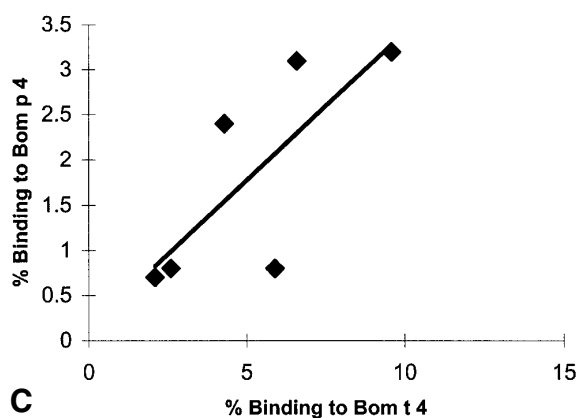
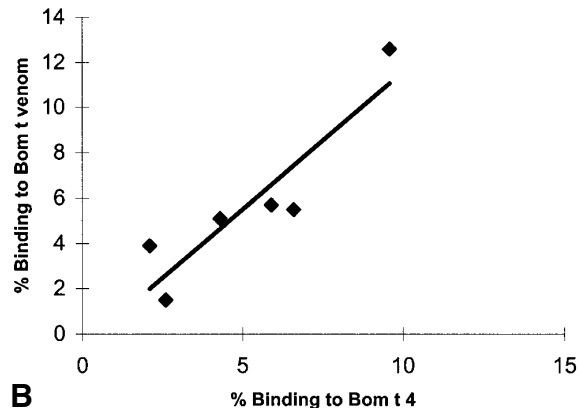
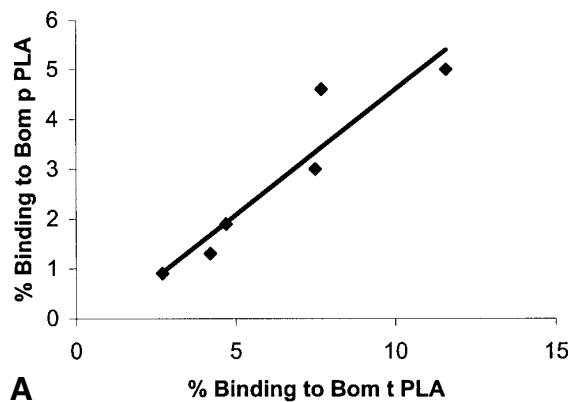


FIG 4. A, Comparison of IgE binding to *B terrestris* and *B pennsylvanicus* phospholipases by sera from *B terrestris* venom-sensitized subjects. B, Comparison of IgE antibody binding to whole *B terrestris* venom and Bom t 4, the venom protease. C, Comparison of IgE binding to *B terrestris* and *B pennsylvanicus* venom proteases.

a Pharmacia (Piscataway, NJ) Mono S column in a gradient of 0 to 1 mol/L NaCl in 0.05 mol/L sodium acetate, pH 5.2.⁴ *B pennsylvanicus* acid phosphatase and hyaluronidase were from our previous study.⁴ Proteins for sequencing were further purified by reversed-phase chromatography.¹⁰ Purities of native proteins were assessed by SDS-PAGE and analysis for phospholipase, protease, hyaluronidase, and acid phosphatase activities.⁴ Protein concentrations were measured by means of the Coomassie Plus Protein Assay (Pierce Chemical Co, Rockford, Ill).

Peptide mapping and amino acid sequencing

The methods for reduction and alkylation and cleavage with modified trypsin, endoproteinase Asp-N, endoproteinase Lys-C, and endoproteinase Arg-C have been described previously.¹⁰ Peptides were separated on a 2.1 × 250-mm HAIL C18 column (Higgins Analytical, Inc, Mountain View, Calif) through use of a gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid at a flow rate of 0.2 mL/min. Peptides were coupled to arylamine disks (Millipore Corp, Bedford, Mass) and sequenced on a Milligen/Biosearch Prosequencer (Millipore Corp). Intact proteins were coupled to 1,4-phenylene diisothiocyanate functionalized disks for sequencing. Protein sequence comparisons were made through use of the BLAST and FASTA programs at the National Center for Biotechnology Information and the Hitachi DNASIS program (Hitachi Software Engineering Co, South San Francisco, Calif).

Models of 3-dimensional structures

Three-dimensional structures were modeled through use of the Swiss-Model system.¹¹ The crystal structure of honeybee venom phospholipase¹² (Protein Data Bank IPOC) was used as a template. Models were visualized and compared through use of Swiss-PdbViewer.¹³

RESULTS

B terrestris venom was first partially separated through use of a benzamidine agarose column to remove the venom protease.⁹ Both the eluate and flow-through pools were then separated through use of a Mono S cation exchange column, as shown in Fig 1. Enzyme activity tests demonstrated the presence of phospholipase A₂ (PLA) and casein hydrolyzing protease as the major components, representing more than 90% of the venom protein; there were also small amounts of hyaluronidase and acid phosphatase. The phospholipase and protease were found to be free of each other and the minor protein components by

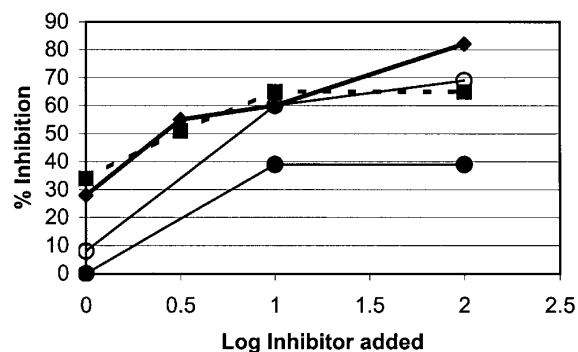


FIG 5. RAST inhibition of IgE binding to *B terrestris* PLA by *B terrestris* PLA (diamonds) and by *B pennsylvanicus* PLA (squares) for an American patient with occupational allergy to *B terrestris* venom and by *B terrestris* PLA (solid circles) and by *B pennsylvanicus* PLA (open circles) for an American patient specifically sensitized to *B pennsylvanicus* or *B fervidus* by a field sting.

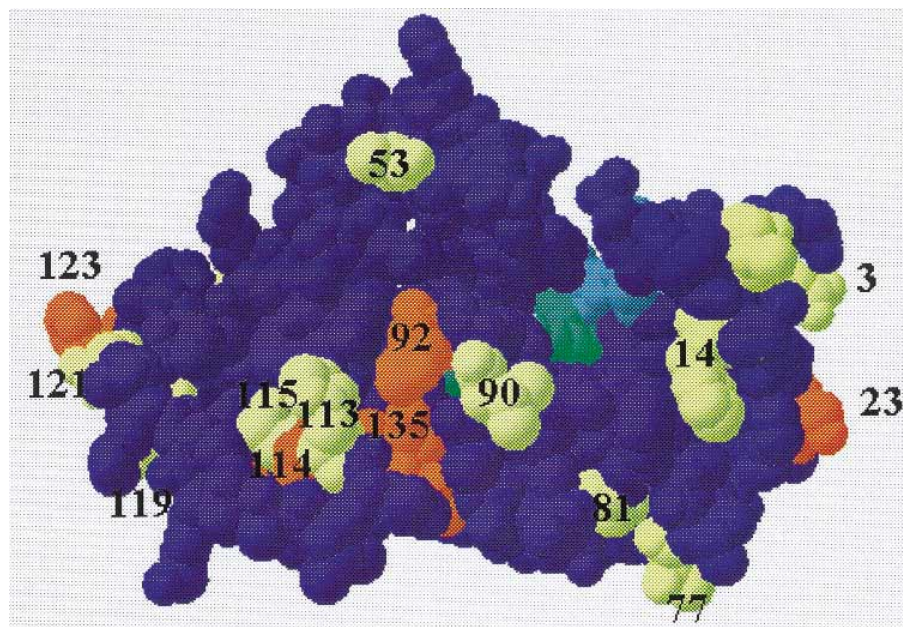


FIG 6. Comparison of the 3-dimensional structures of *B terrestris* and *B pennsylvanicus* venom phospholipases. Dark blue indicates residues identical in both sequences; yellow indicates conservative substitutions; orange indicates nonconservative substitutions. The active site residues in the substrate binding pocket are indicated by green and the calcium ion binding site by light blue.

SDS-PAGE and enzyme activity measurements. The venom protease—Bom t 4, in the international allergen nomenclature—was further characterized by N-terminal amino acid sequencing. The sequence of the first 20 residues, VVGKPAKLGAWPWMVALGF, was identical to that of Bom p 4⁴; however, Bom t 4 was significantly less basic than Bom p 4. The complete amino acid sequence of the phospholipase, Bom t 1, was determined from overlapping peptides from the N-terminal sequence and peptides chromatographically isolated from 4 different enzyme digests. This sequence has been submitted to the Swiss-Prot database (acquisition number P82971) and is compared to the sequences of the American bumblebee phospholipase, Bom p 1,⁴ and honeybee PLA, Api m 1,¹⁴ in Fig 2. The 2 bumblebee phospholipases are 83.8% identical and the *B terrestris* PLA is 52.9% identical to honeybee PLA, with a single insertion of 2 residues after residue 110. N-linked carbohydrate is attached at asparagine 16 in both bumblebee venom phospholipases.

The 6 sera from Europeans occupationally sensitized to *B terrestris* venom were tested for IgE antibodies to *B terrestris* and *B pennsylvanicus* venoms and the 4 purified enzymes from *B pennsylvanicus* venom. For each patient serum, the binding to *B terrestris* venom was equal to or greater than the binding to *B pennsylvanicus* venom. Strong binding was observed to hyaluronidase, phospholipase, and protease, and there was less binding to acid phosphatase, as shown in Fig 3. Higher IgE binding to *B terrestris* PLA than to *B pennsylvanicus* PLA was observed for each of the 6 sera, as shown in Fig 4, A, with a regression line slope of 2.2. IgE binding to purified Bom t 4 was similar to the IgE binding to whole

venom, as shown in Fig 4, B, indicating that the venom protease is a major allergen. Each of the 6 sera bound more strongly to Bom t 4 than to Bom p 4, as shown in Fig 4, C, with a regression line slope of 2.8.

Because these results confirmed the observation of Stapel et al⁵ that there were significant species-specific differences between *B terrestris* and *B pennsylvanicus* venom allergens, RAST inhibition experiments were performed with the purified phospholipases. The curves for inhibition of binding to *B terrestris* PLA by *B terrestris* and *B pennsylvanicus* PLAs for an American subject occupationally sensitized by *B terrestris* venom are shown in Fig 5. Inhibition by *B pennsylvanicus* PLA plateaus at 65%, though the 50% inhibition concentrations are similar. This suggests that *B terrestris* PLA has epitopes not found on *B pennsylvanicus* PLA. Further RAST inhibition of *B terrestris* PLA binding was carried out with 2 sera from European patients and 1 serum from an American patient sensitized with either *B pennsylvanicus* or *B fervidus* who was unreactive to honeybee venom. *B pennsylvanicus* PLA was not able to inhibit binding to *B terrestris* PLA at 50 µg/mL for the first European serum and was significantly less potent with the second serum. Conversely, as is shown in Fig 5, *B pennsylvanicus* PLA was a much more effective inhibitor of binding to *B terrestris* PLA for the American serum, with 69% inhibition at 50 µg/mL and only 39% inhibition by *B terrestris* PLA.

Three-dimensional models of the 2 bumblebee venom phospholipases were constructed by means of the Swiss-Model program,¹¹ the crystallographic structure of honeybee venom phospholipase being used as a template.¹² Because the bumblebee PLA sequences are 47% differ-

ent from those of honeybee PLA and the backbone and functional residues are conserved, very little of the surface is conserved in the model. The 3-dimensional model of *B terrestris* PLA is shown in Fig 6. The residues that differ from *B pennsylvanicus* PLA are colored yellow and orange; the active site of the enzyme is colored green and the calcium ion binding residues are light blue. There are 4 areas of clustered surface differences: 90, 92, 113-114-115, and 135; 119, 121, and 123; 77 and 81; and 3, 14, and 23. The differences at 90, 92, 113-114-115, and 135 include an increase of 2 in the disulfide loop length, an increased positive charge, and an increase of 2 in the aromatic residues. These findings are consistent with the presence of epitopes on *B terrestris* PLA that are different from those on *B pennsylvanicus* PLA.

DISCUSSION

B terrestris venom contains at least 4 allergens, 3 of which—PLA, protease, and hyaluronidase—are major allergens. The hyaluronidase and acid phosphatase were not purified and further studied; this was because of the relatively small quantities from the limited amount of venom available and the difficulty of completely separating the 2 glycoproteins. Insect venom hyaluronidases¹⁵ are relatively conserved and demonstrate cross-reactivity¹⁶ between bees and vespids, so significant differences in IgE binding among bee hyaluronidases would not be expected.

The first studies of bumblebee venom allergy reported a high degree of cross-reactivity with honeybee venom.^{1,4} The initial study from our laboratory used subjects sensitized by field stings,⁴ who are commonly multiply sensitized and reactive to multiple insect venoms.^{7,8} It was noted that 2 bumblebee-reactive individuals reported in that study, 1 of whom has also been included in this study, were unreactive to honeybee venom.

In a study by Kochuyt et al,¹ 6 of 7 patients were CAP-RAST–positive to honeybee venom, and all 5 of the patients who were skin-tested with honeybee venom were positive. Two large local reactions from more than 36 bumblebee stings were reported after honeybee venom immunotherapy.

Stapel et al⁵ demonstrated highly significant differences in IgE reactivity to *B terrestris* and honeybee venoms by a variety of techniques. Their experiments also showed significant differences in immune reactivity to *B terrestris* and *B pennsylvanicus* venoms. The patients in that study and additional patients reported later had weaker or no skin test and RAST reactivity with honeybee venom.⁶

Stern et al³ reported 2 cases of clinical failure of allergen vaccine therapy with honeybee venom in bumblebee-sensitized patients; the results reported in their study support the observations that there is limited cross-reactivity between honeybee and bumblebee venoms in many patients.

Two major allergens, phospholipase and venom protease, are significantly different antigenically. Although a venom protease is found in honeybee venom, its allergenic significance is unknown.¹⁷

There appear to be 2 distinct categories of bumblebee-sensitive patients. The first group has IgE cross-reactive

with honeybee venom allergens. This pattern appears to be more common in patients sensitized by field stings,⁴ though it has been reported in occupationally sensitized subjects.^{1,5} Many subjects with occupational sensitization appear to have bumblebee-specific responses,^{3,5,6} which are less common in subjects sensitized by field stings.⁴ This is somewhat similar to the situation seen in patients sensitized to vespid venoms, many of whom produce antibodies cross-reactive with both yellowjacket and hornet venoms and some of whom produce antibodies reactive with only yellowjacket venom.^{7,8}

These studies also confirm the presence of significant differences between venoms of bumblebees from different groups, represented by the European species *B terrestris* of the subgenus *Bombus* (Latreille) and the North American species *B pennsylvanicus* of the subgenus *Megabombus* (Milliron) or *Fervidobombus* (Skorikov). Both the phospholipases and proteases show significant differences in IgE reactivity. This is supported by the structural studies and 3-dimensional modeling of the phospholipases.

These studies support the recommendations by European workers^{3,5,6} that bumblebee venom from the appropriate species group be used for both the diagnosis and allergen vaccine therapy of individuals with primary bumblebee venom allergy. Unfortunately, these materials are not commercially available in the United States and are only available as an unstandardized individual patient product in some countries of the European Union.

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