

Api m 6: A new bee venom allergen

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Background: Characterization of the primary structure of allergens is a prerequisite for the design of new diagnostic and therapeutic tools for allergic diseases.

Objective: The purpose of this study was the identification and characterization of a low-molecular-weight, IgE-binding, bee venom (BV) allergen.

Methods: BV proteins were separated by using size exclusion chromatography and HPLC. IgE antibody binding to purified proteins was analyzed by means of immunoblotting, and T-cell response was analyzed by means of proliferation assay. Amino acid sequence was determined with 2 approaches, namely Edman degradation and carboxy terminal analysis with mass spectrometry.

Results: Api m 6, which migrated as an 8-kd band in SDS-PAGE, was frequently (42%) recognized by IgE from BV-hypersensitive patients. In addition, PBMCs from BV-hypersensitive patients, as well as from a normal control subject, proliferated in response to this allergen. Api m 6 exists as 4 isoforms of 7190, 7400, 7598, and 7808 d, respectively. Amino acid sequences obtained from HPLC-purified preparations revealed that the isoforms were constituted of a common central core of 67 residues, only differing in the amino- and carboxy-terminal ends. Api m 6 showed no significant sequence homology with known proteins.

Conclusions: We have identified and sequenced a new BV allergen that elicits a strong IgE and T-cell response in a large number of BV-hypersensitive patients. Api m 6 should be considered in the diagnostic and therapeutic approach of BV immunotherapy on the basis of peptides or recombinant proteins. (*J Allergy Clin Immunol* 2001;107:914-20.)

Key words: Api m 6, IgE, bee venom, Hymenoptera, allergens, T cells, immunotherapy, matrix-assisted laser desorption-ionization time of flight mass spectrometry, carboxypeptidase

Because stings from honeybees can cause severe allergic reactions in susceptible individuals, much effort has been put into the purification and characterization of bee venom (BV) components. BV is a complex mixture of glycoproteins, most of them enzymes, and a series of toxic polypeptides.¹ IgE antibodies from BV-hypersensi-

Abbreviations used

BV: Bee venom
DTT: Dithiothreitol
MALDI-TOF: Matrix-assisted laser desorption-ionization time of flight
MW: Molecular weight
PLA₂: Phospholipase A₂
RT: Room temperature
SI: Stimulation index
VIT: Venom immunotherapy

tive patients recognize several of these proteins. Major allergens (recognized by IgE in more than 50% of patients) include phospholipase A₂ (PLA₂), acid phosphatase, hyaluronidase, and allergen C, as well as several proteins of high molecular weights (MWs).²⁻⁷ With the exception of melittin, small polypeptides (<3 kd) do not bind IgE antibodies. Although melittin is the major BV component (50% of dry weight), only a minority of patients have specific IgE antibodies for this polypeptide.⁸ Furthermore, BV contains less-abundant proteins with MWs ranging from 3 to 12 kd, such as adolapin, minimine, and a protease inhibitor.⁹⁻¹¹ These latter molecules have been purified and characterized, but no sequence data are available. None of them was reported to bind to IgE and thus may have no clinical relevance in the allergic reaction.

Standardized BV preparation administered during venom immunotherapy (VIT) provides protection in about 85% to 90% of patients.¹² These preparations contain the allergens in their native form and expose patients to the small risk of anaphylaxis. As a consequence, intensive effort is made to work out strategies for safer specific immunotherapy. Recombinant allergens or synthetic peptides that minimized IgE binding are promising candidates for such approaches.¹³⁻¹⁶ The development of an allergen vaccine consisting of synthetic or recombinant allergens needs, as a prerequisite, complete structural information about the most allergenic proteins.

We here report the characterization and sequence of a new BV allergen, Api m 6. Api m 6 was recognized by IgE antibodies in greater than 40% of BV-hypersensitive patients. This allergen exists as 4 isoforms, Api m 6.01 to 6.04, with MWs of 7190, 7400, 7598, and 7808 d, respectively. No significant homologies to proteins in EMBL, SWISS-PROT, and PDB databases were found. Amino acids up to residue 35 were identical to peptide H3, which was previously purified from BV but not further investigated.¹⁷ However, our results established a clear role for

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Supported by a research grant from Common Program in Medical Research CHUV/EPFL/UNIL.

Received for publication June 12, 2000; revised December 4, 2000; accepted for publication December 26, 2000.

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0091-6749/2001 \$35.00 + 0 1/87/113867
doi:10.1067/mai.2001.113867

this new allergen in BV allergy. Api m 6 should be added to the list of BV allergens to be considered in synthetic preparations for diagnosis and immunotherapy.

METHODS

Patients

Sera and PBMCs were obtained from BV-hypersensitive patients (grade II-IV according to the Mueller¹⁸ classification). All had BV-specific IgE (≥ 0.35 kU/L; CAP System; Pharmacia, Uppsala, Sweden) and positive intradermal skin test results (>0.1 μ g/mL; Pharmalgen; ALK, Hørsholm, Denmark). The research protocol was accepted by the hospital review board. All patients gave written informed consent to the study.

Size exclusion gel chromatography and reverse-phase HPLC

Lyophilized whole BV (*Apis mellifera*; Latoxan, Rosans, France) was dissolved in 50% formic acid at 50 mg/mL. Particles were removed by means of centrifugation and filtration before sample application to a BioRad P-60 column (2.5 $\times$ 100 cm; BioRad, Glattbrugg, Switzerland) equilibrated in 50% formic acid (250 mg was loaded). Acidic conditions were used to minimize melittin tetramer formation.¹⁹ Fractions of 4 mL were collected at a flow rate of 6.5 mL/h. Each fraction was lyophilized, dissolved in 0.02 N acetic acid, and analyzed with SDS-PAGE.²⁰ BV proteins were further purified by means of reverse-phase HPLC by using a C₄ column (250 $\times$ 46 mm; Phenomenex W-Porex 5, Rancho Palos Verdes, Calif). Water-acetonitrile gradient was used for separation (buffer A, water/acetonitrile 90:10 [vol/vol] and 0.1% trifluoroacetic acid; buffer B, water/acetonitrile 10:90 [vol/vol] and 0.1% trifluoroacetic acid).

Amino acid sequence analysis by automated Edman degradation

N-terminal sequence analysis of proteins and proteolytic fragments was carried out with a pulse-liquid phase microsequencer (model 477A; Applied Biosystems, Foster City, Calif) by using standard programs. Proteins were reduced (8 mol/L urea, 0.15 mol/L TRIS-HCl, and 2.5 mmol/L 1,4-dithiothreitol [DTT], pH 8.6) and alkylated (7.5 mmol/L sodium iodoacetate) before desalting on a C₈ reversed-phase column. Alkylated Api m 6.01 and 6.03 protein was incubated with trypsin (sequencing grade; Boehringer Mannheim AG, Rotkreuz, Switzerland) overnight at room temperature (RT; 50 mmol/L TRIS-HCl, pH 8.6); fragments were separated by means of HPLC (C₈ column 5 μ m HAILsil, 2.1 $\times$ 100 mm; Higgins Analytical Inc, Mountain View, Calif).

Sequence analysis and enzymatic digestion

C-terminal sequence analysis of proteins and proteolytic fragments was performed by using matrix-assisted laser desorption-ionization time of flight (MALDI-TOF) mass spectrometry (model voyager-DE RP; PerSeptive Biosystems, Framingham, Mass).²¹ V8 proteinase (endoproteinase Glu-C) was purchased from Promega (Zürich, Switzerland) and used according to the manufacturer's instructions. Endoproteinase Arg-C sequencing grade, endoproteinase Asp-N sequencing grade, carboxypeptidase Y sequencing grade, and carboxypeptidase A were purchased from Boehringer Mannheim AG (Rotkreuz, Switzerland). Enzymatic fragmentation with Arg-C was carried out at either RT or 4°C in 15 mmol/L HEPES buffer (pH 8) with 10 mmol/L DTT. The enzyme/protein ratio was 1:50 (wt/wt). Reaction was stopped by adding matrix solution (saturated solution of sinapinic acid, 10 mg/mL, in acetonitrile/water 30:70 [vol/vol]). Fragments from Asp-N digestion (enzyme/protein ratio of 1:125 [wt/wt] in 15 mmol/L ammonium

acetate buffer, pH 6.5) of reduced proteins (2 mmol/L DTT in water at 37°C overnight) were separated by means of HPLC for further analysis. Determination of free SH groups of cysteines was done by using incubation with N-ethylmaleimide. C-terminal amino acids were determined by using incubation of proteins with carboxypeptidase A (enzyme/protein ratio of 1:10-1:100 [wt/wt] in 15 mmol/L HEPES buffer, pH 7.5) or carboxypeptidase Y (enzyme/protein ratio of 1:10-1:100 [wt/wt] in 15 mmol/L ammonium acetate, pH 6) at 4°C or RT. Experimental conditions had to be optimized for each substrate preparation. Adding matrix solution stopped the reaction.

Database searches

EMBL and SWISS-PROT database searches for protein sequence homology and computer-assisted protein analysis were done with the Wisconsin Package Version 9.1 software (Genetics Computer Group, Madison, Wis).

Immunoblot analysis

Proteins (250 μ g) were separated by using SDS-PAGE (15%) under nonreducing conditions and blotted to PVDF membranes in CAPS/methanol buffer (10 mmol/L 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS) and 10% methanol, pH 11). Membranes were blocked with nonfat milk (5%) in PBS solution containing 0.1% Tween-20 (PBS-Tween) and then incubated with patient sera (1:10 in PBS-Tween) for 24 hours at 4°C. Specific IgE binding was detected by using a biotinylated monoclonal mouse anti-human IgE antibody (Pharmingen, Hamburg, Germany) followed by incubation with streptavidin-conjugated horseradish peroxidase (UBI, Lucerna Chem AG, Luzern, Switzerland). Peroxidase reactivity was visualized by means of enhanced chemiluminescence (Amersham Pharmacia Biotech, Essex, United Kingdom). For direct detection of Api m 6, we produced an anti-Api m 6.03 (7598 d) mAb 5E11 from mice immunized with Api m 6.03 after classic fusion strategies.²² Hybridoma culture supernatant was diluted 1:25,000 in PBS-Tween 1% milk and incubated with membranes for 1 hour at RT. Specific antibody binding was detected with horseradish peroxidase-conjugated sheep anti-mouse Ig antibody (Amersham), and peroxidase reactivity was visualized by means of enhanced chemiluminescence. In the inhibition experiments blots that were loaded with 15 μ g of whole BV were incubated with 1 μ g of Api m 6 HPLC-purified isoforms and incubated with the patient's optimal serum dilution (1:5-1:20) in PBS-Tween 1% milk overnight at 4°C. After extensive washing, blots were treated as described above.

Proliferation assay

PBMCs were isolated from heparinized blood by using gradient density centrifugation (Ficoll, Pharmacia). PBMCs (2 $\times$ 10⁵ cells/well) were cultured in quadruplicates in 96-well plates in RPMI-1640 medium supplemented with 10% AB⁺ serum (Swiss Red Cross, Bern, Switzerland) alone or in the presence of 1 μ g/mL antigen. After 5 days of culture in 5% CO₂ at 37°C, cells were pulsed with 1 μ Ci/well tritiated thymidine (Du Pont NEN products, Boston, Mass) and harvested 18 hours later. Results were expressed as the stimulation index (SI; ie, the ratio of thymidine incorporation in the cultures in the presence of antigen divided by that obtained in the control cultures). All patients' PBMCs responded to tetanus toxoid (10 μ g/mL; Swiss Serum Institute, Bern, Switzerland), which was used as a positive control for proliferation.

RESULTS

Purification of Api m 6 from BV

The pattern of IgE binding to BV proteins varies largely from one BV-hypersensitive patient to another.⁷ When

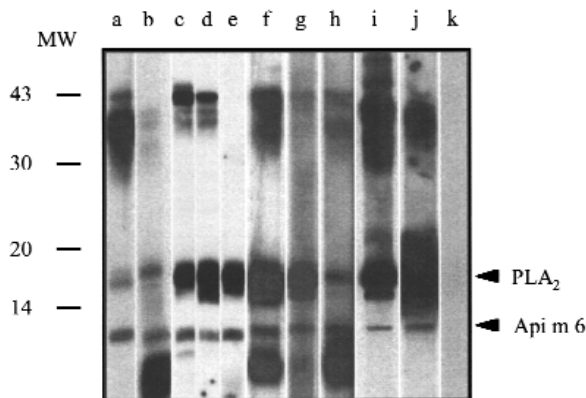


FIG 1. IgE binding to BV allergens. BV proteins were separated by using SDS-PAGE and transferred to PVDF membranes. Lanes a to j, incubation with sera from BV-hypersensitive patients; lane k, control serum. Specific IgE binding was detected by means of chemiluminescence. Positions of PLA₂ and Api m 6 are indicated.

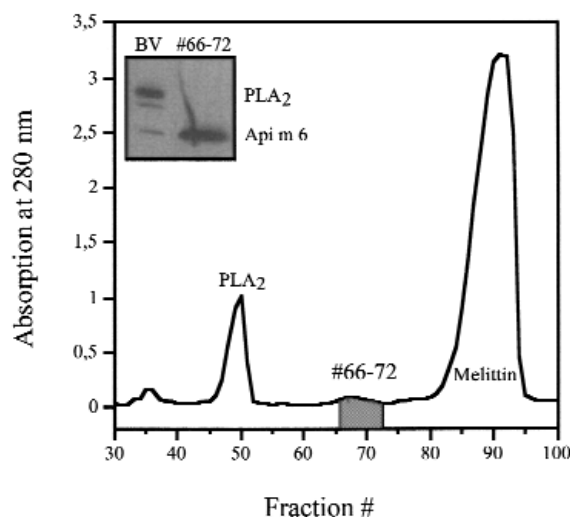


FIG 2. Purification of Api m 6 from whole BV: elution profile and IgE. Api m 6 eluted between peaks of PLA₂ and melittin in fractions 66 to 72 (shaded area). Inset, IgE immunoblot showing that Api m 6 is contained in fractions 66 to 72 in comparison with whole BV.

43 sera were analyzed for BV-specific IgE, 18 (42%) revealed a band at about 8 kD, later referred to as Api m 6. A representative immunoblot with the sera of 10 patients is shown in Fig 1. To purify the Api m 6 band, we separated BV proteins in a first purification step by using size exclusion gel chromatography, and fractions were analyzed by using SDS-PAGE. Api m 6 eluted in fractions 66 to 72 in a broad peak between the peaks of PLA₂ and melittin (Fig 2). MALDI-TOF mass spectrometry analysis of fractions 66 to 72 revealed the presence of 4 proteins with MWs of 7190, 7400, 7598, and 7808 d, respectively. After sequence analysis, these proteins were identified as isoforms of Api m 6 and were numbered according to the rules for the nomenclature of isoallergens: Api m 6.01 (7190 d), Api m 6.02 (7400 d),

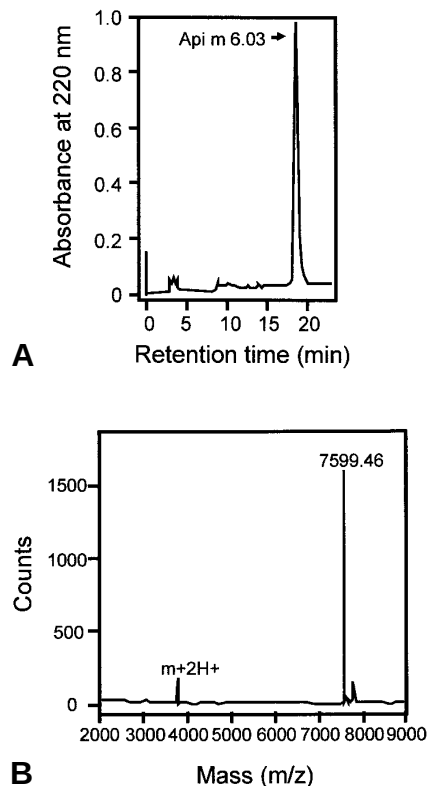


FIG 3. Reverse-phase HPLC profile and mass spectrometry of purified Api m 6.03 (7598 d). **A**, Api m 6.03 eluted at 18.5 minutes from analytical C₄ column (gradient: 1%/2 minutes, see "Methods" section for details). **B**, MALDI-TOF mass spectrometry of Api m 6.03 after HPLC purification.

Api m 6.03 (7598 d), and Api m 6.04 (7808 d).²³ The 4 proteins were further separated by means of reverse-phase HPLC, and pure preparations were obtained after 2 runs. In Fig 3 we show the HPLC and mass spectrum of the Api m 6.03 isoform. All isoforms were purified to the same degree and monitored under the same conditions. All 4 proteins were recognized by IgE from a BV-hypersensitive patient who had positive results for Api m 6 in the initial screening (Fig 4, A). The same cross-reactivity was found with mAb 5E11, which is specific for Api m 6.03 (see "Methods" section; Fig 4, B). Api m 6 could be detected by mAb 5E11 in BV from 3 distinct sources: liquid BV from a bee and 2 lyophilized commercially available preparations (Pharmalgen and Latoxan; Fig 4, D). The purified Api m 6 preparation was used to ascertain the specificity of the detection of the homologous band by means of immunoblotting. As observed in Fig 4, E, incubation of the blot with soluble purified Api m 6 isoforms specifically inhibits the band associated with Api m 6 and not that corresponding to PLA₂.

Furthermore, fractions 66 to 72 containing Api m 6 elicited a weak but significant proliferative response in PBMCs from BV-hypersensitive patients (4 of 6 patients with SIs between 2 and 4) and one BV-nonsensitive patient (SI, 10.6; data not shown). A substantially

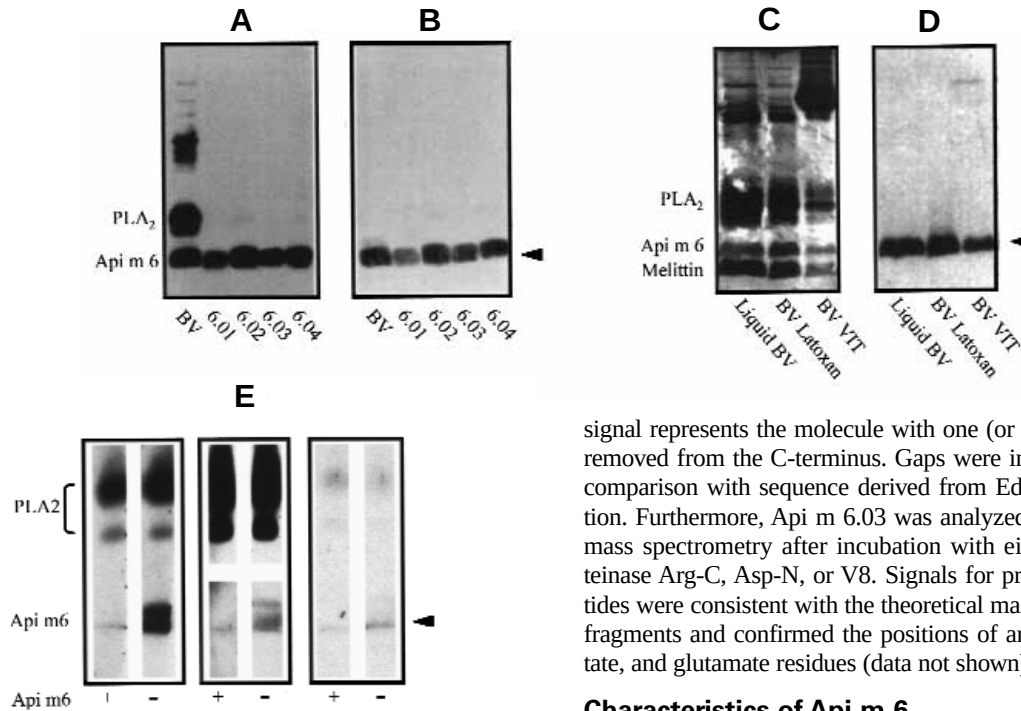


FIG 4. All Api m 6 isoforms were recognized by IgE and mAb 5E11. **A**, IgE immunoblot with serum of a hypersensitive patient positive for the 8-kd band. **B**, Immunoblot with mAb 5E11 (see "Methods" section). Positions of Api m 6 isoforms (arrows) and PLA₂ are indicated. **C** and **D**, Api m 6 is present in different BV preparations (**C**, total protein staining with Protogold; **D**, immunoblot with mAb 5E11). *Liquid BV*, Venom collected from bees; *BV Latoxan*, lyophilized BV collected by means of electrostimulation; *BV VIT*, BV preparation from Pharmedgen-ALK used for VIT (lyophilized powder). **E**, Specific inhibition of immunoblot detection of the band corresponding to Api m 6 with purified Api m 6. For clarity, only the portion of the blot corresponding to low-MW BV proteins is shown.

increased response (from an SI of 3.5 to an SI of 12) was detected for one patient when PBMCs were incubated with purified Api m 6.03.

Amino acid sequence of Api m 6

The complete amino acid sequence of Api m 6.03 was determined by using 2 approaches: N-terminal sequence analysis by means of Edman degradation and C-terminal sequencing with carboxypeptidases in combination with mass spectrometry (Fig 5, A). The first 48 amino acids were resolved by direct sequencing of the reduced and alkylated protein. Overlapping internal segments were obtained by sequencing of HPLC-purified tryptic peptides. C-terminal sequence could only be determined with carboxypeptidases. C-terminal residues were resolved in independent experiments by using either carboxypeptidase Y or A. Long stretches of sequence data obtained from the N-terminal analysis were furthermore confirmed by C-terminal analysis (Fig 5, A). Fig 6 shows sequence laddering of alkylated Asp-N fragment digested with carboxypeptidase Y. Each

signal represents the molecule with one (or more) residue removed from the C-terminus. Gaps were interpretable in comparison with sequence derived from Edman degradation. Furthermore, Api m 6.03 was analyzed by means of mass spectrometry after incubation with either endoprotease Arg-C, Asp-N, or V8. Signals for proteolytic peptides were consistent with the theoretical mass of expected fragments and confirmed the positions of arginine, aspartate, and glutamate residues (data not shown).

Characteristics of Api m 6

BV contains 1% to 2% Api m 6, as deduced from protein recovery after gel filtration with the 4 isoforms present at approximately equimolar amounts. The 4 isoforms, Api m 6.01 to 6.04, share a common central amino acid sequence of 67 residues and differ only in their amino and carboxy terminus by up to 6 amino acids (Fig 5, B). Api m 6.01 (7190 d) and Api m 6.03 (7598 d) differ at the N-terminus by a Phe-Gly-Gly-Phe segment. Otherwise, no difference was found in the 30 N-terminal amino acids sequenced. The total amino acid composition of these 2 isoforms varied only for the same 4 amino acids. Treatment with endoproteases Arg-C, Asp-N, and V8 resulted in the same proteolytic fragmentation pattern when resolved by means of mass spectrometry (data not shown). Api m 6.02 and Api m 6.04 have 2 additional residues, Pro and Leu, at the C-terminus, the order of which has not yet been determined. These 2 residues were found by comparison of amino acid analysis of Api m 6.03 with that of Api m 6.02 and Api m 6.04. Amino acid composition of the 4 isoforms was identical, except for the N- and C-terminal amino acids (data not shown). Furthermore, Api m 6 isoforms had an analogous proteolytic fragmentation pattern when cleaved enzymatically at arginine or aspartate residues and analyzed by means of mass spectrometry (data not shown). These findings strongly suggested a high similarity of their primary structure.

Api m 6, with a basic isoelectric point (pI, 10.05), was not glycosylated and lacked the amino acids methionine, threonine, tryptophan, and histidine. The 10 cysteines were confirmed by comparing the mass of untreated protein with the fully reduced and alkylated form. All cysteines were engaged in disulfide bridges because no free SH groups were detectable after incubation with the

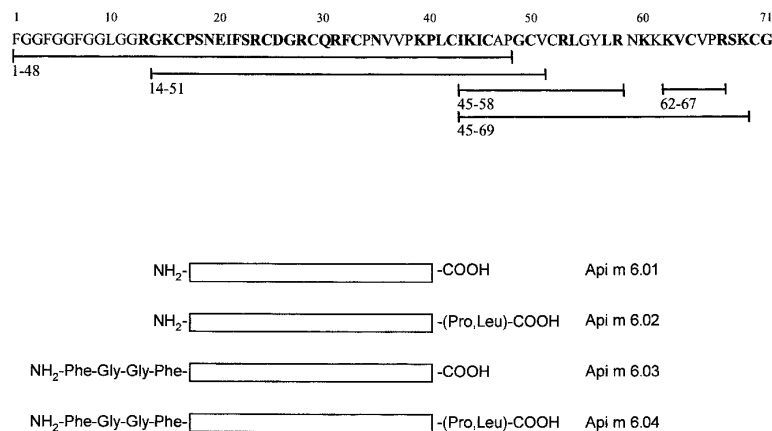


FIG 5. Complete amino acid sequence of Api m 6.03 (7598 d). **A**, Sequence determined by means of automated Edman degradation of the entire protein or tryptic peptides is indicated schematically below sequence annotation. Amino acids sequenced with MALDI-TOF mass spectrometry are accentuated in bold characters. **B**, Schematic representation of Api m 6 isoforms. The order of amino acids in brackets was not determined.

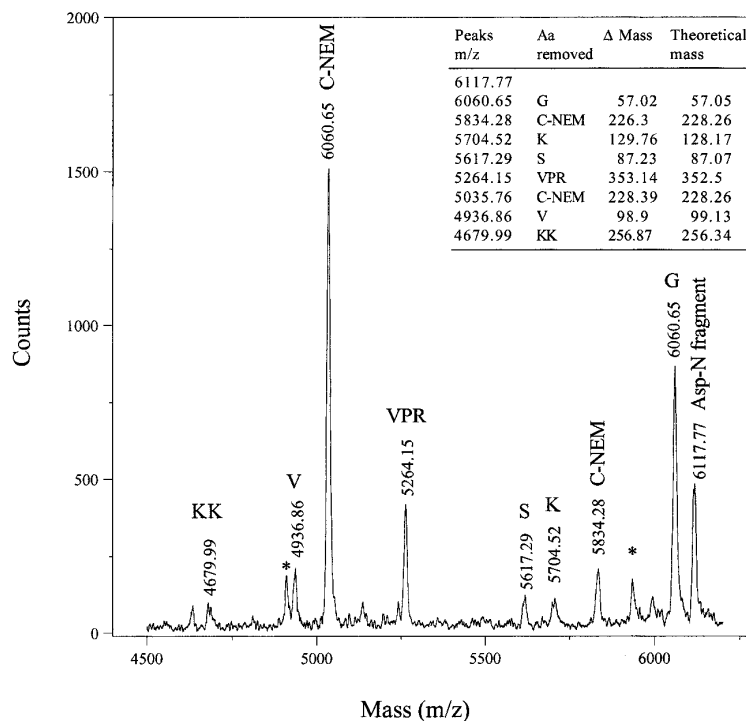


FIG 6. C-terminal ladder sequencing of the alkylated Asp-N fragment of Api m 6.03 (7598 d). The protein was reduced, alkylated with N-ethylmaleimide (NEM; MW, 125.12), and digested with Asp-N, and the C-terminal fragment was purified by means of reverse-phase HPLC. The alkylated fragment (MW, 6117.77) was then incubated with carboxypeptidase Y for 15 minutes at 37°C. Amino acids or peptide fragments marked above the distinct peaks indicate the loss of these residues. The differential mass of each peak was calculated and compared with the theoretical mass (see *table inset*). Peaks marked with an *asterisk* are derived from the Asp-N fragment with one cysteine that was not alkylated.

alkylating agent N-ethylmaleimide (data not shown). Disulfide bridges considerably increase the structural rigidity, rendering Api m 6 resistant to mild reducing and denaturing conditions. Extended incubation times of Api m 6.03 with DTT in the presence of urea were necessary

to enable unfolding and complete reduction of cysteine residues for efficient carboxymethylation suitable for N-terminal sequence analysis. C-terminal sequence analysis and enzymatic cleavage was also only possible after repeated incubation with DTT at 37°C.

DISCUSSION

The initial observation that a BV protein of approximately 8 kd was frequently recognized by IgE from BV-hypersensitive patients led us to isolate and characterize this new allergen. Further analysis of this band disclosed 4 isoforms that strongly bound IgE in a large fraction of patients and elicited a T-cell response. Here we report the characterization and the amino acid sequence of Api m 6.

The 4 Api m 6 isoforms differed in their primary structure only at the amino and carboxy terminus by a maximum of 6 amino acids (Fig 5, B). C-terminal sequencing with MALDI-TOF mass spectrometry proved to be a valid technique, complementing the sequence analyzed by Edman degradation. N-terminal sequencing alone did not yield data for the C-terminal region of the protein.

Much attention was paid in earlier studies to the pharmacologic and bioactive properties of BV, leading to the isolation of various peptides and proteins. Although Api m 6 was readily detectable in whole BV, the only existing record are the N-terminal sequences of 7190-d and 7598-d isoforms that were depicted in a review of BV components.¹⁷ Further studies were not undertaken because those molecules lacked obvious pharmacologic properties. In contrast, we have established a role for Api m 6 in BV allergy. A recent study analyzed the isotype specificity of BV antigen recognition by means of immunoblotting. Proteins of 8, 9, and 10 kd bound IgE.²⁴ Tetrameric melittin was suggested to form the 10-kd band, but no detailed analysis was performed. Other molecules in the same MW range had either distinct amino acid compositions (minimine and adolapin) or differed in the N-terminal amino acid (protease inhibitor).^{9-11,25}

Database searches revealed that Api m 6 contained an epidermal growth factor–like domain signature, but many unrelated proteins share this amino acid motif.²⁶ In addition, no obvious homology to known proteins was found, even when a profile search with the particular cysteine spacing motif, CX₈CX₃CX₃CX₈CX₃CX₃C, was performed. Therefore it was not possible to make functional assumptions on the basis of the primary structure. Preliminary results indicate that BV gland cells produce Api m 6 as a larger membrane protein (M. Astori, et al, unpublished results). We postulate that unidentified proteases may process Api m 6, resulting in its shedding into the venom sac lumen and subsequent formation of the isoforms. Such a mechanism has not yet been described for BV proteins. Most secreted BV proteins are produced as precursor molecules that are cleaved before secretion.²⁷⁻³⁰ We found no evidence for an Api m 6 precursor protein in secreted BV (Fig 4, D). Three distinct venom preparations, including the one used for VIT, were tested for the presence of Api m 6 to account for a possible variation in BV protein content caused by the origin of the venom.³¹ Api m 6 was readily detectable in all 3 preparations.

T-cell response against Api m 6 was detected in the BV-hypersensitive group and a BV-nonhypersensitive volunteer. T-cell priming to Api m 6 occurred therefore in both groups, but there was a marked difference in anti-

body response. Sera from 4 hypersensitive patients with IgE against Api m 6 were also positive for specific IgG and IgG₄ antibodies (data not shown). In contrast, BV-nonhypersensitive individuals (n = 4) had no detectable antibodies to Api m 6.

Isoallergens are commonly found in grass and tree pollens (ie, Amb a I, Bet v I, Lol p I, and Cor a I).³²⁻³⁵ The fact that they are recognized by T cells and IgE antibodies indicates that multiple isoallergens are involved in sensitization. This would make the approach of a vaccine more complex because those multiple isoforms may increase the number of potential stimulating T-cell epitopes.³⁶⁻³⁸ However, Api m 6 isoforms differ only on the amino- and carboxy-terminal ends, allowing the supplementation of a BV vaccine with the synthetic peptide of the common core sequence.

In conclusion, we describe here a new BV allergen with detectable IgE in more than 40% of BV-hypersensitive patients. Thus, strictly speaking, Api m 6 cannot be considered as a major allergen. However, in terms of a tailored immunotherapy, Api m 6 should be included in a future vaccine on the basis of either synthetic peptides or recombinant allergens.

We thank C. von Garnier for critical reading of the manuscript and Y. Chvatchko for suggestions and helpful discussion.

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