

Hymenoptera Venom Allergens

Donald R. Hoffman

*Department of Pathology and Laboratory Medicine, Brody School of Medicine at East Carolina University,
Greenville, NC. E-mail: hoffmand@ecu.edu*

Abstract

Hymenoptera venoms each contain a variety of protein allergens. The major components have all been characterized, and most of the amino acid sequences are known. This article concentrates on the use of contemporary techniques including cloning, mass spectrometry and genomics in the characterization of venom allergens, and newer separation techniques for protein isolation. Examples of the use of these techniques with venom proteins are presented.

Index Entries

Venom; hymenoptera; phospholipase; hyaluronidase; antigen 5; bee; wasp; ant; hornet; yellowjacket.

Introduction

Stinging insect venoms are composed of secreted proteins (some of known enzymatic activity), pharmacologically active peptides, histamine, acetylcholine, catecholamines, and various other substances (1–3). Some venoms contain other organic molecules, such as the alkyl and alkenyl piperidine alkaloids that are the major component of fire ant venom (4). In addition to the secreted proteins, venoms also apparently contain smaller amounts of pro-

teins released by damage to the cells that line the venom duct and reservoir (5). Numerous venom proteins are known to have *N*-linked glycosylation, with a common fucosylated immunogenic polysaccharide (6).

Several recent reviews have concentrated primarily on the properties of the allergenic proteins and peptides in insect venoms (7–9). This article emphasizes examples of contemporary methods for the isolation, characterization, and analysis of allergenic venom proteins.

Bee Venoms

Honeybees

The major protein in honeybee venoms is phospholipase A₂. It has very strong allergenicity by both the injection and inhalation routes (10,11). The mature molecule consists of 134 amino acids with N-linked glycan attached at asparagine 13 (12,13). Phospholipase activity is calcium-dependent, and there is a calcium-binding site near the N-terminal. The three-dimensional structure has been determined from X-ray crystallography (14). Immunoglobulin (Ig)E-binding activity greatly depends on conformation and is greatly reduced by reduction and alkylation (15,16). A recombinant preparation has been studied (16–19). The recombinant was produced in *Escherichia coli* and was then renatured (17). Skin-test activity (16) and histamine-releasing activity (18) were comparable to natural venom phospholipase. The diagnostic specificity was improved because the recombinant molecule was not contaminated with other venom proteins and did not contain the crossreactive N-glycans (19).

B-cell epitopes present on honeybee phospholipase have been investigated with both human IgE and IgG antibodies. The epitopes were found to be almost entirely discontinuous and conformation-dependent in each study (15,16,20). Studies of the T-cell epitopes of phospholipase have shown that short peptides are relatively poor stimulators of response (21–24). The addition of immune stimulators, such as pokeweed mitogen, interleukin-2, or interleukin-4, was needed for a strong response (22,25). Overlapping peptides of 15 amino acids provided minimal stimulation of T-cell proliferation, whereas peptides of 40 to 60 residues provided vigorous stimulation (24,26). The T-cell proliferation response to the three long peptides (residues 1–59, 51–99, and 90–134) increased during early venom vaccine therapy and decreased markedly after 3 to 5 yr of bee venom vaccine therapy (26).

The mixture of three peptides was compared with whole-venom vaccine therapy in a small group of patients, and similar immunological results were found in both groups in terms of proliferative responses, cytokine production, and IgE and IgG₄ antibody responses (27). These results were confirmed, and the safety of the regimen was demonstrated in a recently reported phase I clinical trial of the triple-long overlapping peptide therapy (28).

The phospholipases from two other species of honeybees, *Apis cerana* and *Apis dorsata*, have been studied (29). *A. cerana* phospholipase has 6 amino acid differences, of which 5 are conservative with *Apis mellifera*, whereas *A. dorsata* has 11 differences, of which 10 are conservative. The phospholipases of the two Asian species have 13 amino acid differences, all of which are conservative substitutions. The phospholipases were modeled using the X-ray coordinates for *A. mellifera* phospholipase published by Scott et al. (14) in the SWISS-MODEL program (30). All differences were found to be on the surface of the molecule. Charge differences were present at positions 14, 85, 107, and 115. The modeled three-dimensional surfaces of *A. cerana* and *A. dorsata* phospholipases are superimposed in Fig. 1 (the varying residues are numbered). Notably, all variation is on the surface of the molecule; the backbone and functional residues are conserved. Unpublished studies with sera from East Asian patients demonstrated minor differences in reactivity between *A. cerana* and *A. mellifera* phospholipases.

The hyaluronidase from honeybee venom is the first member of this enzyme family to be studied in detail. It is closely related to vertebrate hyaluronidases, including the sperm hyaluronidases (31). The enzyme belongs to family 56 of the glycosyl hydrolases (32), cleaving the β -1,4 glycosidic bond between N-acetyl-glucosamine and D-glucuronic acid of hyaluronic acid. In venoms, this enzyme activity potentiates infiltration by dissolving the

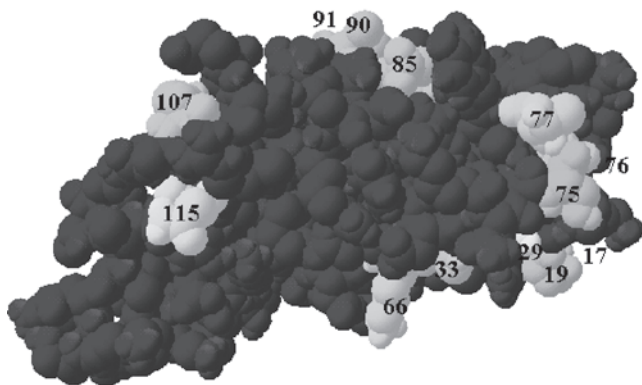


Fig. 1. Three-dimensional models of the surfaces of phospholipases from the Asian honeybees *Apis cerana* and *Apis dorsata*. The lighter residues are those that differ with the residue numbers indicated. Residue 14, which differs in *Apis mellifera* phospholipase, is on the rear surface of the molecule in this view.

extracellular matrix. Hyaluronidase is isolated from venom by multiple chromatography steps, including gel filtration and high-performance cation exchange (10,11); however, removal of the traces of phospholipase requires an antibody affinity purification step (33). There are 350 amino acid residues in the mature protein, with two disulfide bridges and four potential *N*-linked glycosylation sites. Apparently, there are two glycans per molecule. Bee venom hyaluronidase is easily denatured (33). A recombinant molecule has been produced in baculovirus, which has both the enzyme and immunological activity of the native enzyme (34). Hyaluronidase expressed in *E. coli* cells was not biologically active. The three-dimensional structure of bee venom hyaluronidase has been elucidated by X-ray crystallography (35). The basic structure consists of a seven-stranded β -barrel surrounded by 10 α -helices. The substrate-binding groove is large enough to accommodate a hexasaccharide.

Melittin, an amphipathic 26-amino acid peptide, is 30 to 50% of the dry weight of honeybee venom (36). It is produced as promelittin in the venom gland and the membrane-bound

enzyme dipeptidyl peptidase IV sequentially cleaves dipeptides ending in proline or alanine as it travels through the venom duct to the venom sac (37). The conformation of melittin is a pure α -helix (38); at high concentrations in solution, it is present primarily as tetramers. At high concentrations, melittin can directly lyse cells, especially red blood cells (39). The initial pain from bee stings appears to result from the effects of melittin (40,41). Melittin has significantly less allergenic activity than phospholipase and hyaluronidase (10,11,42), with about one-fourth to one-half of individuals allergic to honeybee venom producing IgE antibody. Honeybee venom vaccine therapy causes an increase in the fraction of individuals with melittin-specific IgE.

Kettner et al. (43) isolated a small protein allergen of about 8 kDa and named it Api m 6. This protein was isolated by gel filtration and reversed-phase high-performance liquid chromatography (HPLC). It was sequenced by Edman degradation of enzymatically digested peptides and mass spectrometry using carboxypeptidase to sequentially remove the C-terminal amino acid. Four isoforms were discovered; the largest isoform contained 71 amino acids. Approximately 40% of patients with allergy to honeybee venom demonstrated IgE antibodies to Api m 6.

Several groups of investigators noted an area of IgE antibody reactivity at about 30 to 39 kDa in immunoblot studies of honeybee venom (44–47). A protein was isolated using a combination of gel filtration, cation exchange HPLC, and reversed-phase HPLC on C4 column, and about 66% was sequenced by Edman degradation of tryptic and endoproteinase Glu-C digested peptides (47). The complementary DNA (cDNA) for the molecule was cloned and was found to contain a trypsin serine protease domain and a complement, embryonic sea urchin protein, bone morphogenetic protein (CUB; ref.48) protein-protein interaction domain (47). There are four potential *N*-linked

glycosylation sites, at least one of which is glycosylated. The serine protease has an extremely unusual methionine residue at the entrance to the binding pocket. The enzyme does not bind well to benzamidine-agarose, and the active site may be sterically hindered. Approximately 80% of sera from individuals with allergy to honeybee venom show binding to this glycoprotein.

Honeybee venom is known to contain significant amounts of several enzyme activities, including acid phosphatase (49), α -glucosidase (47), esterases (39), and peptidases. The acid phosphatase is present as both monomers of about 48 kDa and dimers (49–51). The enzyme has been purified by gel filtration and ion-exchange chromatography (49,50) and has been found to be a glycoprotein, with the ability to cause histamine release from sensitized human basophils and to produce a wheal-and-flare reaction after intradermal injection into skin of allergic individuals.

Approximately half of the amino acid sequence was obtained by Edman degradation of peptides prepared from the purified protein (52). It was then possible to assemble most of the acid phosphatase sequence from the honeybee genomic sequence to produce a predicted sequence; however, there was a problem region in the genomic sequence, and the stop codon was missed in the computer-predicted protein sequence. Matrix-assisted laser desorption/ionization–time of flight mass spectrometry was performed on a tryptic digest of the acid phosphatase. Because a preliminary genomic assembly was available for the honeybee, researchers were able to use the MASCOT program (53) to analyze the mass spectrometry data. A sample of the results is illustrated in Fig. 2. The peptides identified by mass spectrometry represented about 52% of the predicted sequence. The genomic sequence was used to design exact-match primers for cDNA cloning. The complete amino acid sequence of the mature enzyme was 373 amino acids, with four potential *N*-linked carbohydrate sites and

two disulfide bonds. The molecule is a typical histidine acid phosphatase similar to insect and mammalian prostatic and lysosomal acid phosphatases. The molecular weight of the protein portion is 43.9 kDa, with an isoelectric point of 5.64. The enzyme is a heat-labile glycoprotein. The cDNA sequence was compared with the genomic-derived sequence, and a region was missing in the problem region of the genomic sequence. The missing stretch of DNA was discovered by searching the genomic sequence fragments, and a corrected assembly was generated. The coding sequence is contained in six exons of the corrected genomic sequence in group un2174 and is shown in Fig. 3. Immunological studies have indicated that the acid phosphatase contains both protein and carbohydrate IgE-binding epitopes (6,52).

Investigators in several laboratories have described several higher molecular weight IgE-binding proteins in honeybee venom (5,44–46,51,54,55). The IgE binding activity has been detected by immunoblot (44,45,54), crossed radioimmuno-electrophoresis (55), and radioallergosorbent test using purified protein (51). Kettner et al. (45) recently used two-dimensional electrophoresis to separate the higher molecular-weight venom proteins and attempted to identify those that bound IgE by N-terminal amino acid sequencing. This yielded a limited amount of data, indicating that some of the known allergens, such as hyaluronidase and acid phosphatase, were heterogeneous. At least one new IgE-binding protein was found, but there was insufficient data for identification. Our laboratory separated the proteins from the high-molecular-weight fraction of honeybee venom using reversed-phase chromatography and attempted to identify proteins by N-terminal amino acid sequencing, as well as amino acid sequencing of internal peptides (5). One of the first proteins identified was about 69 kDa and was found by comparison with the honeybee genome sequence to be a carboxylesterase—probably acetylcholinesterase shown in Fig. 4A. This molecule has been confirmed by mass

1. [gi|48102165](#) Mass: 117717 Score: 100 Expect: 0.00022 Queries matched: 17
similar to CG9451-PA [Apis mellifera]

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
11 - 17	889.50	888.49	888.52	-0.03	0	QINVIFR
18 - 26	1066.50	1065.49	1065.52	-0.03	1	HGDRIPEK
27 - 45	2532.20	2531.19	2531.14	0.06	1	NEMYPKDPYLYYDFYPLER Oxidation (M)
33 - 45	1753.80	1752.79	1752.80	-0.01	0	DPYLYYDFYPLER
54 - 64	1456.80	1455.79	1455.73	0.06	1	MREYQLGQFLR Oxidation (M)
56 - 64	1153.60	1152.59	1152.59	0.00	0	EYQLGQFLR
67 - 89	2634.20	2633.19	2633.18	0.01	0	YGDFLGDIYTEESVSALSSFYDR
67 - 91	2863.40	2862.39	2862.32	0.07	1	YGDFLGDIYTEESVSALSSFYDRTK
92 - 106	1674.00	1672.99	1672.92	0.07	0	MSLQLVLAALYPPNK Oxidation (M)
107 - 122	1984.00	1982.99	1982.98	0.01	0	LQQWNEDLNWQPIATK
126 - 146	2671.30	2670.29	2670.30	-0.01	1	RYEDNIFLPEDCLLFTIELDR
127 - 146	2515.20	2514.19	2514.20	-0.01	0	YEDNIFLPEDCLLFTIELDR
260 - 271	1380.80	1379.79	1379.74	0.05	0	IVYYLGIPSEAR
307 - 324	2069.00	2067.99	2068.00	-0.01	0	FVDESANNLSIEELDFVK
307 - 329	2678.50	2677.49	2677.40	0.10	1	FVDESANNLSIEELDFVKLNLR

1 MTSLIDLKKF SSMSVIAILA MVVGVA/ELK QINVIFRHGD RIPDEKNEMY
51 PKDPYLYYDF YPLERGELTN SGKMMREYQLG QFLRERYGDF LGDIYTEESV
101 SALSSFYDRT KMSLQLVLA LYPPNKLQQW NEDLNWQPIA TKYLRRYEDN
151 IFLPEDCLLF TIELDRVLES PRGKYEFISKY DKLKKKLEEW TGKNITTPWD
201 YYYIYHTLVA EQSYGLTLPS WTNNIFPRGE LFDATVFTYN ITNSTPLKK
251 LYGGPLLRIK TKHMLDVVSG TQKKRKRIYL FSGHESNIAS VLHALQLYYP
301 HVPEYSSSII MELHNIETH YVKIVYYLGI PSEARELQLP GCEVLCPLYK
351 YLQLIENVIP SNEELICKR FVDESANNLS IEELDFVKLN LIRIAGTENK
401 *

Fig. 2. Analysis of mass spectrometric data from bee venom acid phosphatase. The top shows output of the MASCOT program using the National Center for Biotechnology Information nonredundant database. The bottom shows the placement of the peptides identified by mass spectrometry underlined in the final amino acid sequence determined by cDNA cloning. The potential N-linked glycosylation sites are shaded. The slash indicates the signal sequence cleavage determined from N-terminal Edman degradation. (The data are used courtesy of Drs. Daniel Kolarich and Friedrich Altmann.)

tctcgttatagcttttacatataaagcacatactagtttctttcttttctcgtttaattcaatcggtta
 tcttatatttctcctcaaagcgtataatagtgtacatttgaaaattaacattttatacgttaagtaagtgttggc
 acaaagtatatattcattcttcacgtatcaattaatactttacacgtgcaggtatttaccattttaatttat
 taagcttgtctttttcttttttttttttttttttttaatacaaaaacgtgacgatagaatctataaaattg
 gcataaaagatgattatcgatttaattttaaatacgtgataaacggatgaaagattttaaaggatattcgtgt
 tttttttttttttttttttctatcttgcctcgagcttcgagaaaaataatcttttgacaggtggaataaat
 ttaataaaatttcattttctcctcttttgaatttcaggttgaacgggaacaatgatgacttcgcttatcgatct
 taaaaaaatttctcatcgatgagtgtaatcgcgattttggcgatggtggttggcggtccaagcggaacttaa
 caaataaatgtggtgagtaatacaataaagaatttatattttaaattttttcgcgtgaataagatattcgtt
 cgaatcgaactattttaaatttagagaaaaataaataatagaacttggtttgaaattgaaaaaaaagtga
 aaggtgtaattcaattctatatgcgattcgtacaattgcacagatattccggcacggcgataggataccg
 atgagaaaaacgaaatgtatccgaaagatccttatttgattatgatttttatccactggagcgtggcgaa
 ttgactaacgtaagtactttgttctctctctcatcttaggcttatccatgtaaaatatatttttcgatatt
 taattttcacgtatttcttcgattaaattcgatttatccgttttttttaataataattttcgtctaaatatg
 aaaattcaatagttgaggttacgaataatagttcgataaattacttcaataattttataatatgttcaaac
 gtaataataatagtcgaaaataattgtgacaaatagccgagcttatacgaatagcggatcaattatattag
 ctaggataaatgttattaataattatttgaagaaactttatttgagatttttaacttacttttcaattg
 gattaatgtactaactctgttatttttagtcaggtaaaatgcgagaaatcaattggggcaattcttgagag
 agagatatggtgactttttgggagacatttacacggaagaatccgtctcggctctcagctcgttctacgat
 aggacgaaaatgtctctgcaactcgtactcgcggcgtcttatccgccaaataaattgcaacaatggaacga
 agatctgaactggcaaccgatcgcacgaaatatttgcgcgcgtacgaggacaatatcttttggcagaag
 attgtttgttgaagttaaatacaaaaaatatttggaaaaatttcccttcaacttccctttattttaatta
 attattttattttcttttattttaattctctcgttctggttttcgcagatttaccatcgaacttgatagagta
 ttggaatcaccgctggaagatgaattctcaaaatagacaaattgaagaaaaaattggaagaatggac
 cggaaaaaatatcactacgccatgggattattattacatatatcatacactgggtggctgaacaatcgtacg
 gtcttactctgccatcttggaacaataatatattcccagagaggagaattgttcgatgcgacggtatttacg
 tacaacataaccaattcgactcctttgttgaaaaaactttatggaggttaattcgacaataatatttcttct
 tattcgttttaaatccttttcttattaaattttaattcttcttttcttcaattgttagtttttcttctt
 ttttttttttttttttactactgtgatacaaaattcacactaatgttatattttcattattaccgtatc
 agttccacggattctttgattttgcaagtatcaatctttcattctcatattttctgtttctttttttttt
 ttttttttttagaaaagaacttcaatctaataataaataatcgtgtcacttcgtcgtgttgccattttagg
 tccgcttcttcgaatattcaccaagcatatgttagacgtggtatcgggtacgcaaaagaaaaagcgaaaga
 tatacttgttcagtgagcatgaaagtaatatcgcctctgtgttgcaagctcttcaactttattatcctcac
 gttcctgaatatccagttctattataatggagcttcacaatatcgaaggcactcactacgtaaaggtagc
 taataaaaccaatatctatatatttttatagcaaaacaatatattttaaggcatatcttataaattaacctac
 aacctcgatagcggccaatttacgacattgttacgtacattgatccttcgccatatgcaaaatatatatcg
 tctcaattttttattttaataagttaattaacgatgttttttgaaatcgcgagatatgatattgaaaacttt
 tcgaataattttgaggatcatactgaggaacaaattaatcgtattttacgaattattttcattttattcagct
 ttttcgattcttgtttcttcttataatttttaattaccgattaatatcgtttgcagatcgtttactacttgg
 gtatcccgtctgaagcgagagaacttcaattaccgggtgcgaggtactttgccctttgtacaaaatattta
 caattgatagagaacgtgataccatcgaacgaagagttgatctgcgataaaagattcgtcgacgagtcggc
 aaacaatttgcgatcgaagaattagatttcgtgaaattgaacctaaataaggatagcgggtactgagaata
 agtaactattatgggaggattaattaaattgtcaatcacgtgaaaaacgaaatgaagaaaatgaaatatgt
 gtttaaagtattttactttacatgaatgaatgaattttgaaatagttaaatgaatttatcatctaattgata
 ttaattattttatttaattttatcgataatccgatacgcgaaagaaaaaataagagaggaatgtgattcat
 atctctttaactatgttcaaaggtcatcctataagtaatgctatcttttcattttatatccactaacagtat
 tattcggcttcttcttttttcttttttcttaattaaaaaacagtattatttgctaaaattcaacttttagt
 atttttagtattaaaagtattatttaacttttaaaaacatttaacattgtaatttaacaacaagaatggaaa
 tgagcaatttatcgttaattatcgtatttgagattgtgaaaaaagtgcaatcgtttattgtttatttcacaa
 atgtttttttgatcacatttatcacgaatgcaaaaaaagtttatacgtgtaagtttatcacagc
 catactacagggaataacttgattctctgattttacgttgattgtaaaaata

Fig. 3. The corrected genomic sequence of bee venom acid phosphatase. Exons are shaded.

spectrometry (Kolarich, D. and Altmann, F., personal communication), and was also found by Kettner et al. (45). Much of a second protein was determined by amino acid sequencing. This protein is of unknown function, and the cDNA has been cloned. The amino acid sequence is shown in Fig. 4B. It has 204 amino acids, with four N-linked glycosylation sites and no cysteines (5). The isolated protein is rich in carbohydrates. A third protein of about 78 kDa is shown in Fig. 4C was found by amino acid sequencing to be related to hexamerin 2, an arylphorin insect storage protein (5,56). Although there is a large amount of α -glycosidase activity in honeybee venom (47,51), the enzyme has not been isolated. Mass spectrometric analysis of tryptic digests of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-separated honeybee venom high-molecular proteins positively identified the presence of a membrane-bound enzyme, dipeptidylpeptidase IV shown in Fig. 4D, which converts promelittin to melittin in the venom duct (Kolarich, D. and Altmann, F., personal communication).

The identification of these molecules was performed using the honeybee genomic database. All of the known honeybee venom proteins are contained in the present release of the genome 2.0 (January, 2005), although currently, the computerized protein prediction is not highly reliable. Much of the IgE-binding activity of these high-molecular-weight proteins may result from crossreactivity with carbohydrate determinants (57).

Bumblebees

There are two distinct populations of patients with allergy to bumblebees: those sensitized by field stings and those sensitized by occupational exposure in greenhouses (58–63). Venoms from two species of bumblebees have been studied (58,64; *Bombus pennsylvanicus* comes from North America and *Bombus terrestris* is native to Europe (although it is commonly used for pollination in greenhouses). The amino acid

sequences of both phospholipases are known: similarly to honeybee phospholipases, they are phospholipase A₂. The bumblebee proteins each contain an insertion of two amino acids in the short loop between cysteines 105 and 113 of *A. mellifera* phospholipase. The N-linked carbohydrate is at position 16, rather than 13. The *B. terrestris* protein is 52.9% identical to honeybee and 83.8% identical to *B. pennsylvanicus*. Immunological studies show incomplete cross-reactivity between the bumblebee species (58). Most occupationally sensitized patients and some field-sensitized patients show only bumblebee-specific reactivity. In a recent mutation study of honeybee phospholipase (65), by studying the IgE reactivity of sequentially prepared mutants of honeybee phospholipase, 24 mutations and a deletion of 10 amino acids were required to greatly reduce the IgE-binding activity. This is a 25% difference, as well as changes in the backbone structure caused by the deletion.

Bumblebee venoms contain a tryptic protease (58,64), consisting of a single serine protease domain of 243 amino acids. It is enzymatically active at cleaving protein and binds to benzamidine-agarose. The proteases are strong allergens and show partial crossreactivity between the two species (58). They do not crossreact with honeybee venom protease (47). Bumblebee venoms do not contain a significant amount of a melittin-like material but have other surface-active peptides called bombolitins (66), which are not allergenic. Bumblebee venoms contain hyaluronidase and acid phosphatase (64).

Other Bees

Little is known regarding the venoms of other bees. There are both social and solitary stinging bees. Large carpenter bees (*Xylocopa*) and cuckoo bumblebees (*Psithyrus*) are probably similar to the closely related bumblebees. Pence et al. (67) studied a group of 13 patients with reactions to sweat bees (*Halictidae*); only three were skin-test reactive with honeybee

A

MYMLKLSYILLFLGFVKFSWQ/DKQVPKVSTFTGNIRGYKKSRSDRLYEAYEGIPYAQSPVGKFRFQPPRPIKKWS
 KDLSATKKSSVCMQYLMFTTTHGNRVKGSSECLYINIVPVRNNRKPLLPMFWIHGGAFQFASGNEANETLFMDRN
 IVFVAINYRLGPFGLSTGDIVVPGNMGLKDQSMALRWVFNNIKSFGGNPNKITIFGMSAGGASVHYHYLSPMSAGL
 XYIGGISISGVAFCPWAQTKHAPEKAKKLALMKCRTDNTKKMIDCLQSRPARIIAQAVGDFMVNYNPFPTFGPVE
 TYGSNPFISNSPINIINNGQVYDVPWISGVVSKEGLYVDNAKLLWHLNDHWDEIAPYLLDFNYTIPLDQHRQVAKKI
 KNYYLSDPINYDKVESIIQMMSDRLFNIDFEKAVRLQARINKSPVWTTYYSYRAEHSVSEILSGGSTTDYGNIFYN
 KEYFVTLNSIISNVTKPQDLAMQOLLINFYTSFLPNSFEKRSFRSYPSHDHRSIAVTMSTVNIIFSLPLLFLHNR
 SFYW (C-terminal region unknown)

B

MKTGLVLFIAAWFIACHTS/FPGAHDSDSKEERKNVDTVLVLPsierDQMAATFDFPSLSFEDSDEGSNWNWNTLLRP
 NFLDGWYQTLQSAISAHMKKVREOMAGILSRIPQGVVNNKIPEGANTTSTTKIIDGHVVTINETTYTDGSDDYSTLI
 RVRVIDVRPQNETILTTVSSEADSDVTTLPTLIGKNETSTQSSRSVESVEDFDNEIPKNQGDVLT*

C

EYY DTKTADKDFL LKQKKVYNLL YRVAQPALAN ITWYNEGQAW NIEANIDS.....

D

MAIWELFRIRLMVPRVIDQDLERYEPLSEEDHRSARVPFNLEETYDQSFANSFNGTWKTDREILYSDNYVGDRL
 FDVTTGSGTVLLDSSVTADFKA SVMFSFDNSHVAIGHDYVNVFFEGGSDTRRITNTGVPDIVFNGIPDWVYEEVL
 GSPVAFWISPDGRHLAFATFNDTNVRDIVISKYSGPGNSRDQYPNEIRIKYPKNANNIYEEETEGWLRIQPPLYH
 DRYVIVAKLQDSGKAGRFLHATRLEYRNGALVDEDLTPGTCEVISLLLVDHARARLYLGTGLKPKSHKNLYSVQ
 LSGNEPPVCLSCDVLTPEGNRCTYAYAYFSTNGSHYALYAGDPVFIAIVNANHRQISWEENRSLRRKLAARTQP
 IVKNFNVNANGYTNKVKLYLPDFDETKYPLLINVYAGPNTIRITEEATYGFESIIVTNRSVIYGRIDGRGSAYKG
 SKMLFEIYRRLGTVEIEDQIIITRTLQEKYSWIDSNRTGIWGSYGGFSAAMVLATDAESVFKCGISVAPVTSWIYY
 DSLYTERFMGLPTPEDNQSGYNDTDVSRVVEGMRGKMYLIHGTADDNVHYQQTMLNKLAVNSDIMFQQQTYTDEA
 HALGNVFPFLYHTMDRFWANCLGYSH

Fig. 4. Proteins in the high-molecular-weight fraction of honeybee venom. Shading indicates potential N-linked glycosylation sites, underlined residues were verified by amino acid sequencing, and the "/" indicates the end of the initiation sequence for secreted proteins. (A) The amino acid sequence of venom carboxylesterase, possibly acetylcholinesterase; Genbank XP394404 belongs to pfam 00135 carboxylesterases. The C-terminal region is not known. The molecular weight from SDS-PAGE is 68kDa. (B) The amino acid sequence of carbohydrate-rich protein, Genbank AAX33236 and NP001012431. (C) The N-terminal sequence of hexamerin, Genbank XP392867. These proteins exhibit differential splicing and there are a number of forms. The estimated molecular weight is 78 kDa. (D) The amino acid sequence of honeybee venom dipeptidyl peptidase IV, Genbank XP393818. The mass spectrometry data is courtesy of Drs. Friedrich Altmann and Daniel Kolarich. This protein is membrane-bound.

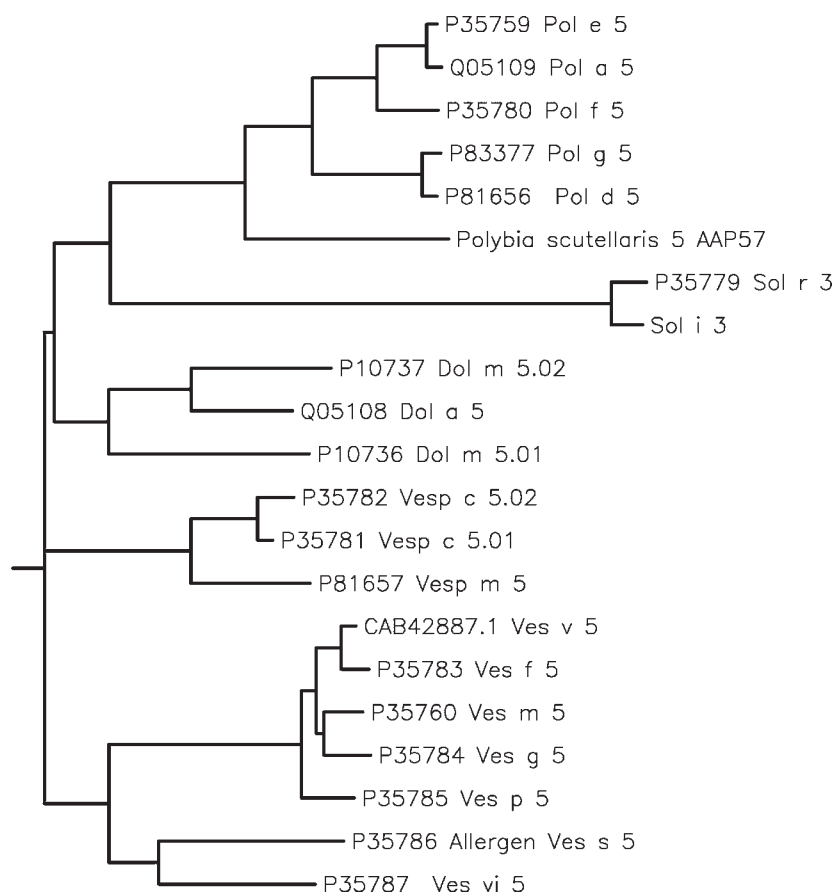


Fig. 5. Phylogenetic tree of antigen-5 sequences from yellowjackets, hornets, paper wasps, and fire ants.

venom. Halictid bees are in a different family from honeybees and bumblebees.

Vespid Venoms

Yellowjackets and Hornets

The venoms of yellowjackets of the genus *Vespula* and hornets of the genus *Dolichovespula* were found to contain three major allergenic proteins: antigen 5, phospholipase, and hyaluronidase (68–71). Two additional proteins with allergenic activity have been described (71) but are incompletely characterized. In 1987, the venoms of the genus *Vespa*—old world hornets—were found to be similar to those of *Vespula* and *Dolichovespula* (72).

Antigen 5 is the most thoroughly studied of the vespid venom allergens (73–77). Amino acid sequences have been revealed from 19 spe-

cies of six genera of wasps. Vespid wasp antigen 5 does not contain carbohydrate. Figure 5 shows a phylogenetic tree of the sequences. Notably, there are two isoforms of some of the hornet proteins (72,73); from limited studies, it appears that these result from gene duplication because both isoforms are found in insects from the same nest.

Several of the molecules have been cloned and expressed (78). The mature molecules of yellowjacket and hornet antigen 5 have 201 to 204 amino acids, with several highly conserved regions. Antigen 5 is a member of a conserved family of proteins found in eukaryotes, including yeast (74), pathogenesis-related proteins of plants (74), secretory proteins of helminths produced during sexual maturation (79), reptile venoms (80), and mammalian cysteine-rich secretory proteins in salivary and reproductive

organs (74). Antigen 5-related proteins are also found in glial cell brain tumors (81).

The three-dimensional structure of yellow-jacket antigen 5 has been solved by X-ray crystallography (77). If the sequence variations seen in Hymenoptera antigen 5 are mapped to the three-dimensional structure, almost all of the changes are found on the surface. The highly crossreactive groups within genera have few changes, although occasional patient sera can recognize differences among the subgenera of *Vespula* (76). Antigen 5 from *Dolichovespula* and *Vespa* is not always crossreactive with antigen 5 from other species of hornets or *Vespula*. Antigen 5 from *Polistes* (paper wasp) is significantly less crossreactive with *Vespula* antigen 5. The antigen 5 homologs from fire ants do not exhibit antigenic crossreactivity with those from vespid wasps, a finding consistent with the low degree of surface conservation and changes in loop lengths (76,77). Occasionally, antigenic crossreactivity has been observed between vespid antigen 5 and homologs from other animals (79); however, this has occurred only with hyperimmune sera.

The phospholipases in wasp venoms are in a different superfamily than those in bee venoms. Vespid wasp phospholipases have A₁B specificity and are members of the lipoprotein lipase superfamily, the pancreatic lipase homologous family, and the RP2 subgroup phospholipases (82). Two approaches have been used to purify the venom phospholipases: affinity chromatography followed by cation exchange chromatography (70) or gel filtration and high-performance cation exchange chromatography (69,71). The phospholipases from seven species representing four genera have been sequenced (83–86). The phospholipases from vespid wasp venoms usually do not contain carbohydrate and have highly homologous regions surrounding the active sites. The three-dimensional structures can be modeled using the X-ray structures of mammalian enzymes as templates (87,88). There are two distinct isozymes of vespid phospholipase,

which are best characterized in *Dolichovespula maculata* (85). The differences are primarily in the N-terminal portion of the molecule. Both isozymes are expressed in single insects. Although no other examples of the second isozyme have been fully sequenced, it has been found in *Vespa* and *Polistes* venoms. In many cases, numerous minor sequence variants have also been noted (85,86).

The crossreactivities of the phospholipases generally follow the phylogeny; closely related species are highly crossreactive, and those that are further removed are less crossreactive (89,90). Table 1 demonstrates the calculated evolutionary distances determined from the amino acid sequence alignment of the known phospholipase A₁B sequences. Rabbit antibodies have been produced that distinguish between the two isozymes of *D. maculata* phospholipase (89), but there is no available evidence to indicate that clinically significant differences exist. Crossreactivity is highly variable among vespid-sensitized patients; a significant fraction show high specificity, and most show varying degrees of crossreactivity among vespids. This indicates that there may be various allergenic epitopes—some are species- or genus-specific and others have varying degrees of crossreactivity (90–92).

The hyaluronidases in vespid venoms are similar to bee venom hyaluronidase (93). The enzymes contain significant amounts of N-linked carbohydrate (94). Crossreactivity has been noted between vespid and bee hyaluronidases (95), a large portion of the reactivity is from the crossreactive carbohydrate determinants (6), but some protein crossreactivity exists.

Two additional minor IgE-binding fractions have been purified from yellowjacket venom (71). Vmac 3, the band between hyaluronidase and phospholipase on SDS-PAGE, appears to be a variant form of hyaluronidase based on protein sequencing results. The high-molecular-weight fraction Vmac 1 has not been well characterized but is known to contain significant amounts of carbohydrate.

Table 1
Calculated Evolutionary Distance Matrix of the Amino Acid Sequences for the Hymenoptera Venom Phospholipases A₁B With Known Sequence

	Vesp c 1	Dol m 1.01	Dol m 1.02	Ves m 1	Ves v 1	Ves s 1	Pol d 1.01	Pol a 1	Sol i 1
Vesp c 1	0.00	0.74	1.31	1.00	1.02	1.14	2.06	1.99	4.44
Dol m 1.01	0.74	0.00	1.18	1.07	1.11	1.34	2.03	1.93	4.38
Dol m 1.02	1.31	1.18	0.00	1.56	1.55	1.78	1.85	1.71	4.94
Ves m 1	1.00	1.07	1.56	0.00	0.12	0.94	2.17	2.01	4.14
Ves v 1	1.01	1.11	1.56	0.12	0.00	0.89	2.10	1.95	4.24
Ves s 1	1.15	1.34	1.78	0.94	0.89	0.00	2.11	1.99	4.42
Pol d 1.01	2.06	2.03	1.85	2.17	2.10	2.11	0.00	0.45	4.87
Pol a 1	1.99	1.93	1.71	2.00	1.95	1.99	0.45	0.00	4.82
Sol i 1	4.44	4.38	4.94	4.14	4.24	4.43	4.87	4.82	0.00

Paper Wasps

Paper wasp venoms contain phospholipase, antigen 5, and hyaluronidase (69,75,76, 96–98). These three proteins are similar to those of other vespid wasps. *Polistes* venoms have additional proteins migrating at 76 and 78 kDa and comigrating with the phospholipase in SDS-PAGE (47,69). The sequences of antigen 5 from North American *Polistes* have 1.5 to 8% differences between species (76); those from European species are also closely related to each other (99,100). Antigen 5 and phospholipases show about a 16% difference in amino acids between North American and European *Polistes* species. This correlates with the radioallergosorbent test inhibition results shown in Fig. 6—that is, there are significant differences in reactivity between venoms from the two groups of *Polistes* (101).

The venom protease allergen has been isolated from *Polistes dominulus*, *Polistes gallicus*, and *Polistes exclamans* by affinity chromatography on benzamidine-agarose (47,99). The complete amino acid and nucleotide sequence has been determined for the *P. dominulus* enzyme. The mature molecule is a typical trypsin-like domain of 244 amino acids with six potential N-linked glycosylation sites, four of which appear to be glycosylated. It has significant IgE-binding activity. Patients sensitized by

European species appear to have much more reactivity to venom protease than patients sensitized by North American species (47).

Many patients with allergy to vespid venoms also exhibit reactivity to paper wasp venoms (91). A significant fraction of these patients have crossreactive antibodies induced by yellowjacket venom (102)—especially those residing in the northeastern United States. However, patients residing in the southeast have a much higher probability of experiencing paper wasp stings and typically exhibit multiple reactivity, where yellowjacket venom cannot completely inhibit the binding to paper wasp venom (91,103).

Ant Venoms

Fire Ants

Venoms from fire ants (*Solenopsis*) contain 90 to 95% oily, strongly basic, water-insoluble N-alkyl and alkenyl-piperidines (104). These alkaloids generate the characteristically sterile pustule that develops at the site of imported fire ant stings (105,106). The allergens are proteins found in nanograms in the aqueous portion of the venom (107). There are four major proteins, and each has been characterized and sequenced. The allergens were prepared from venom collected by electrical stimulation as

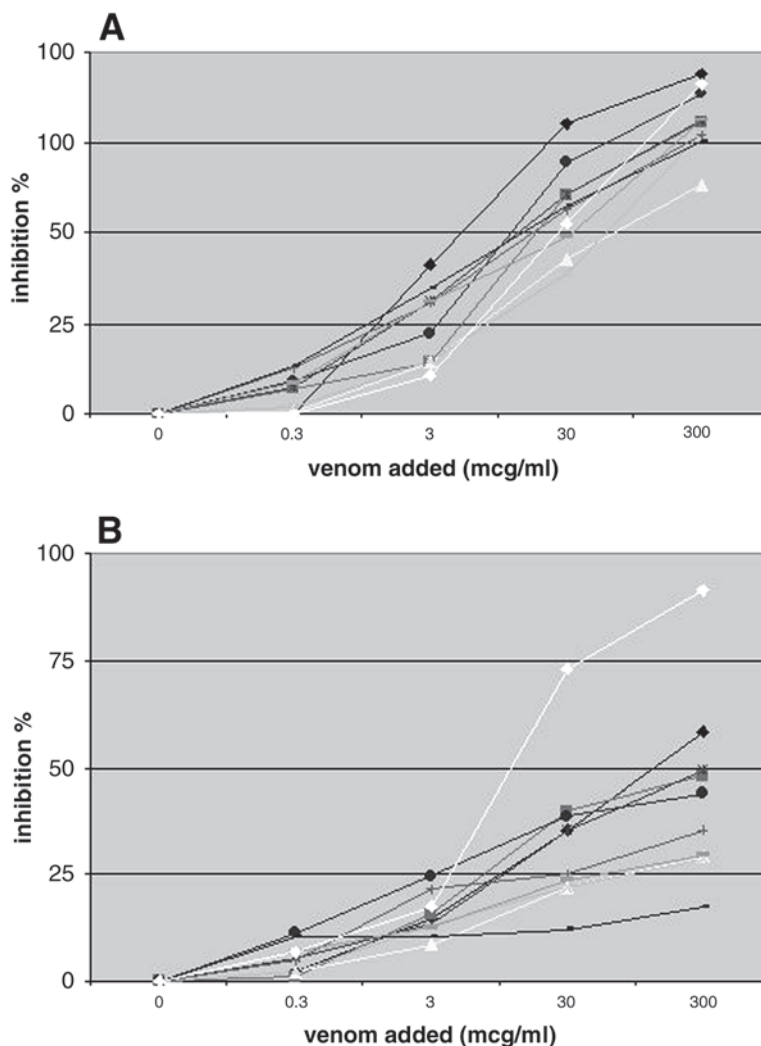


Fig. 6. Radioallergosorbent test inhibition results of binding to *Polistes dominulus* venom by (A) *P. dominulus* venom and by (B) *Polistes exclamans* venom.

well as by laboriously hand-milking (107). The separation was performed by gel filtration on Sephadex G-75 superfine, followed by high-performance ion exchange on a mono S cation exchange column (107). Sol i 1 can be enriched by affinity chromatography on concanavalin A agarose (108), and Sol i 2, 3, and 4 can also be purified by reversed-phase chromatography on a Vydac C4 column.

The allergen with the highest molecular weight, Sol i 1, is a phospholipase A₁B related to those found in vespid wasp venoms that showed about 33 to 38% amino acid identity (108). The region forming the active site domain

of the enzyme is most highly conserved, whereas the outer loops show little similarity. The mature molecule has 309 amino acids with a protein molecular weight of 34.2 kDa. The apparent molecular weight on SDS-PAGE is 37 kDa, and the isoelectric point is 8.51. The molecule contains N-linked carbohydrate attached to at least one of three potential sites. Some patients show crossreactivity between vespid phospholipases and Sol i 1; this crossreactivity can be clinically significant (109). Although a significant portion of this crossreactivity results from the crossreactive carbohydrate determinants, some is caused by protein epitopes.

```
Sol r 2      DIEAQRVLRKDIAECARTLPKCVNQPDPLARVDVWHCAMS KRGVYDNPDP AVVKEKNSK
Sol i 2      DNKELKIIRKDVAECLRTL PKCGNQPDPLARVDVWHCAMA KRGVYDNPDP AVIKERSMK
Sol g 2      DIEKLKILRKDMAECLRTL PKCVNQPDPLVRVDVWHCAMA KGGVFD DSTPA AIKRRHKK
              * :   ::*:** ***** *****_.*****:* ***: . **.:*: *

Sol r 2      MCPKIITDPADVENCKKV SRCVDRETQRPRSNRQKAINITGCILRAGVVEATVLAREK
Sol i 2      MCTKIITDPANVENCKKVASRCVDRETQGPKSNRQKAVNIIGCALRAGVAETT VLARKK
Sol g 2      YCTIAITDPAKVKNC KAVSRCVDKETQRPRSDRQKALNIIACVLRAGVVGTTVLAREK
              *****.***** *****.*****.*****.*****.*****.*****.*****.
```

Sol i 3 is a member of the antigen-5 family (110,111). Sol i 3 has 43 to 50% amino acid sequence identity with vespid antigen 5 (76). Immunological crossreactivity has not been observed between fire ant and vespid wasp antigen 5 (109). Computer modeling of the three-dimensional structure of Sol i 3 using the X-ray structure of Ves v 5 suggests that little of the surface structure is conserved (77). The antigen 5 homologs are highly conserved among *Solenopsis* species (112).

Sol i 2 is the most abundant protein in fire ant venom (107); however, the molecule has relatively low absorbance at 280 nm because it contains only a single tryptophan residue (110). It does not contain carbohydrate. Sol i 2 is not related to any non-fire ant protein in the National Center for Biotechnology Information database, and it has not been possible to find a closely related sequence in the honeybee genome assembly. It is a basic protein of 119 amino acids (110,113) found primarily as disulfide-linked dimers. The IgE-binding activity dependent highly on the native conformation (114), and a fully immunoreactive recombinant protein has been produced in a baculovirus expression system. The amino acid sequence has been determined for the related proteins from *Solenopsis richteri* (112) and *Solenopsis geminata*. These three sequences are compared in Fig. 7; Sol r 2 and Sol g 2 are 78.2 and 72.3% identical to Sol i 2, respectively. The variations are distributed throughout the length of the molecule. A conformation-dependent monoclonal antibody exhibiting species specificity has been pro-

Sol i 4 is structurally related to Sol i 2, with 118 amino acids and no carbohydrate, but it contains one less cysteine-preventing dimerization (107). Although *S. geminata* venom contains a significant amount of a corresponding protein, *S. richteri* venom does not. Sol g 4 has two isoforms that are 97% identical, and these isoforms are 90% identical to Sol i 4.

Despite the sequence differences in the Sol 2 and 4 antigens, IgE antibodies against fire ant venoms are highly crossreactive, and sensitization by any species is a significant risk factor for reactions to stings from other species (112,116). Preliminary studies on the South American species *Solenopsis saevissima* indicated that it also has closely related venom proteins.

Bulldog and Jack Jumper Ants

The primitive ants of the genus *Myrmecia* (bulldog and jack jumper ants) are important causes of anaphylactic sting reactions in Australia (117,118). The venoms of these ants contain phospholipase (119), but the allergens appear to be a complex series of highly basic peptides (120–127). Figure 8 shows the structures of the four known precursor molecules for *Myrmecia* venom allergens. All four of the molecules are similar, and all undergo post-translational processing—probably by the sequential removal of dipeptides (123–127).

Myr p 1

1 MKLSCLLLTL TIIFVLT/IVH APNVEAKDLA DPESEAVGFA DAFGEADAVG EADPNAGLGS
61 VFGRLAR//ILG RVIPKVAKKL GPKVAKVLPK VMKEAIPMAV EMAKSQEEQQ PQ

Myr p 2

1 MKLSCLLLTL AIIFVLT/IVH APNVEAKALA DPESDAVGFA DAVGEADP//ID WKKVDWKKVS
61 KKTCKVMLKA CKFLG

Myr b 3

1 MKLSCLLLTL AIIIVLT/IVH APNVEAKALA DPESDAVGFA DAVGEADPNA// IIGLVSKGTC
61 VLVKTVCKKV LKQG

Myr b 4

1 MKLSCLLLTL AIIFVLT/IVH APNVKAKALA DPESDAVGFA DAVGEADP//FD ITKLNKKLT
61 KATCKVISKG ASMCKVLFDK KKQE

Fig. 8. The precursors of the four known allergens from Australian bulldog and jack jumper ant venoms. The single slash indicates the end of the signal sequences, and the double slash indicates the end of the posttranslational processing. Some of the peptides are found as heterodimers.

The mature peptides show little homology. Additional heterogeneity appears to exist, with as many as 50 peptides present from a single species (126). A major part of the allergenic peptide fractions was found to be a disulfide-linked heterodimer of Myr p 2 residues 49 to 74 and a peptide of unknown sequence with a molecular weight of 2457 Da (126). These peptides possess strong antimicrobial and cytotoxic activities in addition to their potent allergenicity (127). Preliminary studies of sera from patients with fire ant venom and *Myrmecia* venom allergies showed no evidence of crossreactivity.

Harvester Ants

Harvester ants of the genus *Pogonomyrmex* are found in much of North America. Although the ants are common, only occasional allergic reactions to stings have been reported (128). Similarly to fire ants, harvester ants are myrmecine ants; however, they are in different tribes. The

venoms contain phospholipase A and B activities, hyaluronidase, and acid phosphatase (129,130). Crossreactivity with fire ant venom has not been studied.

Ponerine Ants

Venoms of ponerine ants of the genus *Pachycondyla* are important causes of allergic reactions in Asia and the Middle East (117, 131,132). The East Asian ants have also become established in areas of the United States. Immunoblot studies have identified eight IgE-binding bands at 12, 22, 25, 27, 29, 31, 46, and 58 kDa (133,134). The 12-kDa band bound IgE from 85% of sera from allergic individuals. Patients with co-existing allergy to bee venom showed some crossreactivity, but those with only *Pachycondyla* venom allergy did not. Patient sera from Korea showed no crossreactivity to imported fire ant venom (133); however, patients from the Middle East exhibited crossreactivity

to imported fire ant venom in immunoblot studies (135). All 10 sera from patients with allergy to samsum ant (*Pachycondyla sennaarensis*) venom showed binding to the 24-kDa allergen of fire ant venom (Sol i 3), and 3 showed binding to the 13-kDa allergen (Sol i 2 and or 4). It is not known whether this crossreactivity is clinically significant.

Methods for Venom Collection

Honeybee venom is readily available from several commercial sources. A consistent and reliable source of gram amounts of venom has been Champlain Valley Apiaries in Middlebury, VT. Venom is typically collected by using electrical stimulation units, with which the venom is collected on to rubber dental dams and air-dried (136,137). The main contaminant is stinger lancets, although small amounts of fecal contamination and pollen from leg baskets and body hairs can sometimes be found.

Although it is possible to collect vespid venoms in a similar manner, it can be dangerous because yellow jackets in a wide area will react to the release of alarm pheromone and volatile sting substances. Small to moderate amounts of venom can be collected by having live insects sting into small tubes or through membranes (70). For safety, it is recommended that insects be chilled and pinned and handled with the pins while stinging. Hornets may spray venom, and one must also be careful to avoid fecal contamination during collection. Feces are usually yellow or green. Commercial vespid venoms are collected by removing the venom sacs from fresh frozen insects and squeezing out the venom into buffer solution. The sacs should not be crushed or minced beyond the minimum needed to express the venom. Although these preparations do contain some body proteins, they are predominantly venom (138,139).

Fire ant venom can be laboriously collected by having the insects sting into capillary tubes (108). Prototype commercial fire ant venom

was prepared by a proprietary modified electrical stimulation technique.

Purification of Venom Allergens

Venom allergens have been purified using a variety of methods. Usually the first separation step was gel filtration on dextran-based materials including Sephadex G-50, G-75, and G-100. The fractions were then purified using cation-exchange chromatography either on carbohydrate-based or plastic-based resins in conventional or high-performance systems. These methods are described for bee venoms (10,11,33,65), vespid venoms (69,70,72,85,97), and ant venoms (108,124). In selected cases, affinity chromatography methods have been employed: benzamidine-agarose for proteases (47,100), phosphatidylcholine analogs for phospholipases (71,97), and immobilized heparin and concanavalin A for isolation of glycoproteins (71). Reversed-phase high-performance chromatography has been used by several investigators (43,100,115).

High-performance gel filtration materials based on silica, agarose, or polyacrylamide all interacted with materials including both proteins and peptides in the venoms, preventing separation based on size. Chromatograms of *V. maculifrons* antigen 5 and phospholipase on a TSK2000SW column are shown in Fig. 9. No significant separation was observed. High salt concentrations reduced, but did not eliminate, these interactions. The separation of *V. maculifrons* venom on a Superdex 75 column run in 0.5 M NaCl is shown in Fig. 10. An SDS-PAGE gel of the fractions showing smearing of the phospholipase beyond the elution position of antigen 5 is shown in Fig. 11. A 1.7-m column of Sephadex G50F gave the best results in acidic buffer containing 0.15 M NaCl. Sephadex G75 superfine columns were extremely difficult to use with venoms because of compression and plugging. Excellent cation-exchange separations were obtained with Pharmacia Mono S and PolyLC polysulfoethyl A columns. Other

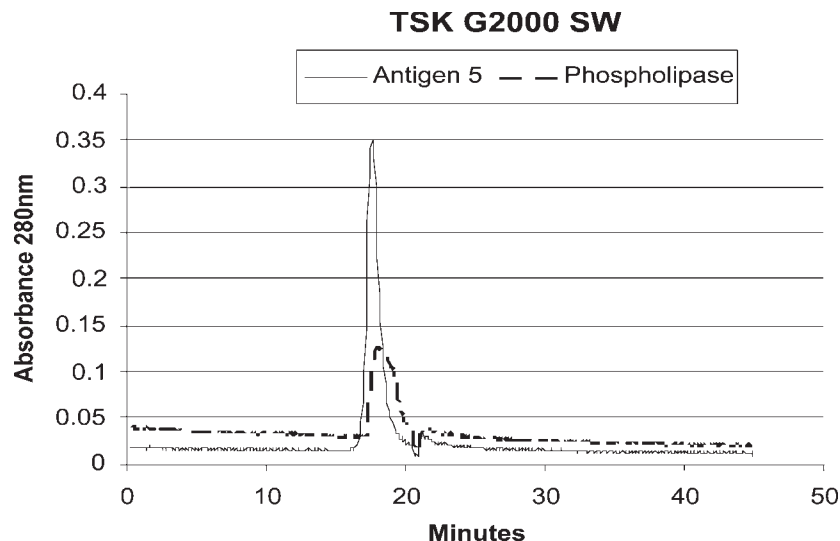


Fig. 9. Chromatograms of *Vespula maculifrons* antigen 5 and phospholipase on a column of TSK2000sw in 0.15 M NaCl and 0.05 M sodium acetate, pH 4.75.

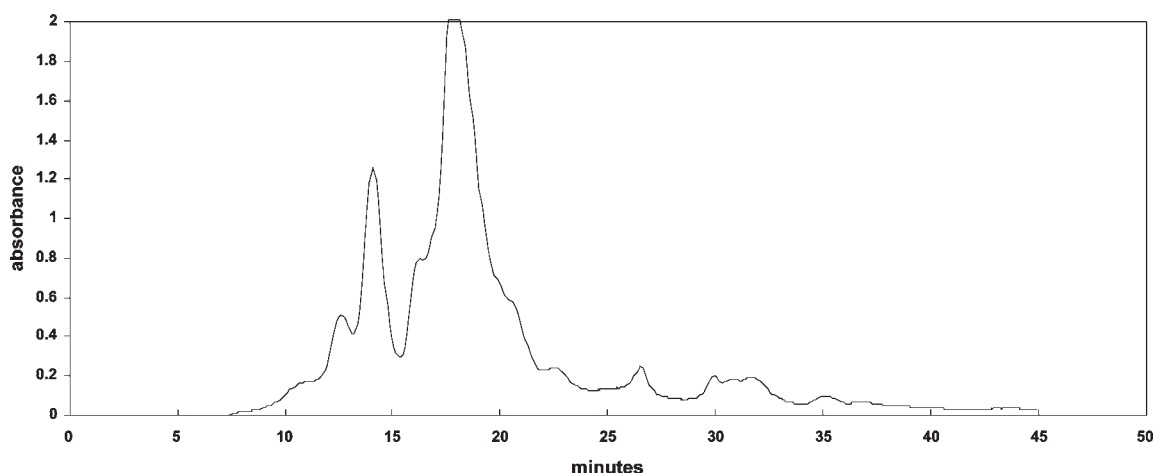


Fig. 10. Chromatogram of *Vespula maculifrons* venom on a Superdex 75 column in 0.5 M NaCl, 0.02 M sodium acetate, pH 5.2. One-minute fractions were collected.

silica-based cation exchangers did not work as well. The use of traditional reversed-phase columns caused varying degrees of denaturation. Perfusion chromatography columns Poros R from Applied Bioscience with the separation shown in Fig. 12 and TSK Gel SuperODS from TosoHaas (115) gave good separations with preservation of immunoreactivity.

Many high-performance gel filtration columns are unsuitable for use with venom proteins; Sephadex remains the media of choice.

Excellent cation-exchange columns and perfusion chromatography columns are readily available.

Recombinant Hymenoptera Venom Allergens

There have been two recent reviews of recombinant Hymenoptera venom allergens (140,141). As mentioned earlier, recombinant honeybee phospholipase (16–19), hyaluronidase (34), and melittin have been studied. Of the

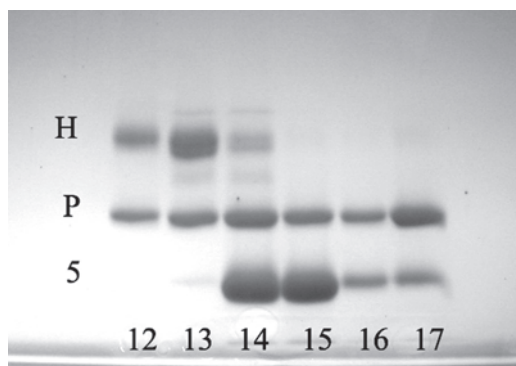


Fig. 11. SDS-PAGE of fractions from the separation shown in Fig. 10. Note the presence of phospholipase in fractions 12-17, and that significant amounts of phospholipase elute after the antigen 5. H, hyaluronidase; P, phospholipase; 5, antigen 5.

vespid allergens, only fully immunoreactive antigen 5 has been produced (78). Of the four imported fire ant allergens, Sol i 2, 3, and 4 have all been produced in immunoreactive form (111,114,142). Some interesting studies of engineered recombinant allergens include the potential use of conformation-changed phospholipase (65) and hybrid molecules of antigen 5 containing yellowjacket and paper wasp regions (97). The immunoreactive recombi-

nants have been produced in baculovirus or yeast expression systems, or in the case of honeybee venom phospholipase, they have been subjected to extensive renaturation procedures. The use of other contemporary techniques for production of recombinant Hymenoptera venom molecules in native conformation has not been reported.

Systematic Nomenclature of Venom Allergens

The venom allergens have been assigned official names by the International Union of Immunological Studies Allergen Nomenclature Subcommittee (143). Honeybee proteins are assigned numbers as follows: 1 to phospholipase A_2 , 2 to hyaluronidase, 3 to acid phosphatase, 4 to melittin, 6 to Api m 6, and 7 to the CUB protease. The number 5 is not used for bees. Other bees and bumblebee proteins use the same numbers as honeybee, with the exception of 4, which is assigned to the venom serine proteases of bumblebees. Vespid venom proteins, including paper wasps, are assigned 1 to phospholipase A_1B , 2 to hyaluronidase, 4 to venom protease, and 5 to antigen 5. Fire ant

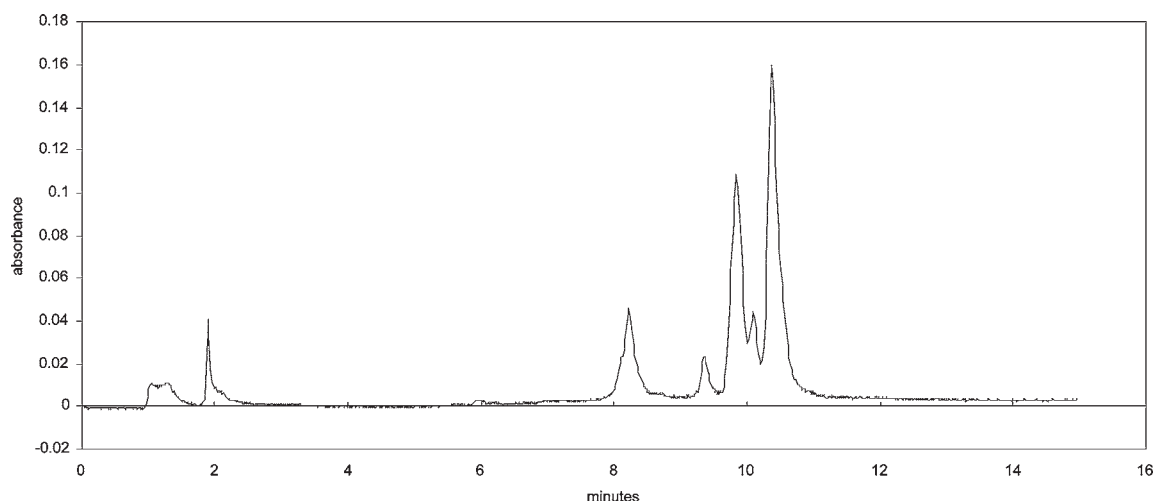


Fig. 12. Perfusion chromatography of a *Vespula maculifrons* venom fraction on a Poros R column. The gradient went from 80% 0.1% trifluoroacetic acid in water to 80% 0.07% trifluoroacetic acid in acetonitrile at 50°C. The peak at about 8 min is antigen 5, the small peak is Vmac 3, followed by hyaluronidase and phospholipase. The hyaluronidase and phospholipase are not completely separated.

venom proteins are assigned 1 to phospholipase A₁, 2 to Sol i 2 homologous proteins, 3 to antigen 5-related proteins, and 4 to Sol i 4 homologous proteins. *Myrmecia* ant venom proteins have been assigned numbers 1 to 4, but recent studies have made it clear that there are numerous forms of the proteins with partial posttranslational processing and that much of the allergenic activity may reside in disulfide-linked heterodimers (126). The nomenclature for the *Myrmecia* proteins needs to be revisited after more thorough investigation of the actual molecular species found in the venoms.

References

- Nakajima, T. (1986), in *Venoms of the Hymenoptera*, Piek T., ed., London: Academic Press, pp. 309–327.
- Banks, B. E. C. and Shipolini, R.A. (1986), in *Venoms of the Hymenoptera*, Piek T., ed., London: Academic Press, pp. 330–416.
- Schmidt, J. O. (1986), in *Venoms of the Hymenoptera*, Piek T., ed., London: Academic Press, pp. 425–508.
- Brand, J. M., Blum, M. S., Fales, H. M., and Mac Connell, J. G. (1972), *Toxicon* **10**, 259–71.
- Schmidt, M., Weimer, E. T., Sakell, R. H., and Hoffman, D. R. (2005), *J Allergy Clin Immunol* **115**, S107.
- Hemmer, W., Focke, M., Kolarich, D., et al., (2001), *J Allergy Clin Immunol* **108**, 1045–1052.
- King, T. P. and Spangfort, M. D. (2000), *Int Arch Allergy Immunol* **123**, 99–106.
- Hoffman, D. R. (2003), in *Monograph on Insect Allergy*, fourth edition, Levine, M. I. and Lockey, R. F. eds., Milwaukee, WI: American Academy of Allergy Asthma and Immunology pp. 37–53.
- King, T. P. and Guralnick M. (2004), *Clin Allergy Immunol* **18**, 339–353.
- King, T. P., Sobotka, A. K., Kochoumian, L., and Lichtenstein, L. M. (1976), *Arch Biochem Biophys* **172**, 661–671.
- Hoffman, D. R. and Shipman, W. H., (1976), *J Allergy Clin Immunol* **58**, 551–562.
- Kuchler, K., Gmachl, M., Sippl, M. J., and Kreil, G., (1989), *Eur J Biochem* **184**, 249–254.
- Kubelka, V., Altmann, F., Staudacher, E., et al., (1993), *Eur J Biochem* **213**, 1193–1204.
- Scott, D. L., Otwinowski, Z., Gelb, M. H., and Sigler, P. B. (1990), *Science* **250**, 1563–1566.
- Hoffman, D. R. (1987), *J Allergy Clin Immunol* **80**, 307–313.
- Muller, U. R., Dudler, T., Schneider, T., et al. (1995), *J Allergy Clin Immunol* **96**, 395–402.
- Dudler, T., Chen, W. Q., Wang, S., et al., (1992), *Biochim Biophys Acta* **1165**, 201–210.
- Forster, E., Dudler, T., Gmachl, M., et al. (1995), *J Allergy Clin Immunol* **95**, 1229–1235.
- Muller, U., Fricker, M., Wymann, D., et al. (1997), *Clin Exp Allergy* **27**, 915–920.
- Schneider, T., Lang, A.B., Carballido, J. M., et al. (1994), *J Allergy Clin Immunol* **94**, 61–70.
- Dhillon, M., Roberts, C. Nunn, T. and Kuo, M. (1992), *J Allergy Clin Immunol* **90**, 42–51.
- Carballido, J. M., Carballido-Perrig, N., Terres, G., et al. (1992), *Eur J Immunol* **22**, 1357–1363.
- Carballido, J. M., Carballido-Perrig, N., Kagi, M. K., et al. (1993), *J Immunol* **150**, 3582–3591.
- Kammerer, R., Kettner, A., Chvatchko, Y., et al. (1997), *Clin Exp Allergy* **27**, 1016–1026.
- Carballido, J. M., Carballido-Perrig, N., Oberli-Schrammli, A., et al. (1994), *J Allergy Clin Immunol* **93**, 758–767.
- Kammerer, R., Chvatchko, Y., Kettner, A., et al. (1997), *J Allergy Clin Immunol* **100**, 96–103.
- Muller, U., Akdis, C. A., Fricker, M., et al. (1998), *J Allergy Clin Immunol* **101**, 747–754.
- Fellrath, J. -M., Kettner, A., Dufour, N., et al. (2003), *J Allergy Clin Immunol* **111**, 854–861.
- Hoffman, D. R. and Schmidt, J. O. (2000), *J Allergy Clin Immunol* **105**, S56.
- Guex, N. and Peitsch, M. C. (1997), *Electrophoresis* **18**, 2714–2723.
- Gmachl, M. and Kreil, G. (1993), *Proc Nat Acad Sci USA* **90**, 3569–3573.
- Henrissat, B. and Bairoch, A. (1996), *Biochem J* **316**, 695–696.
- Kemeny, D. M., Dalton, N., Lawrence, A. J., et al. (1984), *Eur J Biochem* **139**, 217–223.
- Soldatova, L. N., Cramer, R., Gmachl, M., et al. (1998), *J Allergy Clin Immunol* **101**, 691–698.
- Markovic-Housley, Z., Miglierini, G., Soldatova, L., et al. (2000), *Structure Fold Des* **8**, 1025–1035.
- Habermann, E. and Jentsch, J. (1967), *Hoppe-Seyler Z Physiol Chem* **348**, 37–52.
- Kreil, G., Haiml, L., and Suchanek, G. (1980), *Eur J Biochem* **111**, 49–58.
- Terwilliger, T. C., Weissman, L., and Eisenberg, D. (1982), *Biophys J* **37**, 353–361.
- Habermann, E. (1972), *Science* **177**, 314–322.
- Prince, R. C., Gunson, D. E., and Scarpa, A. (1985), *Trends Biochem Sci* **10**, 99.
- Mackler, B. F. and Kreil, G. (1977), *Inflammation* **2**, 55–65.
- Paull, B. R., Yunginger, J. W., and Gleich, G. J. (1977), *J Allergy Clin Immunol* **59**, 334–338.
- Kettner, A., Hughes, G. J., Frutiger, S., et al. (2001), *J Allergy Clin Immunol* **107**, 914–920.
- Jeep, S., Paul, M., Muller, U., and Kunkel, G. (1996), *Allergy* **51**, 540–546.
- Kettner, A., Henry, H., Hughes, G. J., et al. (1999), *Clin Exp Allergy* **129**, 394–401.
- Wood, C. L. and Hoffman, D. R. (1983), *Toxicon* **21**, 291–299.

47. Winningham, K. M., Fitch, C. D., Schmidt, M., and Hoffman, D. R. (2004), *J Allergy Clin Immunol* **114**, 928–933.
48. Bork, P. and Beckmann, G. (1993), *J Mol Biol* **231**, 539–545.
49. Hoffman, D. R. (1977), *J Allergy Clin Immunol* **59**, 364–366.
50. Barboni, E., Kemeny, D. M., Campos, S., and Vernon, C. A. (1987), *Toxicon* **25**, 1097–1103.
51. Hoffman, D. R., Shipman, W. H., and Babin, D. (1977), *J Allergy Clin Immunol* **59**, 147–153.
52. Hoffman, D. R., Weimer, E. T., Sakell, R. H., and Schmidt, M. (2005), *J Allergy Clin Immunol* **115**, S107.
53. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999), *Electrophoresis* **20**, 3551–3567.
54. Roberts-Thomson, P. J., Koh, S., Shepherd, K., et al. (1991), *Asian Pac J Allergy Immunol* **9**, 131–136.
55. Nordvall, S., Uhlin, T., Ohman, S., et al. (1986), *Allergy* **41**, 89–94.
56. Danty, E., Arnold, G., Burmeister, T., et al. (1998), *Insect Biochem Molec Biol* **28**, 387–397.
57. Hemmer, W., Focke, M., Kolarich, D., et al. (2004), *Clin Exp Allergy* **34**, 460–469.
58. Hoffman, D. R., El-Choufani, S. E., Smith, M. M., and deGroot, H. (2001), *J Allergy Clin Immunol* **108**, 855–860.
59. Kochuyt, A. M. van Hoeyveld, E., and Stevens, E. A. M. (1993), *Clin Exp Allergy* **23**, 190–195.
60. de Groot, H., de Graaf-in 't Veld, C., and Gerth van Wijk, R. (1995), *Allergy* **50**, 581–584.
61. Stern, A., Wuthrich, B., and Mullner, G. (2000), *Allergy* **55**, 88–91.
62. Stapel, S. O., Waanders-Lijster de Raadt, J., van Toorenenbergen, A. W., de Groot, H. (1998), *Allergy* **53**, 769–777.
63. de Jong, N. W., Vermeulen, A. M., and de Groot, H. (1999), *Allergy* **54**, 980–984.
64. Hoffman, D. R. and Jacobson, R. S. (1996), *J Allergy Clin Immunol* **97**, 812–821.
65. Buhot, C., Chenal, A., Sanson, A. et al. (2004), *Protein Science* **13**, 2970–2978.
66. Argiolas, A. and Pisano, J. J. (1985), *J Biol Chem* **260**, 1437–1444.
67. Pence, H. L., White, A. F., Cost, K. et al. (1991), *Ann Allergy* **66**, 399–404.
68. King, T. P., Sobotka, A. K., Alagon, A., et al. (1978), *Biochemistry* **17**, 5165–5177.
69. Hoffman, D. R. (1985), *J Allergy Clin Immunol* **75**, 599–605.
70. King, T. P., Alagon, A. C., Kuan, J., et al. (1983), *Mol Immunol* **20**, 297–308.
71. Hoffman, D. R. and Wood, C. L. (1984), *J Allergy Clin Immunol* **74**, 93–103.
72. Hoffman, D. R., Jacobson, R. S., and Zerboni, R. (1987), *Int Archs Allergy Appl Immun* **84**, 25–31.
73. Fang, K. S. Y., Vitale, M., Fehlner, P., and King, T. P. (1988), *Proc Natl Acad Sci USA* **85**, 895–899.
74. King, T. P., Moran, D., Wang, D., et al. (1990), *Protein Seq Data Anal* **3**, 263–266.
75. Lu, G., Villalba, M., Coscia, M. R., et al. (1993), *J Immunol* **150**, 2823–2830.
76. Hoffman, D. R. (1993), *J Allergy Clin Immunol* **92**, 707–716.
77. Henriksen, A., King, T. P., Mirza, O., et al. (2001), *Proteins Struct Func Genet* **45**, 438–448.
78. Monsalve, R. I., Lu, G., and King, T. P. (1999), *Protein Expr Purif* **16**, 410–416.
79. Hawdon, J. M., Jones, B. F., Hoffman, D. R., and Hotez, P. J. (1996), *J Biol Chem* **271**, 6672–6678.
80. Morrisette, J., Kratzschmar, J., Haendler, B., et al. (1995), *Biophys J* **68**, 2280–2288.
81. Rich, T., Chen, P., Furman, F., et al. (1996), *Gene* **180**, 125–130.
82. Fischer, M. and Pleiss, J. (2003), *Nucl Acid Res* **31**, 319–321.
83. Soldatova, L., Kochoumian, L., and King, T. P. (1993), *FEBS Lett* **320**, 145–149.
84. King, T. P., Lu, G., Gonzalez, M., et al. (1996), *J Allergy Clin Immunol* **98**, 588–600.
85. Hoffman, D. R. (1994), *Int Arch Allergy Immunol* **104**, 184–190.
86. Moawad, T. I., Hoffman, D. R., and Zalat, S. (2005), *Acta Biologica Hungarica* **56**, 261–274.
87. Lombardo, D., Chapus, C., Bourne, Y., and Cambillau, C. (1989), *J Mol Biol* **205**, 259–261.
88. Hjorth, A., Carriere, F., Cudrey, C., et al. (1993), *Biochemistry* **32**, 4702–4707.
89. Hoffman, D. R. (1986), *J Allergy Clin Immunol* **78**, 337–343.
90. Hoffman, D. R. (1985), *J Allergy Clin Immunol* **75**, 611–613.
91. Hoffman, D. R. (1981), *Ann Allergy* **46**, 304–309.
92. Hoffman, D. R. and McDonald, C. A. (1982), *Ann Allergy* **48**, 78–81.
93. Lu, G., Kochoumian, L., and King, T. P. (1995), *J Biol Chem* **270**, 4457–4465.
94. Kubelka, V., Altmann, F., and Marz, L. (1995), *Glycoconj J* **12**, 77–83.
95. Jacobson, R. S., Hoffman, D. R., and Kemeny, D. M. (1992), *J Allergy Clin Immunol* **89**, 292.
96. King, T. P., Kochoumian, L., and Joslyn, A. (1984), *Arch Biochem Biophys* **230**, 1–12.
97. King, T. P., Jim, S. Y., Monsalve, R. I., et al. (2001), *J Immunol* **166**, 6057–6065.
98. Monsalve, R. I., Lu, G., and King, T. P. (1999), *Protein Expr Purif* **16**, 410–416.
99. Pantera, B., Hoffman, D. R., Carresi, L., et al. (2003), *Biochim Biophys Acta* **1623**, 72–81.
100. Hoffman, D. R. (1997), *J Allergy Clin Immunol* **99**, S377.
101. Hoffman, D. R., Jacobson, R., and Blanca, M. (1990), *J Allergy Clin Immunol* **85**, 211.
102. Hamilton, R. G., Wisenauer, J. A., Golden, D. B., et al. (1993), *J Allergy Clin Immunol* **92**, 651–659.
103. Hoffman, D. R., Miller, J. S., and Sutton, J. L. (1980), *Ann Allergy* **45**, 276–279.

104. MacConnell, J. G., Williams, R. N., Brand, J. M., and Blum, M. S. (1974), *Ann Entomol Soc Am* **167**, 134–135.
105. deShazo, R. D., Griffing, C., Kwan, T. H., et al. (1984), *J Allergy Clin Immunol* **74**, 841–847.
106. Javors, M. A., Zhou, W., Maas, J. W. Jr., et al. (1993), *Life Sci* **53**, 1105–1112.
107. Hoffman, D. R., Dove, D. E., and Jacobson, R. S. (1988), *J Allergy Clin Immunol* **82**, 818–827.
108. Hoffman, D. R., Sakell, R. H., and Schmidt, M. (2005), *J Allergy Clin Immunol* **115**, 611–616.
109. Hoffman, D. R., Dove, D. E., Moffitt, J. E., and Stafford, C. T. (1988), *J Allergy Clin Immunol* **82**, 828–834.
110. Hoffman, D. R. (1993), *J Allergy Clin Immunol* **91**, 71–78.
111. Schmidt, M., McConnell, T. J., and Hoffman, D. R. (2003), *Allergy* **58**, 342–349.
112. Hoffman, D. R. (1997), *J Allergy Clin Immunol* **100**, 679–683.
113. Schmidt, M., Walker, R. B., Hoffman, D. R., and McConnell, T. J. (1993), *FEBS Lett* **319**, 138–140.
114. Schmidt, M., McConnell, T. J., and Hoffman, D. R. (1996), *J Allergy Clin Immunol* **98**, 82–88.
115. Hoffman, D. R., Jacobson, R. S., Schmidt, M., and Smith, A. M. (1991), *Ann Allergy* **66**, 29–31.
116. Hoffman, D. R., Smith, A. M., Schmidt, M., et al. (1990), *J Allergy Clin Immunol* **85**, 988–996.
117. Shek, L. P., Ngiam, N. S., and Lee, B. W. (2004), *Curr Opin Allergy Clin Immunol* **4**, 325–328.
118. Street, M. D., Donovan, G. R., Baldo, B. A., and Sutherland, S. (1994), *Clin Exp Allergy* **24**, 90–97.
119. Lewis, J. L., Day, A. J., and deLalande, I. S. (1968), *Toxicon* **6**, 109–112.
120. Ford, S. A., Baldo, B. A., Weiner, J., and Sutherland, S. (1991), *Clin Exp Allergy* **21**, 167–171.
121. Donovan, G. R., Baldo, B. A., and Sutherland, S. (1993), *Biochim Biophys Acta* **1171**, 272–280.
122. Donovan, G. R., Street, M. D., Baldo, B. A., et al. (1994), *Biochim Biophys Acta* **1204**, 48–52.
123. Donovan, G. R., Street, M. D., and Baldo, B. A. (1995), *Electrophoresis* **16**, 804–810.
124. Street, M. D., Donovan, G. R., and Baldo, B. A. (1996), *Biochim Biophys Acta* **1305**, 87–97.
125. Donovan, G. R., Street, M. D., Tetaz, T., et al. (1996), *Biochem Mol Biol Int* **39**, 877–885.
126. Davies, N. W., Wiese, M. D., and Brown, S. G. (2004), *Toxicon* **43**, 173–83.
127. Inagaki, H., Akagi, M., Imai, H. T., et al. (2004), *Arch Biochem Biophys* **428**, 170–178.
128. Pinnas, J. L., Strunk, R. C., Wang, T. M., and Thompson, H. C. (1977), *J Allergy Clin Immunol* **59**, 10–16.
129. Schmidt, J. O. and Blum, M. S. (1978), *Science* **200**, 1064–1066.
130. Schmidt, J. O. and Blum, M. S. (1978), *Comp Biochem Physiol* **61C**, 239–247.
131. Dib, G., Guerin, B., Banks, W. A., and Leynadier, F. (1995), *J Allergy Clin Immunol* **96**, 465–472.
132. Cho, Y. S., Lee, Y. M., Lee, C. K., et al. (2002), *J Allergy Clin Immunol* **110**, 54–57.
133. Yun, Y. Y., Ko, S. H., Park, J. W., and Hong, C. S. (1999), *J Allergy Clin Immunol* **104**, 879–882.
134. Kim, S. S., Park, H. S., Kim, H. Y., et al. (2001), *J Allergy Clin Immunol* **107**, 1095–1099.
135. Brummer-Korvenkontio, H., Saarinen, K., Rasanen, L., et al. (2005), *J Allergy Clin Immunol* **115**, S108.
136. Benton, A. W. Morse, R. A. and Stuart, J. D. (1963), *Science* **142**, 228.
137. Morse, R. A. and Benton, A. W. (1964), *Bee World* **45**, 141–143.
138. Wood, C. L. Timmons, B. E. 4th, and Hoffman, D. R. (1983), *Annals Allergy* **51**, 441–445.
139. Mueller, U. Reisman, R. E. Wypych, J., et al. (1981), *J Allergy Clin Immunol* **68**, 254–261.
140. Muller, U. R. (2002), *Allergy* **57**, 570–576.
141. Schmidt, M. and Hoffman, D. R. (2002), *Int Arch Allergy Immunol* **128**, 264–270.
142. Schmidt, M. and Hoffman, D. R. (2001), *J Allergy Clin Immunol* **107**, S112–S113.
143. King, T. P., Hoffman, D. R., Lowenstein, H., et al. (1994), *Bull WHO* **72**, 797–806.