ORIGINAL

The Putative Serine Protease Inhibitor Api m 6 From *Apis mellifera* Venom: Recombinant and Structural Evaluation

Y Michel,[1] M McIntyre,[2] H Ginglinger,[1] M Ollert,[2] L Cifuentes,[2] S Blank,[1] E Spillner[1]

[1]Institute of Biochemistry and Molecular Biology, University of Hamburg, Hamburg, Germany
[2]Clinical Research Division of Molecular and Clinical Allergotoxicology, Department of Dermatology and Allergy Biederstein, Technische Universität München, Munich, Germany

Abstract

Background: Immunoglobulin (Ig) E–mediated reactions to honeybee venom can cause severe anaphylaxis, sometimes with fatal consequences. Detailed knowledge of the allergic potential of all venom components is necessary to ensure proper diagnosis and treatment of allergy and to gain a better understanding of the allergological mechanisms of insect venoms.
Objective: Our objective was to undertake an immunochemical and structural evaluation of the putative low-molecular-weight serine protease inhibitor Api m 6, a component of honeybee venom.
Methods: We recombinantly produced Api m 6 as a soluble protein in *Escherichia coli* and in *Spodoptera frugiperda* (Sf9) insect cells. We also assessed specific IgE reactivity of venom-sensitized patients with 2 prokaryotically produced Api m 6 variants using enzyme-linked immunosorbent assay. Moreover, we built a structural model of Api m 6 and compared it with other protease inhibitor structures to gain insights into the function of Api m 6.
Results: In a population of 31 honeybee venom–allergic patients, 26% showed specific IgE reactivity with prokaryotically produced Api m 6, showing it to be a minor but relevant allergen. Molecular modeling of Api m 6 revealed a typical fold of canonical protease inhibitors, supporting the putative function of this venom allergen. Although Api m 6 has a highly variant surface charge, its epitope distribution appears to be similar to that of related proteins.
Conclusion: Api m 6 is a honeybee venom component with IgE-sensitizing potential in a fraction of venom-allergic patients. Recombinant Api m 6 can help elucidate individual component-resolved reactivity profiles and increase our understanding of immune responses to low-molecular-weight allergens

Key words: Api m 6. *Apis mellifera*. Honeybee venom. Hymenoptera venom. Insect venom allergy. Protease inhibitor. Recombinant allergen. sIgE.

Resumen

Antecedentes: Las reacciones frente a veneno de abeja, mediadas por IgE pueden ser la causa de reacciones anafilácticas graves, siendo a veces de consecuencias fatales para el enfermo que las padece. Un diagnóstico preciso, así como la aplicación de un correcto tratamiento y la posibilidad de conocer el mecanismo inmunológico implicado en las reacciones alérgicas frente a veneno de himenópteros, implica un conocimiento detallado de los componentes de dicho veneno.
Objetivo: El objetivo de este estudio fue evaluar estructural e inmunoquímicamente un componente del veneno de abeja, Api m 6, un inhibidor de la serin proteasa de bajo peso molecular.
Métodos: Para ello se produjo Api m 6 recombinante como una proteína soluble en en *E. coli* y células de insecto Sf9. Se determinó mediante ELISA la IgE específica en pacientes sensibilizados a veneno con dos variantes de Api m 6 producidas procarióticamente. Se obtuvo un modelo estructural de Api m 6 y se comparó con estructuras de otros inhibidores de proteasas.
Resultados: En cuanto a los resultados obtenidos, un 26% de 31 pacientes alérgicos a veneno de abeja mostraban reactividad IgE específica frente a Api m 6 producido procarióticamente, lo que lo convierte en un alérgeno menor pero relevante.
El modelaje de Api m6 revela un plegamiento típico de los inhibidores de proteasa relacionados con la función de este alérgeno. Muestra una alta variabilidad en la distribución de carga de epítopes similar a otras proteínas relacionadas.
Conclusiones: Api m 6 es un componente del veneno de abeja con potencial para sensibilizante en una fracción de pacientes con alergia a veneno de abeja.
Api m 6 recombinante puede contribuir a elucidar el perfil de sensibilización frente a componentes y a conocer mejor la respuesta inmunológica frente a alérgenos de bajo peso molecular en alergia a veneno de abeja.

Palabras clave: Api m 6. Apis mellifera. Veneno de abeja. Veneno de himenóptero. Alergia a veneno de insecto. Inhibidor de proteasa. Alérgeno recombinante. IgE específica.

Introduction

Anaphylaxis mediated by immunoglobulin (Ig) E antibodies in patients who are allergic to hymenoptera venom is one of the most severe hypersensitivity reactions. During the last decade, population-based epidemiological studies have revealed a prevalence of systemic reactions to hymenoptera stings ranging from 0.3% to 8.9%, with anaphylaxis in 0.3-42.8% of cases [1,2]. Honeybee venom (HBV) is a complex mixture of several nonallergenic low-molecular-weight substances and various allergens that induce allergic reactions after honeybee stings [3]. Since all of the compounds in the venom can contribute to sensitization, symptoms, and the success of venom immunotherapy, their detailed characterization is of considerable interest. The low abundance of many of these components makes their availability in recombinant form a prerequisite for detailed immunologic characterization, improved component-resolved diagnosis, and the design of more efficient and safer therapy. Few recombinant HBV allergens are available [4,5]; however, in recent years, significant progress has been made on the identification and recombinant production of venom allergens. The best-known allergens of HBV are Api m 1 (phospholipase A_2), Api m 2 (hyaluronidase), and Api m 4 (melittin), which are found in medium-to-high amounts [3]. Less common allergens, such as Api m 3 (acid phosphatase) [6], Api m 5 (dipeptidyl peptidase IV enzyme) [7], Api m 10 [8-10], and Api m 11 [8,11,12] have also recently been identified, characterized, and recombinantly engineered.

The HBV allergen Api m 6 is a putative low-molecular-weight protease inhibitor, which exists as 4 isoforms [13]. The primary structure of these isoforms shows a common core of 67 residues and varying sequences at the amino and carboxy termini of at most 6 amino acids [13]. The genetic mechanism underlying this variation is unknown, although it has been shown that variations in protein level have a simple genome-level cause [14]. Named Api m 6.01 to 6.04, the isoforms migrate as an 8-kDa band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [13]. Api m 6 comprises a trypsin inhibitor–like cysteine-rich domain containing 10 cysteine residues and no putative N-glycosylation sites and is most likely identical to a 9-kDa protease inhibitor of bee venom described decades ago [15]. Kettner et al [13] purified Api m 6 from whole HBV and demonstrated T-cell proliferation as well as recognition of specific IgE using immunoblotting in approximately 40% of HBV-sensitized patients.

In this study, we describe the cloning and recombinant expression of the HBV allergen Api m 6 in *Escherichia coli* and in insect cells and evaluate the specific IgE reactivity of the prokaryotically expressed protein. The data obtained suggest a relevant role of Api m 6 as a sensitizing venom component in some HBV-allergic patients, since approximately 26% of patients show specific IgE reactivity with Api m 6. We also generated a 3-dimensional model of the mature allergen that highlights its putative role as a protease inhibitor and provides, to our knowledge, the first possible insights into structural functional relationships.

Materials and Methods

Materials

Monoclonal anti-V5 antibody was purchased from Invitrogen. Polyclonal rabbit anti–horseradish peroxidase (HRP) serum, antirabbit IgG alkaline phosphatase (AP) conjugate, and antimouse IgG AP conjugate were obtained from Sigma. Monoclonal AP-conjugated anti-IgE antibody was purchased from BD Pharmingen.

Sera

Sera from HBV-sensitized patients with HBV-specific IgE, positive intradermal skin test results, or both were selected at random from the institutional serum bank. All patients had given their written informed consent for an additional serum

Table 1. Primers Used for Cloning of Api m 6

Primer Name	Primer Sequence[a]	Restriction Site
Cloning of cDNA		
P1for	5′-TTTGGAGGATTTGGAGGATTTGGAGGAC-3′	None
P1back	5′ TCATCCTGGGAGGCATTTAGATCGCGG-3′	None
Cloning for bacterial expression		
P2for	5′-GATC**CATATG**TTTGGAGGATTTGGAGGATTTGGAGGAC-3′	NdeI
P2back1	5′-*GACCGAGGAGAGGGTTAGGGATAGGCTTACC*GGCTGGGAGGCATTTAGATCG-3′	None
P2back2	5′-GGTGGTT**GCTCTTCC**GCA*CGTAGAATCGAGACCGAGGAGAGGGTTAGGG*-3’	SapI
P3for	5′-GATC**GATATC**TTTGGAGGATTTGGAGGATT TGGAGGAC-3′	EcoRI
P3back	5′-GATC**CCGCGG**TCCTGGGAGGCATTTAGATCGCGG-3′	SacII
Cloning for insect cell expression		
P4for	5′-GATC**GATATC**TTTGGAGGATTTGGAGGATTTGGAGGAC-3′	EcoRV
P4back	5′-GATC**CCGCGG**TCCTGGGAGGCATTTAGATCGCGG-3′	SacII

[a]Sequences that specifically bind the Api m 6 coding sequence are underlined, restriction sites are indicated in bold, and sequences coding the V5 epitope are in italics.

sample to be drawn, and the local ethics committee approved all experiments involving human sera.

Cloning of cDNA

The stinger of the honeybee (*Apis mellifera*) with the venom sack and glands attached was separated for isolation of total RNA using peqGoldTriFast (Peqlab Biotechnologie). A detailed description of the primers is given in Table 1. The gene-specific primer P1back and SuperScript III RT (Invitrogen) were used to synthesize cDNA from the isolated total RNA. The cDNA of mature Api m 6 was amplified using *Pfu* DNA polymerase (Fermentas) and the primers P1for and P1back. Subcloning for sequencing was performed using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen).

Cloning and Recombinant Expression of Api m 6 in Bacteria

In order to express Api m 6 in *E coli*, its coding region was amplified in 2 consecutive polymerase chain reactions (PCR) by adding a C-terminal V5 epitope using the primers P2for, P2back1, and P2back2. The PCR product was subcloned into the vector pTXB1 (New England Biolabs) via NdeI and SapI. The vector was further modified by addition of a second chitin-binding domain (CBD). Additionally the Api m 6 coding region was amplified using the primers P3for and P3back, and the PCR product was cloned via EcoRI and SacII into the vector pMalc2x (New England Biolabs), which was modified by addition of a C-terminal V5 epitope. The fusion proteins were expressed and purified according to the manufacturer's recommendations.

Cloning and Recombinant Expression of Api m 6 in Insect Cells

In order to express Api m 6 in insect cells, its coding sequence was amplified using the primers P4for and P4back. The PCR product was subcloned via EcoRV and SacII into the vector pIB/V5-His (Invitrogen), which was modified by addition of a melittin signal sequence, an N-terminal 10-fold His-Tag, and a SacII restriction site [6]. The vector containing Api m 6 was used to transfect *Spodoptera frugiperda* (Sf9) cells (Invitrogen) after applying Cellfectin transfection reagent (Invitrogen). Cells were selected for stable integration of the vector by addition of 80 mg/mL of blasticidin S (Invitrogen) to the medium. The medium of confluent stably transfected insect cell cultures was collected, concentrated, and used for Western blotting.

Western Blotting

Recombinant allergens were separated by SDS-PAGE and immobilized onto nitrocellulose membranes. Anti-V5 epitope monoclonal antibody (mAb) was applied according to the manufacturer's recommendations, and human sera were diluted 1:5 with 5 mg/mL bovine serum albumin in phosphate-buffered saline (PBS). Bound antibodies were visualized via corresponding AP-conjugated secondary antibodies and nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indolyl phosphate.

Immunoreactivity of Patient Sera With Recombinant Proteins

Specific IgE immunoreactivity of human sera was assessed using enzyme-linked immunosorbent assay (ELISA) with 384-well microtiter plates (Greiner) coated with purified recombinant proteins (20 μg/mL) at 4°C overnight and blocked with 40 mg/mL milkpowder in PBS. Sera were then diluted 1:2 with PBS and incubated in a final volume of 20 μL for 4 hours at room temperature. After 4 washes in PBS, bound IgE were detected with a AP-conjugated antihuman IgE mAb. After a further 4 washes in PBS, 50 μL of substrate solution (5 mg/mL 4-nitrophenylphosphate [AppliChem]) per well was added. The plates were read at 405 nm. The lower end functional cut-off, indicated as a line, was calculated as the mean of the negative controls plus 2 SDs. Reactivity values only slightly higher than the cutoff were excluded. For ELISA procedures with anti-V5 epitope mAb and HRP antiserum, the antibodies were applied according to the manufacturer's recommendations and bound antibodies visualized using the corresponding AP-conjugated secondary antibodies as described above.

Construction of a Homology Model of Api m 6

A homology model of Api m 6 was constructed using the local meta-threading server (LOMETS) to predict protein structure [16] and the *A mellifera* chymotrypsin/cathepsin G inhibitor-1 (AMCI-1) as a template. The confidence score of the model is "high," indicating high reliability. Antigenic determinants/conformational epitopes of Api m 6 were predicted using the conformational epitope prediction server [17].

Other Methods

Standard molecular biology procedures were performed according to established protocols [18].

Results

Recombinant Expression and Characterization of Api m 6

The coding region of the longest variant, Api m 6.04 [13], was amplified from venom gland cDNA. The mature protein consists of 73 amino acids, has a theoretical molecular mass of 7.8 kDa, and contains no putative N-glycosylation sites. Expression in *E coli* as maltose-binding protein (MBP) fusion protein and purification using affinity chromatography yielded soluble protein with an apparent molecular weight of approximately 60 kDa (Figure 1A). In parallel, Api m 6 without a fusion partner was prokaryotically produced using CBD fusion followed by autocatalytic intein-mediated cleavage [19], resulting in pure and soluble target protein released from the intein-CBD tag (Figure 1B). Visualization using the V5 epitope tag showed that the recombinant allergen has an apparent molecular weight of approximately 12 kDa (Figure 1A, B).

Both Api m 6 variants reacted with a monoclonal anti-V5 epitope antibody in the immunoblot study (Figure 1A). ELISA corroborated data on protein identity (Figure 1C). The absence of reactivity between both proteins and a polyclonal

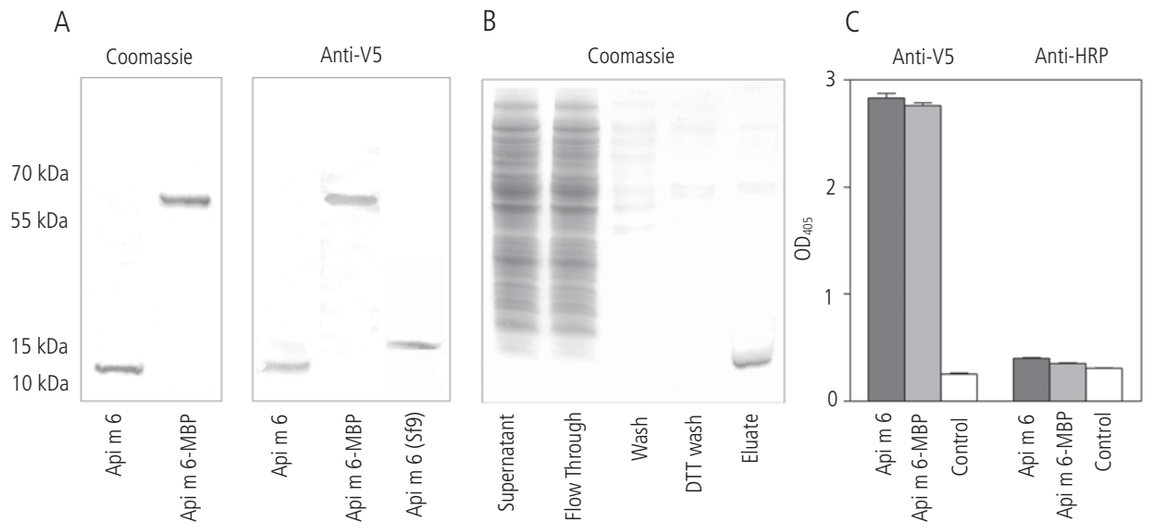

Figure 1. Recombinant expression, purification and characterization of Api m 6. A, SDS-PAGE and immunoblot analyses of Api m 6 with and without an MBP fusion partner recombinantly produced in *Escherichia coli* or Sf9 insect cells visualized by Coomassie blue staining or monoclonal anti-V5 epitope antibody. B, SDS-PAGE analysis of the purification of prokaryotically produced Api m 6 without a fusion partner using the strategy of CBD fusion followed by autocatalytic intein-mediated cleavage, which resulted in pure target protein released from the intein-CBD tag. C, Immunoreactivity of recombinant Api m 6 and Api m 6-MBP produced in *E coli* using enzyme-linked immunosorbent assay with monoclonal anti-V5 epitope antibody and polyclonal HRP antiserum. Results are presented as triplicates. SDS-PAGE, indicates sodium dodecyl sulfate polyacrylamide gel electrophoresis; MBP, maltose-binding protein; Sf9, *Spodoptera frugiperda*; CBD, chitin-binding domain; HRP, horseradish peroxidase.

HRP antiserum specific for α–1,3-core fucosyl residues—the underlying principle of cross-reactive carbohydrate determinants (CCDs)—demonstrates, expectedly, that the recombinant allergens are devoid of any CCD reactivity (Figure 1C). Moreover, Api m 6 was produced by stable transfection of Sf9 insect cells with an apparent molecular weight of approximately 15 kDa in the immunoblot study (Figure 1A). However, the expression level in Sf9 cells proved to be very low; consequently, both variants of prokaryotically produced Api m 6 were used for further specific IgE reactivity analyses.

Taken together, these findings demonstrate that Api m 6 can be produced prokaryotically as a soluble protein either with or without a fusion partner. Since prokaryotically produced proteins are devoid of CCD reactivity, recombinant Api m 6 variants seem to be suitable target molecules to assess their relevance as proteinogenic allergens.

Screening of Patient Sera for IgE Reactivity With Api m 6 Variants

In order to evaluate the IgE immunoreactivity of Api m 6, individual sera from 31 randomly selected patients with a clinical history of insect venom allergy were assayed for Api m 6–specific IgE antibodies using ELISA after applying Api m 6–MBP and nonfused Api m 6 produced in *E coli*. All patients were recruited during daily clinical practice and had sIgE for HBV, a positive result in intradermal skin tests with HBV, or both (Table 2).

Of the 31 sera, 8 (25.8%) showed IgE reactivity with both Api m 6 variants (Figure 2A). Although most reactive sera showed comparable reactivity with Api m 6-MBP and nonfused Api m 6 (Figure 2A, B), 1 serum exhibited dramatically reduced reactivity to the nonfused Api m 6. Since Api m 6 is devoid of any CCD-based cross-reactivity and contains no putative N-glycosylation sites, its reactivity obviously includes specificity for the protein only. These data were confirmed by the reactivity of a pool of serum from HBV-allergic patients with Api m 6–MBP in immunoblotting (Figure 3).

Sequence Alignment of Api m 6 With Serine Protease Inhibitors

Api m 6 contains a trypsin inhibitor–like (TIL) domain, which may act as a protease inhibitor in the venom. Interestingly, a small protease inhibitor isolated from HBV decades ago corresponds to Api m 6 in terms of molecular weight and the absence of the amino acids threonine, methionine, and histidine [20]. Figure 4 shows an alignment of the Api m 6 mature sequence with the *A mellifera* chymotrypsin/cathepsin G inhibitor-1 (AMCI-1), a protein of 56 amino acids isolated from honeybee hemolymph [21], and with the *Ascaris suum* chymotrypsin/elastase inhibitor (C/E-1), a protein consisting of 63 amino acids [22]. The sequence identity of Api m 6 with AMCI-1 and C/E-1 was 33% and 29%, respectively. Basically, this similarity is due to the presence of 10 cysteines in the TIL domain, which form 5 disulfide bonds pairing the cysteine residues 1-7, 2-6, 3-5, 4-10, and 8-9. Moreover, the TIL domain

Table 2. Demographic and Clinical Data

Patient No.	Anaphylaxis Grade[a]	Skin Test[b]		sIgE–Extract[c]		sIgE–Allergens[d]	
		HBV, µg/mL	YJV, µg/mL	il, kU_A/L	i3, kU_A/L	Api m 6, OD_{405nm}	Api m 6-MBP OD_{405nm}
1	2	0.01	0.001	3.15	4.46	0.3328	0.3869
2	2	0.01	0.001	32.9	14.4	0.9075	0.6418
3	1	0.1	0.0001	1.76	16.4	0.3039	0.3472
4	2	0.01	0.01	1.09	35.1	0.9598	1.6909
5	2	0.1	Negative	0.963	<0.1	2.0292	1.4795
6	1	0.01	0.01	5.46	3.64	0.5112	0.3894
7	2	0.1	0.0001	14.1	20	0.3533	0.4238
8	3	0.0001	0.001	13.8	17.8	1.4632	1.2015
9	2	0.01	Negative	0.62	0.63	0.3603	0.4638
10	1	0.1	0.01	5.94	2.14	0.3547	0.4093
11	3	0.001	0.0001	1.44	6.04	0.8112	1.0451
12	2	0.01	0.001	25.9	>100	0.3315	0.3390
13	1	0.1	0.01	0.163	4.34	0.4633	0.5758
14	3	0.0001	0.001	1.47	7.060	0.3811	0.3983
15	1	0.001	Negative	13.4	1.58	0.3718	0.6371
16	1	0.001	0.01	0.436	0.191	0.4119	0.4606
17	2	0.01	Negative	0.62	0.63	0.3346	0.3269
18	2	0.0001	Negative	60.8	0.521	0.3906	0.3715
19	2	0.001	Negative	27.6	<0.1	0.4329	0.4403
20	2	0.01	Negative	6.59	0.224	0.4605	0.5270
21	2	0.001	0.1	10.2	14.5	0.3815	0.4293
22	2	0.0001	Negative	23.8	2.19	0.8313	0.9925
23	2	0.001	Negative	35.9	0.889	0.4268	0.3969
24	2	0.01	Negative	81.4	3.31	0.6578	1.7865
25	2	0.0001	0.01	1.89	0.19	0.8104	0.8468
26	3	0.001	0.001	1.33	0.467	0.4978	0.4702
27	3	0.01	0.01	3.88	0.4	0.5199	0.6451
28[e]	2	ND	ND	20.5	1.42	0.4269	0.4254
29[f]	3	ND	ND	3.54	6.75	0.3450	0.3749
30	1	0.01	0.0001	5.200	17.900	0.4982	0.5644
31[e]	ND	ND	ND	13.800	8.290	0.4928	0.5596

Abbreviation: HBV, honeybee venom; MBP, maltose-binding protein; ND, not determined; YJV, yellow jacket venom.
[a]According to Ring and Messmer [33]
[b]The lowest venom extract concentration that gave a positive result in intradermal skin testing after applying a 10-fold concentration range from 0.1 to 0.0001 µg/mL for each of the venoms. Intradermal tests were rated positive when the wheal size was >5 mm in diameter with surrounding erythema.
[c]As tested in either UniCAP250 or Immulite2000 sIgE assays.
[d]As tested in an immunoglobulin E enzyme-linked immunosorbent assay using a lower cutoff of 0.55, as described in the Methods section.
[e]No skin test was performed, but these patients were medicated in the emergency department for a frank clinical reaction after a honeybee sting.
[f]Skin test was not evaluable owing to a clinical history of urticaria factitia for this patient.

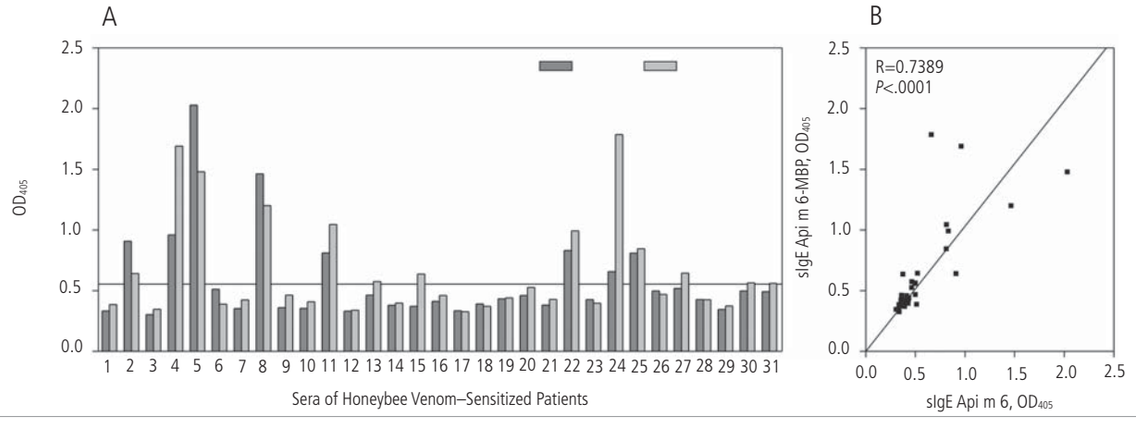

Figure 2. IgE immunoreactivity of individual patient sera with Api m 6 variants. A, Immunoglobulin E reactivity with prokaryotically produced Api m 6 and Api m 6-MBP fusion protein was assessed by ELISA with 31 sera of honeybee venom–sensitized patients. The lower end functional cut-off of the ELISA is represented by a solid line. B, Correlation of the IgE reactivities obtained with the 2 prokaryotically produced Api m 6 variants was assessed using Pearson correlation analysis. ELISA indicates enzyme-linked immunosorbent assay. MBP, maltose-binding protein; Ig, immunoglobulin.

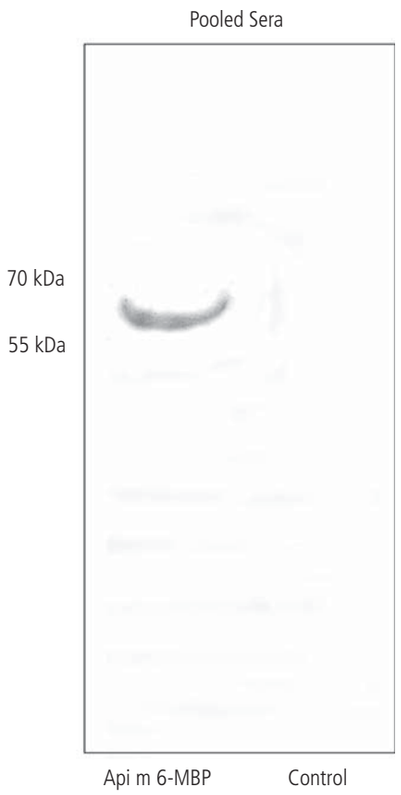

Figure 3. Immunoreactivity of Api m 6 with pooled sera of honeybee venom–allergic patients. Sodium dodecyl polyacrylamide gel electrophoresis and immunoblot analysis of purified Api m 6-MBP visualized by pooled sera of 100 patients with a clinical history of honeybee venom allergy and anti-human IgE mAb conjugated to alkaline phosphatase. MBP alone served as a control. MBP indicates maltose-binding protein.

of all 3 proteins contains 4 ß strands at identical positions. In contrast to Api m 6, C/E-1 and AMCI-1 contain 2 and 1 short α helices, respectively.

Generation of a 3-Dimensional Model of Api m 6

To obtain further insights into the potential function of Api m 6, the structure of AMCI-1 in nuclear magnetic resonance imaging [23] was used as a template to generate a structural model of Api m 6 based on LOMETS to predict protein structure [16]. The model of Api m 6 (Figure 5A) was compared with the AMCI-1 template (Figure 5B) and with C/E-1 (Figure 5C), the structure of which was solved by crystallization [24]. Obviously, Api m 6 and both the protease inhibitors exhibit a common fold, which is dominated by an exposed binding loop including the putative protease binding site and showing a typical canonical conformation that is essential for the biologic activity of protease inhibitors. Further characteristic elements of all 3 structures are the antiparallel ß strands forming the base of the inhibitory loop and several turns. Given the lack of extensive secondary structural elements, both the binding loop and the scaffold built by the ß sheets are stabilized by the presence of 5 disulfide bonds. Notably, the protein loop exhibits a strongly exposed lysine (K44) (Figure 5A) constituting the P1 position, which is important for enzyme binding and a potential recognition motif, together with positions P_3 and P_3', which are occupied by cysteines (C42, C46). The sequential P_3-P_3' segment of the binding loop of canonical inhibitors represents the primary contact region and the flanking sequences form the so-called secondary contact region, which can also participate in enzyme binding [25]. In contrast to the 2 other protease inhibitors shown, the coulombic coloring of the Api m 6 surface is strongly dominated by a positive charge (Figure 6A). This observation is in line with the isoelectric point of Api m 6 of 9.7, which is relatively high when compared to C/E-1 (5.2) and AMCI-1 (4.8) and gives Api m 6 a strong basic character. As suggested by conformational epitope prediction, the differentially charged molecule surface has no impact on the density or distribution of epitopes that apparently cover the whole surface of all 3 molecules (Figure 6B).

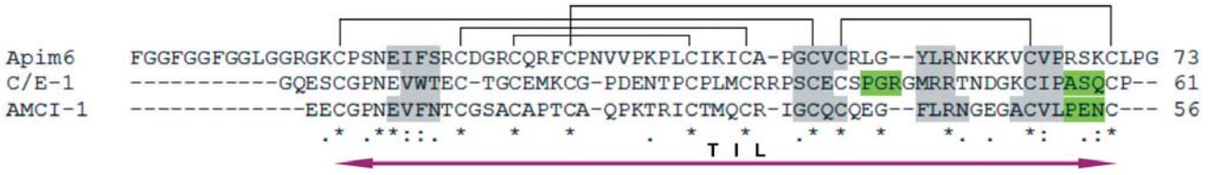

Figure 4. Alignment of the amino acid sequences of Api m 6 (GenBank accession no. ABD51779), the *Ascaris suum* chymotrypsin/elastase inhibitor (C/E-1) (PDB ID 1EAI chain D), and the *Apis mellifera* chymotrypsin/cathepsin G inhibitor-1 (AMCI-1) (PDB ID 1CCV). Asterisks, colons, and periods indicate fully conserved, strongly similar, and weakly similar residues, respectively. Sequences forming ß strands are highlighted in grey and helical regions in green. The pairing cysteine residues 1-7, 2-6, 3-5, 4-10, and 8-9 are connected through black brackets, and the TIL domain is illustrated by a purple arrow.

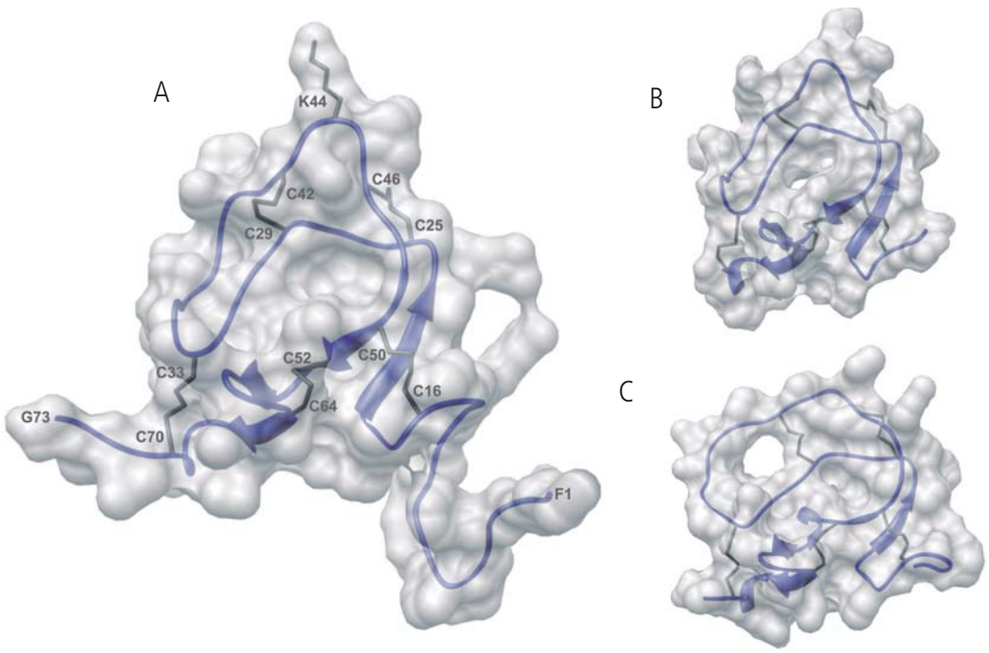

Figure 5. Structure of Api m 6 and related proteins. A, Molecular model of Api m 6 in comparison with the solved structures of the *Apis mellifera* chymotrypsin/cathepsin G inhibitor-1 (AMCI-1) (B) and the *Ascaris suum* chymotrypsin/elastase inhibitor (C). The modeling was performed using the structure of AMCI-1 (PDB ID 1CCV) as a template. Lysine 44 of Api m 6 corresponds to the important inhibitory P1 position of the binding loop.

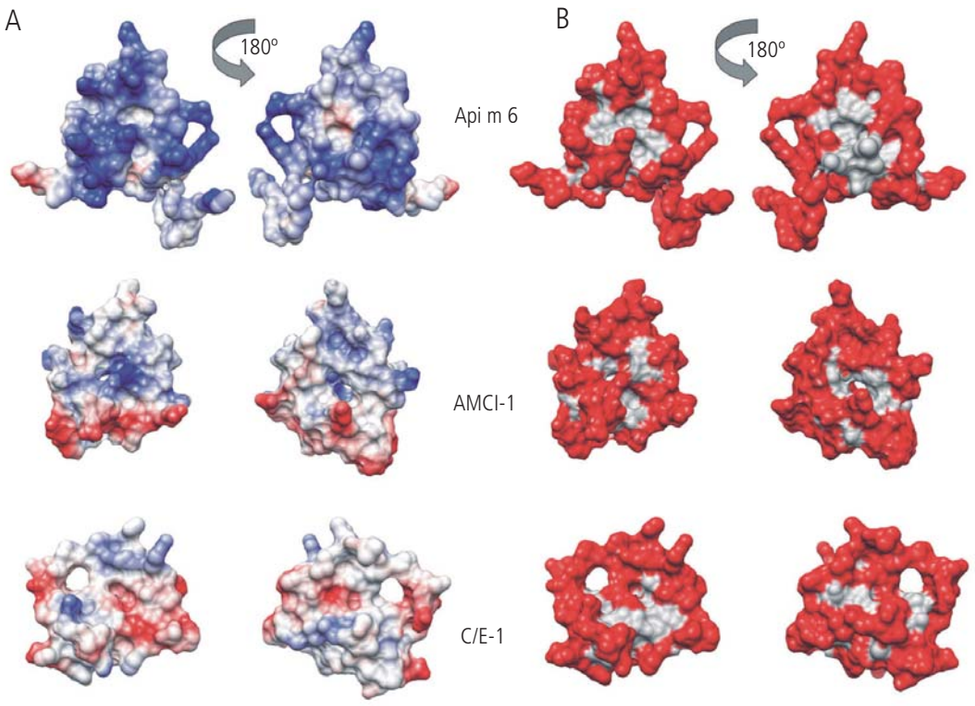

Figure 6. Electrostatic potential and predicted epitopes of Api m 6 and related proteins. A, Coulombic surface coloring indicates the electrostatic potential, ranging from basic (blue) to acidic (red) surface properties. B, Predicted conformational B cell epitopes of Api m 6, AMCI-1, and C/E-1 are highlighted in red. Epitopes were predicted using the conformational epitope prediction server. AMCI-1 indicates *Apis mellifera* chymotrypsin/cathepsin G inhibitor; C/E-1, *Ascaris suum* chymotrypsin/elastase inhibitor.

Discussion

Much progress has been made on the identification and characterization of novel hymenoptera venom allergens: however, relatively little is known about low-abundance components and their allergenicity, sensitizing potential, clinical relevance, and function in venom. In order to design component-resolved diagnostic approaches and advanced therapeutic strategies, detailed knowledge of the molecular composition of hymenoptera venoms and in-depth characterization of the immunological properties of the individual components are imperative. Here, we report the results of the first recombinant and structural evaluation of the HBV allergen and putative protease inhibitor Api m 6.

Api m 6 was identified some years ago after separation of HBV [13], and it accounts for 0.8% to 2% of the composition of HBV [13,20]. A set of 4 isoforms has been identified [13], and a rudimentary analysis of the genetic origin of this protein variation has been performed [14]. Moreover, using immunoblot, it was demonstrated that native purified Api m 6 shows distinct IgE reactivity with patient sera [13]. We describe the generation of Api m 6 as a recombinant protein produced in *E coli* and Sf9 insect cells and the determination of specific IgE reactivity using ELISA. We also created a 3-dimensional model of Api m 6 in order to assess its function as a putative protease inhibitor in venom and highlight its special role as a low-molecular-weight allergen.

We obtained soluble recombinant Api m 6 using stably transfected Sf9 insect cells; however, the expression rate was so low that purification from culture supernatant was not feasible. It might be speculated that, in some cases, biologically active hymenoptera proteins might impede expression in systems such as insect cells, owing to intracellular binding or interaction with endogenous host proteins. Given the absence of predicted N-glycosylation sites in Api m 6, which in other proteins are often essential for correct folding, the protein appeared to be suitable for production in *E coli*. Two variants of Api m 6 were successfully expressed: an MBP fusion protein, as well as a nonfused protein carrying the V5 epitope using the strategy of CBD fusion followed by autocatalytic intein-mediated cleavage [19]. Both protein variants were obtained in soluble form with the expected molecular weight.

Using ELISA and the different Api m 6 variants, we detected specific IgE reactivity in 26% of HBV-allergic patients. This finding is consistent with the results obtained by Kettner et al [13], who classified Api m 6 as a minor allergen after recording specific IgE reactivity of approximately 40% with purified natural Api m 6 in immunoblot. It remains to be determined whether other venom components, such as the low-molecular-weight but high-abundance component melittin, contribute to the slightly higher IgE reactivity. Together, these results clearly demonstrate the role of Api m 6 as an IgE-sensitizing component in HBV-allergic patients. Despite its low molecular weight, Api m 6 has a noticeable sensitizing effect; however, further studies are needed to address the potential of such small molecules to induce pronounced effector cell activation and thus clinical symptoms.

In 1973, Shkenderov [15] separated HBV using gel filtration and observed that the isolated melittin peak inhibited trypsin activity. As melittin does not account for this effect, the responsible inhibitor was isolated and described as a basic protein of approximately 9 kDa that, in terms of molecular weight and given the absence of the amino acids threonine, methionine, and histidine, most likely corresponds to Api m 6. The function of Api m 6 has not been completely resolved, although its structure suggests that it is a protease inhibitor, much in the same way as the trypsin inhibitor–like cysteine-rich domain, which is usually indicative of serine protease inhibition [25] and typically contains 10 cysteine residues forming 5 disulfide bonds. This strong cross-linking is typical for inhibitors, and the topology of the conserved disulfide bonds is usually well preserved within a single family [25], as observed in inhibitors such as the *Ascaris* family of serine protease inhibitors [22,26,27] and AMCI-1 [23]. Considering the sequence homology of Api m 6 with AMCI-1 and C/E-1, Api m 6 is highly likely to be a serine protease inhibitor in the class of canonical inhibitors. These are usually small proteins that bind to proteases through an exposed convex binding loop, which is similar in all known inhibitor structures [28,29] and is complementary to the active site of the enzyme. The 3-dimensional structures of Api m 6, AMCI-1, and C/E-1 all exhibit an explicit canonical loop conformation, resulting from the extensive system of disulfide bonds that builds a stabilizing scaffold inside the molecules. Several inhibitor families lack both a hydrophobic core and an extensive secondary structure [25].

In principle, the canonical conformation can be achieved by different sequences of unrelated proteins, and canonical inhibitors are the most distinct example of convergent protein evolution [30]. Nevertheless, several families have amino acid sequences in the binding loop that show clear amino acid preferences. For the *Ascaris* family, a cysteine is present at position P_3 [25], as in Api m 6. To a large extent, the amino acid at position P_1 determines protease inhibitor association energy in canonical inhibitors [25], and its dominant role for interaction has been demonstrated [31,32]. In Api m 6, this position is occupied by a lysine residue that is fully exposed and could therefore be embedded in the S_1 pocket of a corresponding enzyme. The 3-dimensional model of Api m 6 generated in this work and the comparison with known protease inhibitors elucidate several putative sequential and structural characteristics that are common to serine protease inhibitors and strongly support the inhibitory function of Api m 6. Despite a variant surface charge distribution, epitope prediction suggests a mechanism of interaction with antibodies, which is similar to the 2 low-molecular-weight inhibitors shown here.

In summary, we produced Api m 6 from *A mellifera* venom for the first time in soluble form in *E coli* and assessed its allergenic potential in recombinant form. The results obtained clearly demonstrate an IgE-sensitizing potential in a fraction of HBV-allergic patients, thus indicating that Api m 6 is a relevant allergen. Given the small molecular size of this allergen, further studies on its ability to induce effector cell activation will reveal the coherence of IgE sensitization and clinical relevance. Moreover, structural modeling approaches demonstrate a typical canonical serine protease inhibitor fold and provide the first potential structural concepts for Api m 6. Although the natural function and putative binding partners are not known, the recombinant HBV allergen Api m 6 has the potential to give structural insights into allergenic molecules and to contribute to a more detailed understanding of immune responses to low-molecular-weight allergens.

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Manuscript received February 13, 2012; accepted for publication May 23, 2012.

Simon Blank and Edzard Spillner

Institute of Biochemistry and Molecular Biology
Department of Chemistry, University of Hamburg,
Martin-Luther-King-Platz 6, 20146 Hamburg, Germany
E-mail: blank@chemie.uni-hamburg.de
spillner@chemie.uni-hamburg.de