

Hymenoptera venom protease allergens

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Background: Recent studies have shown the presence of additional allergenic proteins in honeybee and paper wasp venoms. Both venoms contain serine protease enzymes. **Objective:** We isolated and obtained complete sequences of honeybee and Mediterranean paper wasp venom proteases, both of which have significant IgE binding activity. The structures are compared with bumblebee venom protease. **Methods:** Venom proteases were chromatographically isolated from venoms and partial amino acid sequences determined. RT-PCR and rapid amplification of cDNA ends methods were used to clone cDNA, and complete sequences were determined for honeybee and a paper wasp venom protease. **Results:** The venom proteases are all serine proteases of the trypsin type. The honeybee protease contains a complement, embryonic sea urchin protein, bone morphogenetic protein interaction domain as well as a linker and propeptide sequence, and a unique methionine residue near the active site. It has IgE binding activity. The paper wasp protease is a single trypsin domain and is an important allergen. The framework residues are poorly conserved among honeybee, bumblebee, and paper wasp enzymes. **Conclusions:** The 3 venom serine proteases have significant IgE binding activities. The structures are poorly conserved even among the *Apidae*, suggesting little cross-reactivity among the protein portions. The paper wasp venom proteases are important allergens. (J Allergy Clin Immunol 2004;114:928-33.)

Key words: Honeybee, venom, protease, allergen, CUB domain, paper wasp, bumblebee

Abbreviations used

CUB: Complement, embryonic sea urchin protein,
bone morphogenetic protein
RACE: Rapid amplification of cDNA ends

wasps contain additional proteins.^{7,8} Studies of IgE binding activities of venoms from European and North American species of *Polistes* have shown significant differences in specificities.⁹⁻¹¹ Further studies of paper wasp venoms have led to the isolation of a venom protease from both North American and European species.^{10,11}

In this article, we report on the isolation, purification, cloning, sequencing, and characterization of venom serine protease allergens from the honeybee, *Apis mellifera*, and paper wasp, *Polistes dominulus*. The proteins are shown to have IgE binding activity. The amino acid sequences and computer models of the three dimensional structures of honeybee, bumblebee and paper wasp venom serine proteases are compared.

METHODS

Materials

Honeybee venom was purchased from Champlain Valley Apiaries (Middlebury, Vt). *B pennsylvanicus* and *P dominulus* venoms were donated by Vespa Laboratories (Spring Mills, Pa). Honeybees and *Polistes exclamans* wasps were collected locally and flash-frozen at -70°C . *P exclamans* venom was collected from dissected venom sacs. *P dominulus* insects were collected in the northeastern United States and donated by Vespa Laboratories. *Polistes* antigen 5's and *B pennsylvanicus* protease were prepared as previously described.^{1,8}

Human sera were used with the approval of the East Carolina University Institutional Review Board. Sera from patients allergic to European *Polistes* were provided by Dr P. Campi of Florence, Italy, and Dr M. Blanca of Malaga, Spain. Sera were stored at -20°C . until used.

Isolation of the protease from bee venom

The high-molecular-weight fraction on honeybee venom was isolated by gel filtration on Sephadex G-50 (Amersham-Pharmacia Biotech, Piscataway, NJ) and further separated by gel filtration on Sephadex G-75 superfine.¹²⁻¹⁴ The fractions with α -glucosidase activity were pooled and separated by ion exchange chromatography on a Pharmacia Mono S column.⁸ The fifth and largest peak was the 39-kd protein. This peak was pooled and concentrated and further purified on a Vydac C4 reversed phase column (The Separations Group, Hesperia, Calif) by using an acetonitrile gradient in 0.1% trifluoroacetic acid. The largest peak was pooled and concentrated.

An important serine protease allergen, Bom p 4, was reported in the venom of the bumblebee, *Bombus pennsylvanicus*, by our laboratory in 1996.¹ A similar allergen has been reported from the venom of *Bombus terrestris*.² An allergenic protein of approximately 39 kd has been noted in honeybee venom by several laboratories.^{3,4} Although venoms from yellow jackets and hornets contain only 3 major proteins,^{5,6} venoms from *Polistes*

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TABLE I. Primers

Common	
Oligo (dT) 181	TCTGAATTCTCGAGTCGACATCTTTTTT TTTTT
Oligo (dT) 182	TCTGAATTCTCGAGTCGACATCT
5' anchor (BRL) (CUA) ₄	GGCCACGTCGACTAGTTAC (GGGII) ₃ G
Honeybee	
Ap 3' 1	AAYGARTTYCCNATGATGGC
Ap 201r	TCCATYTGRCANGCRTCYT
Ap anP2	GTAAAACTCGTATGACCCCAACCCA
Ap 5' 2	GCTATCGTAGTTGGTGAACATGATTGGAGCAGT
Ap 460	CATTGCGTTAACCATTATATTGCCAT
Ap 5'a	GGCGAAATATATTATTTACAATCCTAG
Paper wasp	
PDOM 1	ATHAAYGARTTYCCNATGGT
PDOM 3	TCRTTYTGRCANGCRTC
EPDP 6	ACGATTATACGACAGATACGGAAAC
EPDP 7	TAAAAATGGAAGACAACTGGTCCAAC
PDEXPFWD	GAGCTCGAGAAAAGAGAGGCTGAAGCTA TAGTAAATG GGTGAAACAGAAAT
PDEXPREV	GAGTCTAGATTAATGATGATGATGATGA TGATCCGCT TGCAGTATGTTTCTC

Isolation of paper wasp venom proteases by affinity chromatography

Paper wasp venom was dissolved in 0.05 mol/L Tris-HCl, pH 7.4, and poured through a benzamidine-agarose column (Sigma Chemical Co, St Louis, Mo). The column was extensively washed with buffer and buffer containing 0.15 mol/L NaCl until no detectable protein was found in the washings. The protease was eluted with freshly prepared 0.1 mol/L benzamidine in buffer. The protein containing fractions were concentrated by diafiltration and extensively dialyzed to remove the benzamidine.^{15,16}

SDS-PAGE

Both 1-dimensional and 2-dimensional electrophoresis were performed as previously described.⁸

Amino acid sequence determination

Peptide mapping and amino acid sequencing was performed as previously described.^{17,18}

Cloning of the proteases

A mellifera and *P dominulus* proteases were cloned by using methods similar to those previously reported.^{19,20} The primers used are shown in Table I. The mRNA was isolated by using the Promega (Madison, Wis) polyATtract system. ELONGase (Invitrogen, Carlsbad, Calif) was used for the PCR reactions and Superscript (Invitrogen) for the reversed transcription reactions. Insert constructs were made by using the Promega pGEM-T Easy Vector System. Cloning was performed with the Invitrogen Topo TA Cloning Kit. The 3' rapid amplification of cDNA ends (RACE) was performed with primer 181 or 182 and the 5'-RACE with the Invitrogen abridged anchor primer. The sequence of the honeybee venom protein was verified by PCR+1 reaction.²¹ All sequences were verified by sequencing independently derived clones.

DNA sequencing and analysis

DNA sequencing was performed at the Biomolecular Research Facility of the University of Tennessee at Knoxville. Data processing and analysis were performed by using GCG software (Accelrys, San Diego, Calif) and National Center for Biotechnology Information online resources. Leader sequences were detected with Signal P.²²

RAST

The RAST was performed by using our standard procedure.²³ Specific binding is expressed as percent of total counts bound after subtraction of mean binding of negative sera. IgE immunoblots were carried out as previously described.²⁴

Three-dimensional structure modeling

Three-dimensional models were constructed by using Swiss-Model software (Swiss Institute of Bioinformatics, Geneva, Switzerland) with a variety of serine proteases as templates.²⁵

RESULTS

Isolation and structure of honeybee venom protease

After the 4-stage chromatographic purification described in methods, the bee venom protease gave a single broad band at 39 kd in SDS-PAGE and a single spot in 2-dimensional electrophoresis. Most of the IgE binding and enzyme activity was lost after the reversed phase chromatography step. The protein was reduced and alkylated. Then digests were prepared with trypsin and endoproteinase Glu-C. Separated peptides were sequenced. Degenerate primers for PCR were prepared from the amino acid sequence. When aligned with the sequence determined from mRNA cloning, the amino acid sequenced peptides represented 66% of the mature peptide sequence, including 43 amino acids in the complement, embryonic sea urchin protein, bone morphogenetic protein (CUB) domain and all 31 residues of the linker-propeptide region.

By using RT-PCR and RACE, the mRNA for the entire protein was cloned from before the initiation codon to the poly-A tail. The sequence has GenBank accession number AY127579. The translated sequences are shown in Figs 1 and 2. The molecule contains a typical tryptic protease domain of 245 amino acids with a serine protease catalytic triad, aspartic acid specificity residue, and a highly unusual methionine at the entrance to the binding pocket at position 347. There is an initial 35-residue leader sequence, followed by an 88-residue CUB protein-protein association domain²⁶ and 31 residues of linker and propeptide. There are 4 potential N-linked glycosylation sites, at least 1 of which is glycosylated. This protein has been assigned the name Api m 7 by the International Union of Immunological Societies Allergen Nomenclature Subcommittee.

FIG 1. Alignment of the protease domains of honeybee, bumblebee, and Mediterranean paper wasp venom proteases. The dots indicate insertions to maximize alignment.

FIG 2. Complete amino acid sequences of the honeybee and Mediterranean paper wasp venom proteases and the mature peptide of bumblebee venom protease. The CUB domain is underlined. The active site residues are shown in *bold*, the tryptic specificity residues are shown in *lower-case bold*, and the residue at the entrance to the binding groove is marked by a *triangle*. Symbols underneath sequences are (*) identical residues in all 3 sequences, (:) similar conservative substitutions, and (.) related residues.

TABLE II. Specific IgE binding to *P exclamans* venom proteins

	American patients		Italian patients	
	Protease % Specific binding	Protease/ Ag5	Protease % Specific binding	Protease/ Ag5
1	1.1	0.52	5.0	2.63
2	2.0	0.15	23.2	2.05
3	1.8	0.53	6.6	3.88
4	3.6	0.51	18.5	10.88
5	1.6	0.13	6.1	2.77
6	9.8	0.3	15.4	1.18
7	1.5	0.09	5.0	2.38
8	2.5	0.29	10.8	2.00
9	1.1	0.06	7.2	3.27
10	3.4	0.41	10.7	3.82
11	2.1	0.11	13.5	1.19
12	5.0	0.14	8.1	3.37
13	3.6	0.2	16.2	1.69
14	4.1	0.29	4.8	2.09
15	2.6	0.87		
16	1.1	0.25		
17	12.2	0.58		
18	2.9	0.19		
19	7.4	0.63		
Mean	3.65	0.33	10.8	3.09
	SD = 3.00	SD = 0.22	SD = 5.8	SD = 2.41

Isolation and characterization of paper wasp venom protease

Proteases from *P dominulus* and *P exclamans* venoms were prepared by benzamidine affinity chromatography. The proteins were found to be free of phospholipase and antigen 5 by both SDS-PAGE and enzyme activity testing. The *P dominulus* protein was reduced and alkylated and subjected to proteolytic enzyme digestions to prepare peptides for amino acid sequencing. These sequences were used to design degenerate primers for mRNA cloning. The amino acid sequences determined represented 86% of the sequence of the mature enzyme. The mRNA sequence has been deposited in GenBank with accession AY285998. The translated sequence is shown in Figs 1 and 2. The mRNA had a leader sequence of 19 amino acids, a propeptide sequence of 14 amino acids, and a tryptic enzyme sequence of 244 amino acids. There are 6 potential N-linked glycosylation sites, 4 of which appear to be glycosylated in the natural molecule. This protein has been assigned the name Pol d 4 by the IUIS Allergen Nomenclature Subcommittee.

The enzymes from *P exclamans* and *Polistes gallicus* venoms are similar, but their complete sequences have not been determined.

IgE binding activities of venom proteases

IgE binding to the honeybee venom protease could not be measured by RAST because the purified protein was denatured after the reversed phase chromatography step. The fraction from the ion exchange chromatography still

TABLE III. Specific binding of IgE from sera of patients allergic to Italian paper wasp venom to venom proteases from *P dominulus* and *P exclamans*

Serum	<i>P dominulus</i> % Specific binding	<i>P exclamans</i> % Specific binding
A	1.5	3.1
B	14.6	14.9
C	1.1	1.8
D	8.1	8.6
E	3.3	2.2
F	8.8	9.7
G	6.0	1.3
H	11.3	11.3
I	13.8	10.5
J	1.6	2.3
K	2.3	4.4
L	3.0	5.1
M	8.7	6.8
N	5.2	3.4
Mean	6.4	6.1
SD	4.6	4.3
Regression coefficient $R = 0.90$		Slope = 0.97

contained residual hyaluronidase and other venom proteases. IgE binding was readily detectable to the 39-kd band in immunoblot studies in 80% of the sera studied. Representative blots have previously been illustrated by Kettner et al.³

Direct RAST binding results to purified *Polistes* venom proteases are presented in Tables II and III. By using *P exclamans* protease and antigen 5, sera from southern European patients sensitized by European *Polistes* showed higher average IgE binding to protease than to antigen 5, whereas the reverse was observed for sera from North American patients, who were not exposed to European *Polistes*. The mean protease to antigen 5 binding ratio was 3.09 for Italian sera and 0.33 for North American sera. Similar IgE binding was observed with Italian patients' sera to both *P dominulus* and *P exclamans* proteases with a linear regression $R = 0.90$ and a slope of 0.97. The protease from *P gallicus*, a sister species to *P dominulus*, has also been demonstrated to have IgE binding activity.¹¹ RAST inhibition studies have demonstrated significant differences in specificity between IgE antibodies from patients sensitized by European *Polistes* and those from patients sensitized by North American *Polistes*.⁹⁻¹¹

Comparisons of the venom proteases

The protease domains are all members of subfamily S1A, clan PA(S), enzyme S01, trypsin EC 3.4.21.4.²⁷ The protease domains are 41.5% identical for paper wasp and honeybee over 248 residues; 31.8% identical for paper wasp and bumblebee over 245 residues; and 33.1% identical for honeybee and bumblebee over 242 residues. As can be seen in Figs 1 and 2, the identity is almost entirely in the highly conserved residues of the S1A

family. Many of the peptide loops have variable lengths. The proteases also differ at the entrance to the binding pocket, where the bumblebee enzyme has the typical glycine residue, the paper wasp enzyme has the less common asparagine, and the honeybee enzyme has the unique methionine, as shown in Fig 2. A methionine in this position is reported only in another hypothetical honeybee CUB protease, GenBank XM_392669. Attempts to construct 3-dimensional models by using the Swiss-Model server with a variety of trypsin templates were successful for the paper wasp and bumblebee enzymes but not for the honeybee protease. Because of the variable loop lengths, the molecular models were superimposable only for the active site regions. We have been unable to find a suitable template for the protease domain of the honeybee venom allergen. Because of the significant differences in 3-dimensional structure, it is improbable that there is much antigenic cross-reactivity among protein portions of the honeybee, bumblebee, and paper wasp enzymes. The CUB and linker domains are not found in the mature peptide sequences of either bumblebee or paper wasp venom enzymes.

DISCUSSION

Serine proteases are found in bumblebee,^{1,2} honeybee, and paper wasp venoms. There is no evidence that significant amounts of protease are found in either yellow jacket or hornet venoms.^{5,6,8} The enzymes from bumblebee and paper wasp venoms are typical trypsins with a single serine protease domain. The honeybee venom protease contains a CUB domain in addition to a trypsin domain, as indicated in Fig 2. This domain mediates protein-protein interactions during development and in interacting protein cascades like the classical complement pathway.²⁶

The 3 venom proteases show a high degree of conservation of the serine protease and trypsin conserved residues but little of the framework residues. Loop lengths vary among the 3 enzymes. The honeybee and bumblebee enzymes are not closely related to each other by sequence. The structures of the 3 enzymes appear different enough that little immunologic cross-reactivity of the protein portions would be expected. They each probably have a different function in the venom.

The honeybee and paper wasp enzymes have been found experimentally to contain N-linked carbohydrate. Some of the IgE reactivity may be a result of this cross-reactive carbohydrate determinant.^{28,29} Although approximately 80% of honeybee reactive sera demonstrate binding to the venom protease by immunoblot, there appear to be differences with binding to hyaluronidase and the higher-molecular-weight proteins.³ The bumblebee enzyme can hydrolyze casein,¹ and the paper wasp enzyme binds to benzamidine, indicating that they possess proteolytic activity.

The enzyme activity of the honeybee protein is unknown. It does not bind well to the benzamidine agarose

column. There is a bulky methionine residue (Fig 2) at the entrance to the substrate pocket, which may be important in determining the specificity of the protease. This residue is found only in another honeybee CUB protease in GenBank. The bumblebee and paper wasp enzymes have more typical residues, glycine and asparagine, at this position.

Immunologically, the protease enzymes from North American and European bumblebees, *B pennsylvanicus* and *B terrestris*, appear to be related.^{1,2} The enzymes from North American and European paper wasps, *P exclamans*, *P dominulus*, and *P gallicus*,¹¹ are highly cross-reactive, as shown in Table III. There are significant amounts of protease in venoms from all 3 of the genera studied. In SDS-PAGE, the paper wasp enzyme migrates with the phospholipase, and the honeybee enzyme is a broad band below the hyaluronidase and acid phosphatase bands. The venom proteases appear to be significant allergens, but a definitive evaluation of their importance requires the production of carbohydrate free recombinant molecules with native conformation.

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