

Molecular cloning and expression in insect cells of honeybee venom allergen acid phosphatase (Api m 3)

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Background: Acid phosphatase (Api m 3) is a major allergen in honeybee (*Apis mellifera*) venom, and its availability as a recombinant protein may facilitate the development of improved diagnostic tests and immunotherapies.

Objective: One objective is the determination of the complete primary structure of Api m 3 and to obtain recombinant Api m 3 on the basis of expression in insect cells. Another objective is the quantitative analysis of patient serum IgE antibody reactive to recombinant Api m 3.

Methods: The cloning of Api m 3 from venom gland cDNA and its expression as a full-length protein in eukaryotic insect cells is described. The immunoreactivity of serum IgE antibodies of honeybee venom-sensitized patients to recombinant Api m 3 was determined in an enzyme immunoassay.

Results: PCR amplification generated a 1122-bp DNA fragment whose identity as the coding sequence of Api m 3 was verified by several means. Recombinant Api m 3, expressed in *Trichoplusia ni* cells, showed an expected molecular weight and enzymatic activity at pH 4.5. Analysis of tryptic fragments of purified recombinant Api m 3 by mass spectrometry confirmed its identity. In immunoassays, recombinant Api m 3 is specifically recognized by IgE antibodies of pooled serum in Western blots and by 37% of the individual sera of honeybee venom-sensitized patients in ELISA analysis.

Conclusion: The availability of recombinant Api m 3 provides a tool for both the development of improved diagnostic tests and the design of safer and more effective immunotherapeutic approaches for honeybee venom allergy.

Clinical implications: The recombinant venom allergen Api m 3 is a key element in the search for an optimized component-

resolved approach to honeybee venom allergy with regard to both the development of superior diagnostic tests and the improvement of allergen immunotherapy. (*J Allergy Clin Immunol* 2006;117:848-54.)

Key words: Honeybee venom, Api m 3, acid phosphatase, *Apis mellifera*, recombinant allergen, expression, insect cells

Honeybee stings may cause life-threatening and sometimes fatal IgE-mediated anaphylactic reactions in human beings. Venom immunotherapy is effective in the majority of patients with honeybee venom allergy, but treatment failures do occur. Systemic allergic side effects to immunotherapy injections have been observed in 20% to 40% of patients and, according to a sting challenge test during venom immunotherapy, 10% to 20% of patients were not protected by honeybee venom immunotherapy and continued to develop generalized allergic symptoms.^{1,2} Thus, there is considerable interest in improving safety and efficacy of honeybee venom immunotherapy. The use of recombinant allergens for the development of safer and more efficacious treatment modalities may provide such an improvement,³ but only a limited number of honeybee venom allergens are available as recombinant proteins.^{4,5}

The 3 most potent honeybee venom allergens responsible for IgE-mediated allergic reactions are phospholipase A₂ (Api m 1), hyaluronidase (Api m 2), and acid phosphatase (Api m 3).⁶ Melittin (Api m 4), a basic peptide consisting of 26 amino acids, is considered a minor allergen.⁷ Phospholipase A₂ and hyaluronidase have been cloned and expressed in bacteria and in baculovirus-infected insect cells.⁸⁻¹¹ Their structures have been elucidated by x-ray crystallography.^{12,13} Acid phosphatase was purified from honeybee venom and partially characterized more than a decade ago,¹⁴⁻¹⁹ but attempts to clone and express this important allergen have not been successful.^{20,21}

In this study, we developed a completely different—*in silico*—approach for cloning of Api m 3 by using sequence information from published Api m 3 peptides¹⁹ to scan the published honeybee genome.²² Two genomic regions with possible open reading frames were identified and used for the design of appropriate primers. With honeybee venom gland cDNA as template, PCR yielded the gene coding for Api m 3.²³ Expression in eukaryotic insect cells yielded a protein that exhibits the expected structural

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Abbreviations used

MALDI-TOF-MS: Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry

NTA: Nitrilo-triacetic acid

sIgE: Specific IgE

and functional features and is recognized by IgE from honeybee venom sensitized patient sera. Because of these characteristics, recombinant Api m 3 represents a potentially valuable tool for the improvement of current diagnostic tests and immunotherapy of honeybee venom allergy.

METHODS

Materials

Three groups of sera were selected at random from the institutional serum bank: (1) sera with a positive specific IgE (sIgE) test to honeybee venom ($i1 \geq 0.35$ kU/L; $n = 40$); (2) sera with a negative sIgE test to honeybee venom ($i1 < 0.35$ kU/L) but a strongly positive test to vespid venom ($i3 \geq 50$ kU/L; $n = 10$); and (3) sera with a negative sIgE test to both honeybee and vespid venom ($i1$ and $i3 < 0.35$ kU/L; $n = 9$). All patients had given their informed written consent to have an additional serum sample drawn. Whole honeybee venom was from Sigma (Taufkirchen, Germany).

Cloning of cDNA

Total RNA was isolated from the separated stinger of a honeybee (*Apis mellifera*) with attached venom sack and additional glands. Total RNA was isolated by using a kit (peqGold TriFast; peqlab Biotechnologie, Erlangen, Germany). RevertAid M-MuLV Reverse Transcriptase (Fermentas, St Leon-Rot, Germany) was used to synthesize cDNA from the isolated total RNA with an oligo-dT oligonucleotide primer of 20-bp length. RNaseOut recombinant ribonuclease inhibitor (0.5 μ L; Invitrogen, Karlsruhe, Germany) was added to the standard 20- μ L reaction mix containing 5 μ L honeybee venom gland tissue RNA. Reverse transcription was performed at 42°C for 60 minutes. First-strand cDNA was used as template for PCR amplification of Api m 3 DNA sequences. The PCR reaction was performed with recombinant *Pfu* DNA polymerase (Fermentas) using the primers *Api m 3 for* and *Api m 3 back*. Primers for Api m 1, Api m 2, and Api m 4 were also used for amplification of the respective products (see this article's Table E1 in the Online Repository at www.jacionline.org). DNA from the PCR reaction was isolated from 1% agarose gels (peqGOLD universal agarose, peqlab) using the QIAEX II gel extraction kit (Qiagen, Hilden, Germany). Subcloning for sequencing was performed using the TOPO TA Cloning Kit (Invitrogen) with pCR 2.1-TOPO vector. Because of use of *Pfu* DNA polymerase, an additional TA-elongation reaction step with AGS Gold *Taq* DNA Polymerase (AGS Hybaid, Heidelberg, Germany) was introduced. The ligated DNA was transformed into *Escherichia coli* of the strain TG1 by electroporation (2-mm cuvettes, EasyJect+; Eurogentec, Seraing, Belgium) and selected on ampicillin agar plates.

The sequencing reaction was performed with BigDye Terminator Cycle Sequencing Kit from ABI (Applied Biosystems Applera, Darmstadt, Germany). Sequence runs were performed on ABI 377 instruments. Sequencing primers were *M13/Uni for* and *M13/Uni rev* (see this article's Table E1 in the Online Repository at

www.jacionline.org). After sequencing of selected subcloned cDNA clones and verification of the sequence, the clone was used for secondary amplification with *Pfu* DNA polymerase. The PCR product was subcloned into the *EcoR* V and *Sac* II digested expression vector after restriction digest with *Sac* II.

Insect cell expression vector

To construct the insect expression vector pIB/Mel opt-H10, the vector pIB/V5-His (Invitrogen) was modified. A melittin signal sequence for secretion of the recombinant protein was added and the Kozak sequence was optimized for higher expression rates.²⁴ The melittin signal sequence was amplified from total bee venom gland RNA, synthesized as described using the primers *melt leader for* and *melt leader back* (see this article's Table E1 in the Online Repository at www.jacionline.org). The fragment was cloned between the *Hind* III and *Bam*HI site of the parent vector. A tag containing vector was used with the primers *10xHis for* and *10xHis back* to generate a first template. The amplicon was used as second template with primer SOE Xa to be further modified to attach a *EcoR* V site at the 3'-end. The sequence containing the 10x Histidine-tag and Factor Xa cleavage site was cloned between the *Bam*HI and *EcoR* V site of the modified vector. For all PCR steps *Pfu* DNA polymerase was used.

Expression and purification of Api m 3

High Five insect cells (Invitrogen) were used for expression. DNA was purified from bacterial cultures using the EZNA Plasmid Miniprep Kit II (peqlab). For transfection of purified DNA into cells, the reagent Cellfectin (Invitrogen) was used. Insect cells were grown in serum-free medium (Express Five SFM, containing 16.5 mmol/L glutamine and 10 μ g/mL gentamycin; Invitrogen). Cells were selected for stable integration of the recombinant product by addition of 80 μ g/mL Blasticidin S (Invitrogen) antibiotic to the medium.

Medium of confluent stably transfected insect cell expression cultures was collected. The supernatant was adjusted to pH 7.8 and centrifuged at 4000g for 5 minutes. Aliquots of 10 to 20 mL medium were applied to a nickel-chelating affinity matrix (nitrilo-triacetic acid [NTA]-agarose, Qiagen). The column was washed with 10 mL NTA-binding buffer (50 mmol/L sodium phosphate, pH 7.6, 500 mmol/L NaCl) and pre-eluted with NTA-binding buffer containing 20 mmol/L imidazole. The recombinant protein was eluted from the matrix with 10 mL NTA-binding buffer containing 400 mmol/L imidazole. Purification was confirmed by SDS-PAGE and silver staining.

Immunoreactivity of human sera with recombinant Api m 3

To detect specific IgE immunoreactivity of undiluted human sera with purified recombinant Api m 3, ELISA plates (NUNC, Wiesbaden, Germany) were coated with 100 μ L purified recombinant Api m 3 (1 μ g/mL) or, as a positive control, purified native Api m 1 (1 μ g/mL) (Latexan, Valence, France) at 4°C overnight. For all steps, an ELISA reagent set was used (BD Biosciences, Heidelberg, Germany). Bound IgE was detected with a biotinylated mouse antihuman IgE (BD Biosciences) together with horseradish peroxidase-conjugated avidin (both diluted 1:250). Color was developed with 100 μ L substrate solution per well for 30 minutes in the dark. Finally, 50 μ L stop solution was added, and plates were read at 450/570 nm. To standardize the serum samples, a human IgE standard was run on each plate by using murine anti-IgE (10 μ g/mL) as capture antibody and human myeloma IgE (Calbiochem-Merck, Darmstadt, Germany) over a concentration range of 31.25 to 4000 pg/mL (100 μ L per well). All samples were assayed in duplicate. The cutoff of the ELISA was calculated as the mean of the negative controls plus 2 SDs.

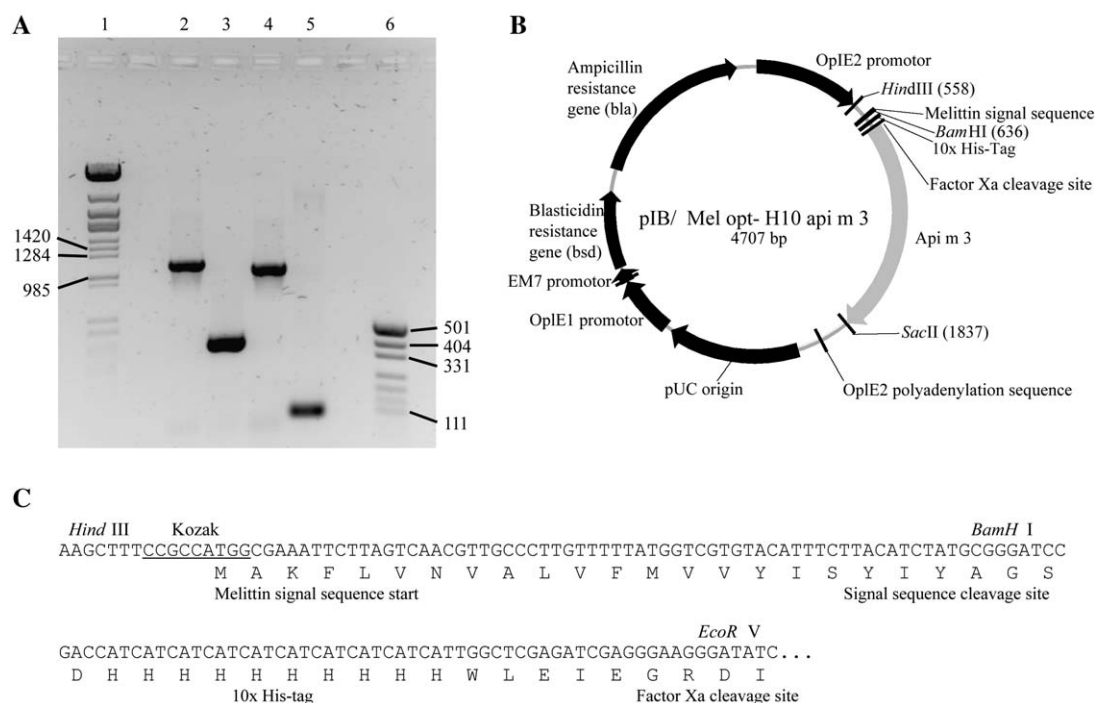


FIG 1. Api m 3 cDNA cloning. **A**, RT-PCR products from honeybee venom cDNA. *Lane 2*, Api m 3; *lanes 3 to 5*, Api m 1, Api m 2 and Api m 4, respectively; *lanes 1 and 6*, molecular weight standards (in bp). **B**, Map of expression vector pIB/Mel opt-H10 carrying the Api m 3 gene. **C**, Modified cloning site of the expression vector.

Other methods

Proteins were separated on 10% SDS-PAGE under reducing conditions. Transfer to nitrocellulose membrane (Protran; Schleicher & Schuell BioScience, Dassel, Germany) and subsequent immunostaining for sIgE reactive allergens were performed by using a kit (AlaBLOT; DPC Biemann, Bad Nauheim, Germany) according to the manufacturer's instructions as described.²⁵ Enzymatic activity of the recombinant protein was confirmed in a liquid assay for acid phosphatase activity using p-nitrophenyl phosphate as substrate.¹⁹ Verification of the identity of the recombinant Api m 3 was performed by analysis of tryptic fragments by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) using standard protocols. Statistical analysis of the ELISA data was performed by using *t* test for unpaired samples, linear regression analysis, and χ^2 test with the software packages Sigma Plot and SPSS (Systat Software, Erkrath, Germany).

RESULTS

cDNA cloning and sequence analysis

Using nucleic acid sequences derived from published peptide sequences,¹⁹ small virtual probes of partial deduced sequences were applied to scan the honeybee genome for Api m 3 sequences.²² Two genomic sequences matched the probes. Scanning of these regions with bioinformatic tools^{26,27} revealed possible open reading frames. Furthermore, scanning for a potential signal peptide cleavage site²⁸ indicated the putative 5'-terminus of the sequence coding for the Api m 3 protein. Primers designed on the basis of the identified genomic sequences were

used to amplify the gene from bee venom gland cDNA. PCR amplification generated a DNA fragment of the expected size, 1122 bp in length (Fig 1, A, lane 2). The identity of this DNA fragment with the coding sequence for Api m 3 was verified by several lines of evidence as described below. For control of the venom gland cDNA, the genes for Api m 1, Api m 2, and Api m 4 were amplified. The sizes (Fig 1, A, lanes 3-5) and the sequences (not shown) of the DNA fragments were identical to those reported.

The Api m 3 nucleotide sequence (see this article's [Fig E1](#) in the Online Repository at www.jacionline.org) encodes a 373-amino acid polypeptide with a calculated molecular mass of 44,012 d, which is in accordance with the reported apparent molecular weight (approximately 45 kd) of native Api m 3.¹⁸ The 10x histidine-tag and a Factor Xa cleavage site fused to the N-terminus for convenient purification of the recombinant protein increase the molecular mass to 46,641 d ([Fig 1, C](#)). The deduced amino acid sequence is composed of 4 putative glycosylation sites of the sequence Asn-Xaa-Ser/Thr ([Fig 2](#)). The motif RHGXRX within the Api m 3 sequence ([Fig 2](#)) provides evidence for an acid phosphatase because this sequence has been found in the active site of high-molecular-weight acid phosphatases analyzed so far by x-ray crystallography.²⁹⁻³¹ The Api m 3 sequence also contains other amino acid residues including His255 and Glu256, which have been reported to be essential for the enzymatic activity of acid phosphatases.^{32,33} The RHGXRX motif as well as the residue His255 are also present in acid phosphatases

FIG 2. Alignment of Api m 3 to acid phosphatase sequences. Shown is the alignment of Api m 3 to different insect acid phosphatases with significant homology. Amino acids necessary for acid phosphatase activity and for glycosylation are shaded in *grey*.

To study further the immunoreactivity of recombinant Api m 3, individual patient sera were assayed by ELISA for specific IgE antibodies to Api m 3. Fig 4, A, shows the reactivity in the sera of 40 patients who were characterized by a positive sIgE test to honeybee venom. The lower positive/negative cutoff of the recombinant Api m 3 sIgE ELISA was at 0.1 (OD_{450nm}) (Fig 4, solid line). A standard for total human IgE was run on each plate (Fig 4, D). Overall, 15 of 40 (37.5%) patient sera had detectable sIgE to recombinant Api m 3. All 40 sera showed various degrees of sIgE ELISA reactivity to native Api m 1 (not shown), which is in accordance with the high prevalence of allergen-specific IgE to Api m 1 in bee venom allergy.³⁴ In contrast with Api m 1-specific IgE, which showed linear correlation to honeybee venom-specific IgE, no such correlation was present for recombinant Api m 3-specific IgE. For control, sera from patients with no detectable sIgE to honeybee venom were used. Of these 19 patients, 10 patients were highly sensitized to *Vespa* spp venom but nonreactive toward honeybee venom (Fig 4, B), and 9 were individuals lacking serologic

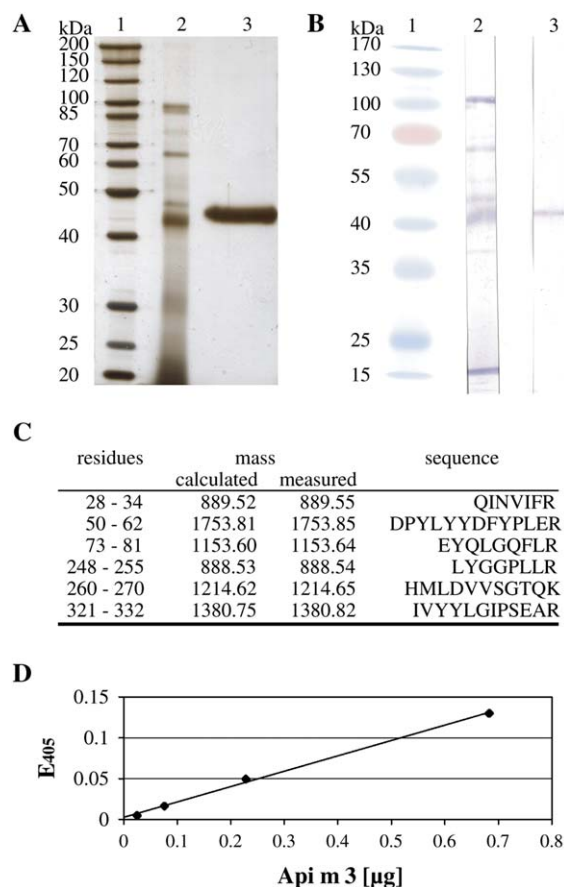


FIG 3. Api m 3 expression, purification, and identification. **A and B,** Silver-stained SDS-PAGE gel and IgE immunoblot with recombinant Api m 3. Lane 1, Molecular weight standards; lane 2, whole honeybee venom; lane 3, purified recombinant Api m 3. **C,** Tryptic fragments of recombinant Api m 3 identified by MALDI-TOF-MS. **D,** Enzymatic activity of purified recombinant Api m 3.

IgE reactivity to both hymenoptera venoms (Fig 4, C). Only 1 serum out of the group of 19 control sera showed reactivity with recombinant Api m 3, which was significantly different from the group of honeybee venom-sensitized individuals ($P < .01$; χ^2 test). A t test for unpaired samples comparing recombinant Api m 3 reactivity of the honeybee venom-sensitized group and the control group was significant ($P = .0131$).

DISCUSSION

On the basis of reported peptide sequences,¹⁹ cloning of honeybee venom acid phosphatase proved to be difficult. Therefore, we decided to follow a different approach for cloning of Api m 3. An Api m 3 DNA fragment—projected *in silico*—could be amplified by PCR from a honeybee venom gland cDNA.²³ Alignment of the sequence to the reported peptide fragments¹⁹ and the identification of sequence motifs important for the catalytic activity of high molecular weight acid phosphatases provided evidence for the identity of this DNA fragment with

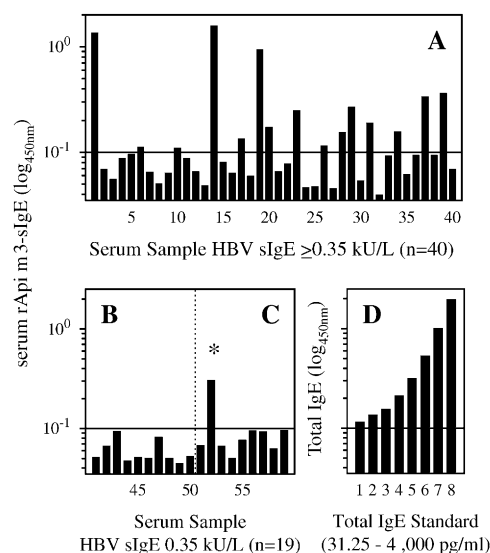


FIG 4. Immunoreactivity of individual sera with recombinant Api m 3. ELISA assay measuring the IgE antibody reactivity with rApi m 3 (log OD 450 nm). **A,** Honeybee venom (HBV)-sensitized patients (1-40). **B,** HBV-negative patients with IgE reactivity to vespid venom (41-50). **C,** Hymenoptera venom-negative patients (51-59). Sample 52 is marked with an asterisk. **D,** Serial standard for total human IgE (31.25-4000 pg/mL).

the Api m 3 coding sequence. Furthermore, during the preparation of this manuscript, an almost identical coding sequence for honeybee venom acid phosphatase differing in only 4 bases was identified by others.^{35,36} The reported differences in the 4 bases have no effect on the amino acid sequence, thus leading to an identical Api m 3 protein.

The deduced amino acid sequence of Api m 3 exhibits several characteristics that are common to high-molecular-weight acid phosphatases.³⁷ High-molecular-weight acid phosphatases hydrolyze phosphomonoesters in a 2-step mechanism involving an active site histidine.³⁸ The catalytic histidine is part of the characteristic amino acid sequence motif RHGXRXP, which is present in the active sites of all high-molecular-weight acid phosphatases analyzed by x-ray crystallography so far.²⁹⁻³¹ This sequence motif is also found in Api m 3 and related acid phosphatases from *Drosophila* species (Fig 2). Also present are His255 and Glu256, both of which have been identified by mutational analysis studies as essential for the enzymatic activity of acidic phosphatases.³¹⁻³³

Because honeybee venom acid phosphatase is a glycoprotein^{16,18} composed of 4 potential glycosylation sites as demonstrated in this study, eukaryotic insect cells were used as the expression system for recombinant Api m 3. Glycosylation can have profound effects on the binding of specific antibodies and appears to be functionally important for recombinant allergens—when the natural counterpart is a glycoprotein—by supporting intermediate folding forms and stabilizing the 3-dimensional structure of glycoproteins.³⁹⁻⁴¹ For example, the IgE-binding capacity of recombinant honeybee venom hyaluronidase expressed in *E coli* was strongly reduced compared with the native

allergen.^{11,42} When expressed in the baculovirus system, IgE binding proved to be similar to that of native hyaluronidase.¹¹ Carbohydrates may also represent IgE epitopes. For Api m 1, which is a glycoprotein,¹⁶ a fucose-containing carbohydrate chain has been reported to be an important IgE epitope,⁴³ although others questioned the relevance of carbohydrates for the allergenic activity of Api m 1.⁴⁴ Api m 3 also contains the necessary sequence information for glycosylation that provided the rationale for selecting an expression system allowing insect-specific glycosylation. The glycosylation pattern provided by other eukaryotic cells, for example by mammalian cells, has been reported to be different,⁴⁵ and at this point a potential effect of a different glycosylation on folding and epitope presentation of recombinant Api m 3 cannot be excluded.

The enzymatic activity of recombinant Api m 3 suggests proper folding of the protein. Furthermore, the immunoreactivity of recombinant Api m 3 in immunoblots and in ELISA analyses with IgE from honeybee venom-sensitized individuals demonstrates the presence of human IgE epitopes. As evident from Fig 4, more than 1/3 of the randomly selected honeybee venom sIgE-positive sera proved to be reactive with recombinant Api m 3. Previous studies using purified native Api m 3 demonstrated sIgE antibodies to Api m 3 in 60% of the sera of patients with honeybee venom allergy.³⁴ Possible reasons for the discrepant numbers between our study using recombinant Api m 3 and the study by Kemeny et al³⁴ using purified native Api m 3 may include the selection criterion for the patient sera, a differential sensitivity of the immunoassays for the detection of Api m 3 sIgE, loss of IgE binding epitopes in the recombinant Api m 3 allergen compared with its native counterpart, or residual contamination of purified natural Api m 3 with trace amounts of other prevalent allergens such as Api m 1 and/or Api m 4.

Of particular interest within our data is serum sample 52 among the group defined by a negative serologic result to honeybee venom (Fig 4, C). This patient had a clear-cut positive sIgE result in the recombinant Api m 3 ELISA. He reported to our clinic with a history of an anaphylactic reaction after a *Hymenoptera* sting. The offending insect was not identified. He had an elevated basal tryptase level (43.2 µg/L) suggesting occult mastocytosis, a negative intradermal test with honeybee and vespine venom, but a positive cellular allergy test with honeybee venom (increased sulfidoleukotriene release and basophil CD63 expression). A re-evaluation of the serum sample with a different sIgE system that is able to measure sIgE down to 0.1 kU/L under routine conditions⁴⁶ confirmed the presence of honeybee venom-specific IgE antibodies at a concentration of 0.23 kU/L. Thus, it can be speculated that a positive serologic result to honeybee venom could be missed because of a possible underrepresentation of Api m 3 in allergen test extracts. Similar observations were previously reported in latex allergy, in which spiking of natural extracts with recombinant Hev b 5 helped to increase sIgE test sensitivity.⁴⁷

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1  GAA CTT AAA CAA ATA AAT GTG ATA TTC CGG CAC GGC GAT AGG ATA CCC GAT GAG AAA AAC GAA ATG TAT
70  CCG AAA GAT CCT TAT TTG TAT TAT GAT TTT TAT CCA CTG GAG CGT GGC GAA TTG ACT AAC TCA GGT AAA
139 ATG CGA GAA TAT CAA TTG GGG CAA TTC TTG AGA GAG AGA TAT GGT GAC TTT TTG GGA GAC ATT TAC ACG
208 GAA GAA TCC GTC TCG GCT CTC AGC TCG TTC TAC GAT AGG ACG AAA ATG TCT CTG CAA CTC GTA CTC GCG
277 GCG CTC TAT CCG CCA AAT AAA TTG CAA CAA TGG AAC GAA GAT CTG AAC TGG CAA CCG ATC GCC ACG AAA
346 TAT TTG CGC CGC TAC GAG GAC AAT ATC TTT TTG CCA GAA GAT TGT TTG TTA TTT ACC ATC GAA CTT GAT
415 AGA GTA TTG GAA TCA CCG CGT GGA AAG TAT GAA TTC TCG AAA TAT GAC AAA TTG AAG AAA AAA TTG GAA
484 GAA TGG ACC GGA AAA AAT ATC ACT ACG CCA TGG GAT TAT TAT TAC ATA TAT CAT ACA CTG GTG GCT GAA
553 CAA TCG TAC GGT CTT ACT CTG CCA TCT TGG ACA AAT AAT ATA TTC CCG AGA GGA GAA TTG TTC GAT GCG
622 ACG GTA TTT ACG TAC AAC ATA ACC AAT TCG ACT CCT TTG TTG AAA AAA CTT TAT GGA GGT CCG CTT CTT
691 CGA ATA TTC ACC AAG CAT ATG TTA GAC GTG GTA TCG GGT ACG CAA AAG AAA AAG CGA AAG ATA TAC TTG
760 TTC AGT GGA CAT GAA AGT AAT ATC GCC TCT GTG TTG CAC GCT CTT CAA CTT TAT TAT CCT CAC GTT CCT
829 GAA TAT TCC AGT TCT ATT ATA ATG GAG CTT CAC AAT ATC GAA GGC ACT CAC TAC GTA AAG ATC GTT TAC
898 TAC TTG GGT ATC CCG TCT GAA GCG AGA GAA CTT CAA TTA CCC GGC TGC GAG GTA CTT TGC CCT TTG TAC
967 AAA TAT TTA CAA TTG ATA GAG AAC GTG ATA CCA TCG AAC GAA GAG TTG ATC TGC GAT AAA AGA TTC GTC
1036 GAC GAA TCG GCA AAC AAT TTG TCG ATC GAA GAA TTA GAT TTC GTG AAA TTG AAC CTA ATA AGG ATA GCG
1105 GGT ACT GAG AAT AAG TAA

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FIG E1. Nucleotide sequence of Api m 3. Shown is the sequence of the processed gene with native N-terminus (1122 base pairs corresponding to 373 amino acids).

Peptide No. (Ref. 19)	Sequences of published peptide fragments
1	ELKQINVIFRHGDRIPDEKNEMYPKKLEEWTDK
2	FVDESANNLSIEEIDFVK
3	LQQWNEDLNWQPIATK
4	GKYEFSKR
5	YNIFAGTWK
6	LYGGPLLRDNYVGDER
7	DITTPKDYYYIYHTLVAENEYSSCIIMEYHNIEGTHYVKIVYY LGIPSEARELQLPGCEVLCPLEKYLQLIENVIPSNEELICDKR

			*	20		40		*	
Api m 3 :	ELKQINVI	FRHGDRIPDEKNEMYPKDPYLYDFYPLR	GELTNSGKMREY :	50					
Ref (19):	ELKQINVI FRHGDRIPDEKNEMYPK KLEEWTDK								
	Peptide	1							
		60		*		80		*	100
Api m 3 :	QLGQFLRERYGD	FLGDIYTEESVSALSSFYDRTKMSLQLVLAALYP	PNKL :	100					
Ref (19):									L
			*	120		*		140	*
Api m 3 :	QQWNEDLNWQPIATKYLRRYEDNIFLPEDCLLF	TIELDRVLES	PRGKYEF :	150					
Ref (19):	QQWNEDLNWQPIATK							GKYEF	
	Peptide	3						Peptide	4
		160		*		180		*	200
Api m 3 :	SKYDKLK	KKLEEW	TGKNITTPWDYYIYHTLVAEQSYGLTLPSWTNNIFP :	200					
Ref (19):	SKR								
			*	220		*		240	*
Api m 3 :	RGELFDATVFTYNITNSTPLLKKLYGGPLLRI	FTKHMLDVVSGTQ	KKKRK :	250					
Ref (19):									LYGGPLL RDNVVGDER
					Peptide	6			
		260		*		280		*	300
Api m 3 :	IYLFSGHESNIASVLHALQLYYPHVPEYSSSIIMELHNIEGTHYVKIVYY :	300							
Ref (19):	DITTPKDYYYIYHTLVAENEYSSCIIMEYH NIEGTHYVKIVYY								
		Peptide	7						
			*	320		*		340	*
Api m 3 :	LGIPSEARELQLPGCEVLCPLYKYLQLIENVIPSNEELICDKRFVDESAN :	350							
Ref (19):	LGIPSEARELQLPGCEVLCPL E KYLQLIENVIPSNEELICDKRFVDESAN								
								Peptide	2
		360		*					
Api m 3 :	NLSIEELDFVKLNLI	RIAGTENK- :	373						
Ref (19):	NLSIEEIDFVK								

FIG E2. Alignment of published peptides to the deduced Api m 3 sequence. **A**, Peptides as described¹⁹ and labeled according to the original alignment. **B**, Alignment of published peptides to the translated Api m 3 sequence. Not all peptides could be aligned successfully; nonmatching stretches are highlighted in *light grey*.

TABLE E1. Oligonucleotide primer sequences

Name	Length	R-site*	Sequence
Api m 1 for	21mer	—	5'-ATA ATA TAT CCA GGA ACG TTA
Api m 1 back	30mer	<i>Sac</i> II	5'-GAC <u>CGC GGT</u> CAA TAC TTG CGA AGA TCG AAC
Api m 2 for	23mer	—	5'-CCC GAC AAC AAC AAA ACC GTA CG
Api m 2 back	30mer	<i>Sac</i> II	5'-GAC <u>CGC GGT</u> CAC ACT TGG TCC ACG CTC ACG
Api m 3 for	21mer	—	5'-GAA CTT AAA CAA ATA AAT GTG
Api m 3 back	32mer	<i>Sac</i> II	5'-AAC <u>CGC GGT</u> TAC TTA CTT ATT CTC AGT ACC CG
Api m 4 for	22mer	—	5'-GGA ATT GGA GCA GTT CTG AAG G
Api m 4 back	29mer	<i>Sac</i> II	5'-AGC <u>CGC GGC</u> TAA CCC TGT TGC CTC TTA CG
M13/Uni for	22mer	—	5'-GTA AAA CGA CGG CCA GTG CCA A
M13/Uni rev	22mer	—	5'-CAG GAA ACA GCT ATG ACC ATG A
melt leader for	33mer	<i>Hind</i> III	5'-GGA <u>AAG CTT</u> TCC GCC ATG GCG AAA TTC TTA GTC
melt leader back	27mer	<i>Bam</i> H I	5'-CGG <u>GAT CCC</u> GCA TAG ATG TAA GAA ATG
10xHis for	24mer	<i>Bam</i> H I	5'-CTG AAT AGC GCC <u>GGA TCC</u> GAC CAT
10xHis back	24mer	—	5'-CCC TCT AGA CTC GAG CCA ATG ATG
SOE Xa	32mer	<i>Eco</i> R V	5'-GGG <u>ATA TCC</u> CTT CCC TCG ATC CCT CTA GAC TC

**R-site* refers to restriction enzyme sites embedded in the primer sequence. If a restriction site is present in a primer, the position is underlined in the sequence.