

The major royal jelly proteins 8 and 9 (Api m 11) are glycosylated components of *Apis mellifera* venom with allergenic potential beyond carbohydrate-based reactivity

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Summary

Background As hymenoptera venoms are one of the allergen sources causing the highest incidence of anaphylaxis and sometimes fatal consequences, the detailed characterization of all venom allergens is imperative for design of component-resolved diagnostic approaches and improved intervention strategies.

Objective Our aim was the immunochemical characterization of major royal jelly proteins (MRJP) 8 and 9, both components identified in honeybee venom (HBV) and putative allergens.

Methods Both MRJPs were recombinantly produced as soluble differentially glycosylated proteins providing a defined degree of reactivity to cross-reactive carbohydrate determinants (CCD) in insect cells. Allergen-specific IgE (sIgE) reactivity of HBV-allergic patients was analysed by ELISA and immunoblotting.

Results MRJP8 and MRJP9 were identified as venom components by MS-based proteomic analyses. In a population of 47 HBV-allergic patients, reactivities with CCD-carrying MRJPs were in the range of 56% (61%), underlining the contribution of CCDs to allergen-binding. Beyond CCD-reactivity, 15% of patients showed sIgE reactivity with MRJP8 and 34% with MRJP9 respectively. These reactivities roughly in the range of Api m 2 render the MRJPs minor, but important allergens.

Conclusion and Clinical Relevance The glycosylated MRJP8 and MRJP9 of HBV have IgE-sensitizing potential in HBV-allergic patients beyond CCD reactivity and have to be considered as allergens, which might be potentially important for a fraction of venom allergic patients. They are valuable tools to elucidate individual component-resolved reactivity profiles of venom allergic patients and to provide insights into the role of particular venom components. Due to their allergenic properties, MRJP8 and MRJP9 were designated as isoallergens Api m 11.0101 and Api m 11.0201 respectively.

Keywords *Apis mellifera*, cross-reactive carbohydrate determinant, honeybee venom, hymenoptera venom, insect venom allergy, major royal jelly proteins, MRJP, sIgE

Abbreviations AP, alkaline phosphatase; CCD, cross-reactive carbohydrate determinant; HBV, honeybee venom; MRJP, major royal jelly protein; MS, mass spectrometry; NTA, nitrilo-triacetic acid; sIgE, specific IgE; VIT, venom immunotherapy.

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Introduction

Generalized systemic reactions to the venom of stinging hymenoptera have been recognized as a potentially fatal condition mediated by IgE antibodies in venom

allergic patients. The best known honeybee venom (HBV) allergens include phospholipase A₂ (Api m 1), hyaluronidase (Api m 2) and the basic 26 amino acid peptide melittin (Api m 4) [1], all constituting medium to higher abundance proteins with amounts of 12%, 2%

and 50%, respectively, of dry weight in the venom [2]. In recent years significant progress has been made in identification of additional HBV compounds of lower abundance. Recently, we could clone and express the acid phosphatase (Api m 3) [3]. Moreover, with the dipeptidylpeptidase enzymes allergen C (Api m 5) and its vespid homologue Ves v 3, we could describe a novel class of hymenoptera venom enzymes [4]. Moreover, we could demonstrate that the lower abundance allergen Api m 10 is a major allergen of HBV and of considerable interest for diagnostic as well as therapeutic purposes [5]. Other proteins include a putative protease inhibitor (Api m 6) [6], peptidases, esterases and proteases [7], some of which are listed in the databases and currently assessed for their allergenic potential (Api m 7–9). As all components may contribute to allergic sensitization, allergic symptoms and success of venom immunotherapy (VIT), their detailed characterization and assessment of their allergic potential is a prerequisite for the understanding of the molecular and allergological mechanisms of hymenoptera venoms.

In general, recombinant approaches are well suited to assess the allergenicity and the clinical relevance of such venom compounds. Thereby, the expression system should meet the requirements of folding and post-translational modifications, all of which are potentially important for the establishment of conformational epitopes [8]. In particular, glycan structures can contribute significantly to biochemical and structural characteristics of proteins and constitute the underlying principle of cross-reactive carbohydrate determinants (CCD), a peculiarity of hymenoptera and plant allergens interfering with diagnostic approaches [9–11].

Characterization of all venom proteins and putative allergens without the interference of CCDs is of considerable interest for the design of component-resolved diagnostic approaches and novel therapeutic strategies. Recently, by proteomic approaches, the major royal jelly proteins (MRJPs) 8 and 9 were demonstrated to be components of HBV [12, 13]. However, until now, no information concerning the allergic potential of both venom proteins and a potential role as new allergens of HBV is available.

The MRJPs are a family of highly homologous proteins, so far only identified in the genus *Apis*, although sharing a common origin with yellow-like proteins that are common in arthropods [14]. The honeybee genome encodes for nine MRJP genes arranged in a tandem array. Although all protein products have been identified, little is known about their function. They constitute ~90% of total proteins of royal jelly (RJ) [15–18], the substance produced, secreted and fed to developing larvae by nurse bees. The RJ is fed to all larvae only within the initial 3 days of development and then only to those designated to become queens pointing to its

dual role in nutrition and caste determination [14]. Moreover, all MRJPs show distinct temporal, spatial, sexual and caste-specific gene expression patterns in several honeybee organs, including the brain [14, 19]. Although scarce information about structure function relationships is available, e. g. a C-terminal fragment of MRJP1 was identified as an antimicrobial peptide [20], MRJPs seem to have evolved diverse physiological and developmental functions since their origin [14].

In this study, the allergenicity of MRJP8 and MRJP9 was addressed. The coding sequences of both proteins were cloned from venom gland cDNA and recombinant expression as differentially glycosylated proteins established by the baculovirus-mediated infection of insect cells. Sensitization to both putative allergens without interference of cross-reactive carbohydrates in a population of HBV-allergic patients was analysed via allergen-specific IgE measurement. The obtained data demonstrate the sensitizing potential of MRJP8 and MRJP9 and suggest a relevant role as allergens in HBV allergy.

Materials and Methods

Materials

Anti-V5 antibody was purchased from Invitrogen (Karlsruhe, Germany). Polyclonal rabbit anti-HRP serum as well as anti-rabbit-IgG alkaline phosphatase (AP) conjugate and anti-mouse IgG AP conjugate were obtained from Sigma (Taufkirchen, Germany). The monoclonal AP conjugated anti-IgE antibody was purchased from BD Pharmingen (Heidelberg, Germany). The MUXF-HSA conjugate was kindly provided by Siemens Healthcare Diagnostics (Los Angeles, CA, USA).

Patients

Sera from HBV-sensitized patients with HBV-specific IgE and/or positive intradermal skin test results were selected at random from the institutional serum bank. All patients had given their informed written consent to draw an additional serum sample, and all experiments applying human sera were approved by the local ethics committee.

Protein biochemistry

Four hundred microgram of *Apis mellifera* venom dissolved in 30 µL 5 × PAGE loading dye were subjected to SDS-PAGE. Bands in the range of 50–60 kDa were excised, the proteins digested in-gel by trypsin (Roche Diagnostics, Penzberg, Germany) and resulting peptide fragments were sequenced on a Waters Micromass QToF2 mass spectrometer (Waters, Milford, MA, USA)

by tandem mass spectrometry according to the manufacturer's instructions.

Cloning of cDNA

Total RNA was isolated from the separated stinger with attached venom sack and glands of honeybee (*A. mellifera carnica*) using peqGold TriFast™ (Peqlab Biotechnologie, Erlangen, Germany). SuperScript III RT (Invitrogen) and the gene-specific primers 5'-TCAAG-GAGATATGCAACGAGTATTCTTTATC-3' and 5'-TCAAA-GGAAAATTGAGAAAAAATTTGTTTTAGG-3' were used to synthesize cDNA of MRJP8 and MRJP9, respectively, from the isolated total RNA. The cDNA of mature MRJP8 was amplified using *Pfu* DNA polymerase (Fermentas, St. Leon-Roth, Germany) and the primers 5'-GTTACAGAC-ATTCATTCGAGAAATTTGAC-3' and 5'-TCA AGGAGA-TATGCAACGAGTATTCTTTATC-3'. The cDNA of mature MRJP9 was amplified using the primers 5'-CATTATTC-TTTGAGAGATTTCAAGGC-3' and 5'-TCAAAGGAAAAT-TGAGAAAAAATTTGTTTTAGG-3'. Subcloning for sequencing was done using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) with the pCR-Blunt II-TOPO vector.

Cloning and expression in insect cells

Subcloned MRJP8 and MRJP9 cDNA was used for secondary amplification of the coding region using *Pfu* DNA polymerase using the primers 5'-GATCTC-TAGAGTTACAGACATTCATTCGAGAAATTTGAC-3' and 5'-GATCGCGCCGCTCAAGGAGATATGCAACGAGTATTCTTTATC-3' for MRJP8 and 5'-GATCTCTAGACATTATTCTTTGAGAGATTTCAAGGC-3' and 5'-GATCGCGCCGCTCAAAGGAAAATTGAGAAAAAATTTGTTTTAGG-3' for MRJP9 respectively. The PCR product was subcloned via *Xba*I and *Not*I into the digested baculovirus transfer vector pAcGP67B (BD Pharmingen, Heidelberg, Germany) which was modified by addition of a N-terminal 10-fold His-tag, V5 epitope as well as a *Xba*I restriction site.

Recombinant baculovirus production

Spodoptera frugiperda cells (Sf9) (Invitrogen) were grown at 27°C in serum-free medium (Express Five SFM; Lonza, Verviers, Belgium containing 10 µg/mL gentamycin; Invitrogen, Karlsruhe, Germany). Cell density was determined by haemocytometer counts, cell viability was evaluated by staining with Trypan Blue. Recombinant baculovirus was generated by cotransfection of Sf9 cells with BaculoGold bright DNA (BD Pharmingen, Heidelberg, Germany) and the baculovirus transfer vector pAC-GP67-B containing MRJP8 or MRJP9. High titre stocks were produced by three rounds

of virus amplification and optimal multiplicity of infection for protein expression was determined empirically by infection of Sf9 cells in 100 mL suspension flasks (1.5×10^6 cells/mL in 20 mL suspension culture) with serial dilutions of high titre virus stock.

Expression in baculovirus-infected insect cells

High titre stocks of recombinant baculovirus was used to infect 400 mL suspension cultures of Sf9 or HighFive cells (Invitrogen) (1.5×10^6 cells per mL) in 2000 mL flasks. For protein production the cells were incubated at 27°C and 110 rpm for 72 h.

Protein purification

The supernatant of baculovirus-infected cells was collected, adjusted to pH 8, centrifuged at $4000 \times g$ for 5 min and applied to a nickel-chelating affinity matrix [nitrilo-triacetic acid (NTA)-agarose; Qiagen, Hilden, Germany]. The column was washed with binding buffer (50 mM sodium phosphate, pH 7.6, 500 mM NaCl) and pre-eluted with NTA-binding buffer containing 20 mM imidazole. The recombinant protein was eluted from the matrix using NTA-binding buffer containing 300 mM imidazole. Purification was confirmed by SDS-PAGE.

Western blotting

For immunoblot procedures, the purified recombinant allergens were separated by SDS-PAGE and immobilized onto nitrocellulose membranes. Human sera was diluted 1 : 10 with 5 mg/mL bovine serum albumin in PBS and applied to the corresponding Western blots. Visualization of bound IgE was then performed with anti-human IgE mAb conjugated to AP and nitroterazolum blue chloride/5-bromo-4-chloro-3-indoyl phosphate, according to the recommendations of the manufacturer. For Western blot procedures with anti-V5 epitope mAb and anti-HRP antiserum, the antibodies were applied according to the recommendations of the manufacturer and bound antibodies visualized via corresponding secondary antibodies conjugated to AP as described above.

Immunoreactivity of patient sera with recombinant proteins

For assessment of specific IgE immunoreactivity of human sera in ELISA, 384 well microtiter plates (Greiner, Frickenhausen, Germany) were coated with purified recombinant proteins (20 µg/mL) at 4°C overnight and blocked with 40 mg/mL milkpowder in PBS. Thereafter, human sera was diluted 1 : 2 with PBS and incubated in a final volume of 20 µL for 4 h at room temperature.

After washing four times with PBS, bound IgE were detected with a monoclonal AP-conjugated anti-human IgE antibody (BD Pharmingen, Heidelberg, Germany) diluted 1 : 1000. After washing four times with PBS, 50 µL of substrate solution (5 mg/mL 4-nitrophenyl-phosphate; AppliChem, Darmstadt, Germany) per well was added. The plates were read at 405 nm. The lower end functional cut-off indicated as lines was calculated as the mean of the negative controls plus two SDs. Reactivities only slightly higher than the cut-off were excluded. For ELISA procedures with anti-V5 epitope mAb and anti-HRP antiserum, the antibodies were applied according to the recommendations of the manufacturer and bound antibodies visualized via corresponding secondary antibodies conjugated to AP as described above.

Other methods

Molecular biology standard procedures such as PCR, DNA-restriction, ligation, transformation and plasmid-isolation were performed according to established protocols [21].

Results

Recombinant expression and characterization of MRJP8 and MRJP9

Systematic mass spectrometry (MS)-based proteomic analyses of *A. mellifera* venom proteins yielded three tryptic fragments from the molecular mass range of 50–60 kDa that could be assigned to major royal jelly protein 8 (MRJP8), and eight tryptic fragments that could be assigned to major royal jelly protein 9 (MRJP9) (Fig. 1), rendering them putative allergens with theoretical molecular masses of 45.1 kDa and 46.3 kDa respectively. Peptides of both proteins were recently identified by others, thereby supporting our data [12, 13]. For recombinant expression of both proteins, which share a sequence identity of 62% on protein level (Fig. 1), their coding sequences were amplified from HBV gland cDNA. The mature MRJP8 consists of 400 amino acids and contains six putative N-glycosylation sites, the mature MRJP9 consists of 403 amino acids with three putative N-glycosylation sites respectively.

Subsequently, both proteins were produced recombinantly by the Baculovirus-mediated expression in Sf9 (*S. frugiperda*) and HighFive (*Trichoplusia ni*) insect cells providing a varying degree of α 1,3-core fucosylation and, hence, CCD-based cross-reactivity. MRJP8 and MRJP9 were obtained from both cell lines in soluble and secreted form with yields of approximately 5–10 µg/mL of culture supernatant and showed a molecular weight of 60–65 kDa and 55–60 kDa respectively

(Fig. 2A). Discrepancies between theoretical molecular weights and apparent molecular weights observed in SDS-PAGE and immunoblot, which are more pronounced in the case of MRJP8, underline the contribution of the carbohydrates to the overall molecular characteristics.

Although the proteins produced in both Sf9 and HighFive cells are glycosylated, the use of a rabbit HRP antiserum, specific for α 1,3-core fucosyl residues, demonstrates that only the proteins produced in HighFive cells show a pronounced CCD-based cross-reactivity whereas Sf9-produced proteins do not (Fig. 2A, right panel). This expression strategy was described previously for other recombinant allergens [4, 5, 22, 23]. The ELISA analyses corroborated the data obtained in immunoblotting (Fig. 2B).

Taken together, these data demonstrate that insect cells are suitable hosts for the production of MRJPs. The proteins produced in Sf9 cells represent adequate target molecules to assess their relevance as proteinogenic allergens beyond carbohydrate-based cross-reactivity.

IgE immunoreactivity of differentially glycosylated MRJP8 and MRJP9

To evaluate the IgE immunoreactivity of the differentially glycosylated MRJPs produced in either Sf9 or HighFive insect cells, individual sera of patients with a clinical history of an allergic reaction after a stinging event were analysed by ELISA for specific IgE (sIgE) antibodies. The MUXF-HSA, a conjugate that provides the core-fucosylated glycotope from pineapple stem Bromelain was included as a marker for sIgE directed against CCDs. All patients were recruited during daily clinical practice and had sIgE for HBV (i1) and/or showed a positive result in intradermal skin test with HBV.

Of the 47 sera 25 (53.2%) showed IgE reactivity with MRJP8 produced in HighFive cells (Fig. 3A). As proteins produced in these cells exhibit CCD-based cross-reactivity, their specific reactivity includes protein- as well as CCD-specificity and mimics the natural situation. It is, however, not possible in such an approach to distinguish between these two modes and to evaluate true protein reactivity. Using Sf9-derived, CCD-devoid proteins lacking α 1,3-core fucose residues only seven sera (14.9%) showed pronounced IgE reactivity with MRJP8 (Fig. 3A). All other sera that reacted with the HighFive cell-derived protein exclusively exhibited pronounced reactivity with the CCD-marker MUXF. Overall 24 sera (51.1%) were reactive with MUXF-HSA and additionally showed reactivity with MRJP8 and MRJP9 from HighFive cells. The protein-specific IgE reactivity with MRJP9 was more pronounced as 16 sera (34%) showed

MRJP8	--MIRWLLMLGITGTCQGVTDIHSRNLT--NSLKVIYEWKYIDYDFGSDKEKRAAIQSGDY	57
MRJP9	MSFNIWLLILYFSIVCQAKAHYSLRDFKANIFQVKYQWKYFDYNFGSDEKRAAIQSGEY	60
	: * * : : : . * . . : . * : : * : : * * : : : * : : : : : * : : : : *	
MRJP8	NYTMNYLLDTDQWGDKTFVIMKFNGVPSSLNVITNKTGNGGPLLAPYPDWTWAKNENC	117
MRJP9	NYKNNVPIDVDRWNGKTFVTILRNDGVPSSLNVISNKGIGNGGPLLEPYPNWSWAKNQNCS	120
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MRJP8	GITSAYKIEIDMCDRLWVLDSGLINNVRVSCPPQLLVFDLNTSQLLKQVKIPHDIAVNTT	177
MRJP9	GITSVYRIAIDEWDRWLVDNG--ISGETSVCPQSQIVVFDLKNKSKLLQVKIPHDIAINST	179
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MRJP8	TEKGALVTLVSQLLSCEVNGSTLVYIGDNEGFALIIYNNSDNSFQRLTSSTFASDPRYTT	237
MRJP9	TGKRNVVTPIVQSFDYNN---TWVYIADVEGYALIIYNNADDSFQRLTSSTFVYDPRYTK	236
	* * : * * * : . : * * * . * * : : : * : : : : * : : : : * . * . *	
MRJP8	FTINGESFTLQSGIFGMALSPLTQNLYYSSALSSHNLNLYVNTTEQFVKSQYQANNVHYQGKE	297
MRJP9	YTINDESFSLQDGILGMALSHKTQNLYYSSAMSSHNLNLYVNTKQFTQKGKQANDIQYQAS	296
	: * * . * : : * . * : * : * * * : : * : : : : * : : : : * : : : : *	
MRJP8	NILWTQASAKGISDNGVLFFGLVGDTSLACWNNENRLLDRRNIEVVAKNKETLQAITGLKV	357
MRJP9	DILWTQASAKAISSETGALFFGLVSDTALGCWNNENRPLKRRNIEIVAKNNDTLQFISGKI	356
	: * : : * : : * . * . * : : * . * : : * : : * . * : : * : : * : : * : *	
MRJP8	KKRISFILVHGFPLEYEYVLAVSNRIQKVIYG--FDFNDVNFRILIANVNDLIKNTRCISP	416
MRJP9	IKQISSNIYE--RQNNHEYIWVSNKYQKIANGDLNFNEVNFRIINAPVNLIRYTRCENP	414
	: : * * : . : * : : * : : * : : * : : : : * : : : : * * : : * : *	
MRJP8	-----	
MRJP9	KTNFFSIFL 423	

Fig. 1. Alignment of MRJP8 and MRJP9. Alignment of database-derived sequences of MRJP8 (Genbank accession AAR83734) and MRJP9 (Genbank accession AAY21180.1) reveals an identity of 62% on protein level. Peptides identified by mass spectrometry are underlined. Signal sequences are italicized and putative glycosylation sites are highlighted in grey.

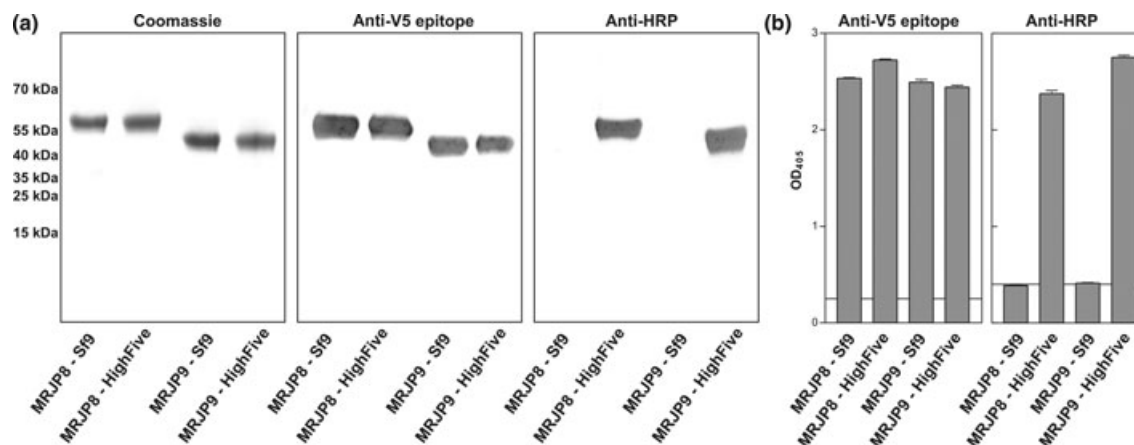


Fig. 2. Recombinant expression of MRJP8 and MRJP9 in insect cells. (A) SDS-PAGE and immunoblot analysis of MRJP8 and MRJP9 recombinantly produced in Sf9 or HighFive insect cells visualized by either Coomassie blue staining or monoclonal anti-V5 epitope antibody and HRP antiserum. (B) Immunoreactivity of recombinant MRJP8 and MRJP9 in ELISA using the anti-V5 epitope antibody and the HRP antiserum. Results are presented as triplicates.

specific reactivity with the Sf9 cell-derived protein and 29 (61.7%) with the HighFive cell-derived protein (Fig. 3A).

In sum, four sera (8.5%) were reactive with MRJP9 exclusively, five sera were reactive with proteinic epitopes of MRJP9 and in addition showed CCD-reactivity. Seven of the sera (14.9%) showed reactivity with MRJP8 and MRJP9 on protein level, six thereof exhibit-

ing additional CCD-reactivity and none of the sera showed MRJP8-specific IgE exclusively. Thirteen sera (27.7%) were solely CCD-reactive and the remaining 18 sera (38.3%) reacted with neither the MRJPs nor with CCDs (Fig. 3B).

To illustrate the distinct recognition patterns, the IgE reactivity of selected sera is shown in ELISA as well as by immunoblotting (Figs. 4A and B). Serum 1

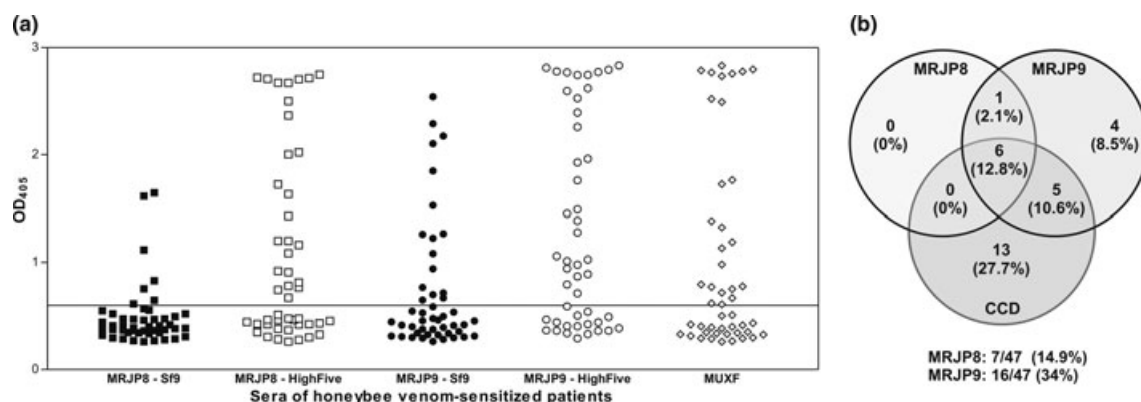


Fig. 3. IgE immunoreactivity of individual honeybee venom-sensitized patient sera with recombinant MRJP8 and MRJP9, produced in either Sf9 or HighFive insect cells. (A) The IgE reactivity was assessed by ELISA with 47 sera of honeybee venom-sensitized patients. MUXF-HSA conjugate served as a CCD marker. The lower end functional cut-off of the ELISA is represented by a solid line. (B) Schematic representation of obtained sIgE reactivities with proteinic epitopes of MRJP8 and MRJP9 as well as with CCDs.

specifically recognizes proteinic epitopes of MRJP8 and MRJP9 as indicated by the missing reactivity with the CCD-marker MUXF-HSA. In contrast, sera two and three also exhibit reactivity with proteinic epitopes of both proteins, but apparently CCDs contribute to this binding as suggested by the reactivity observed with MUXF-HSA and the pronounced reactivity with the HighFive cell-derived proteins. Serum 4 recognizes proteinic epitopes of MRJP9 exclusively, as indicated by the missing reactivity with MUXF-HSA and both variants of MRJP8. Serum 5 is reactive with CCDs and proteinic epitopes of MRJP9 as evident from the missing reactivity with CCD-free MRJP8 from Sf9 cells in ELISA and Western Blot. Finally, serum 6 is reactive with MUXF-HSA and the HighFive-derived MRJP8 and MRJP9, but not with the Sf9-derived non-fucosylated allergens, indicating exclusive CCD-reactivity.

These data demonstrate that the MRJP8 and MRJP9 from HBV are components with IgE-sensitizing potential beyond carbohydrate-based cross-reactivity in a population of HBV-allergic patients. Moreover, the contribution of CCDs to pronounced IgE reactivity was demonstrated using differentially glycosylated allergens.

Discussion

This is the first study focusing on the IgE-sensitizing potential of MRJPs as components of HBV. In the last years, several new proteins have been identified in HBV, mostly by proteomic approaches, and many, if not all of them proved to be important allergens. Hence, detailed knowledge is imperative for the design of component-resolved diagnostic approaches and advanced therapeutic strategies. Peptides of MRJP8 and MRJP9 have already been identified some years ago and their expression in the venom gland was verified by RT-PCR

and the presence of signal peptides in both molecules [12, 13]. Herein, we analysed HBV by MS/MS-based strategies in the range of 50–60 kDa and, in addition to peptides of the acid phosphatase Api m 3, we also detected peptides of both MRJPs.

Apart from venom, the major source of MRJPs is the RJ itself, which is used as a health tonic, especially in Asia, and is commonly found in a variety of products including cosmetics, food supplements and beverages as well as in commercial medical products [24]. There are several reports of anaphylaxis and asthma due to the ingestion of RJ [25–30]. The prevalence of skin test reactivity to RJ among adult asthmatic patients in Hong Kong was 16.8% [31] and approximately 7% among hospital employees, whereby a positive association between skin test reactivity and atopy was observed [32]. In the latter study, 18 different IgE-binding proteins of RJ were identified by immunoblotting and one component with a molecular weight of 55 kDa, which might correspond to one or different MRJPs, was detected by all the reactive sera. In another study, MRJP1 and MRJP2 were identified as major allergens of RJ [24]. Although an uncommon source of allergy [33, 34], one of the major allergens of honey was identified as MRJP1 [35, 36] and the IgE reactivity of proteinic epitopes demonstrated [36]. A clinically relevant cross-reactivity between HBV and RJ or honey so far remains object of speculation. The sequence identity of the nine MRJPs, ranging between approximately 40% and 70%, however, makes it likely that at least some immunological epitopes are shared. One study focused on a comparison of IgE reactivity with HBV and RJ and no direct relationship was observed, although 38% of sera from patients with HBV allergy were additionally reactive with a RJ solid phase [29]. Unfortunately, in this study, interference by CCDs was not taken into account, a problem that is emphasized by the finding

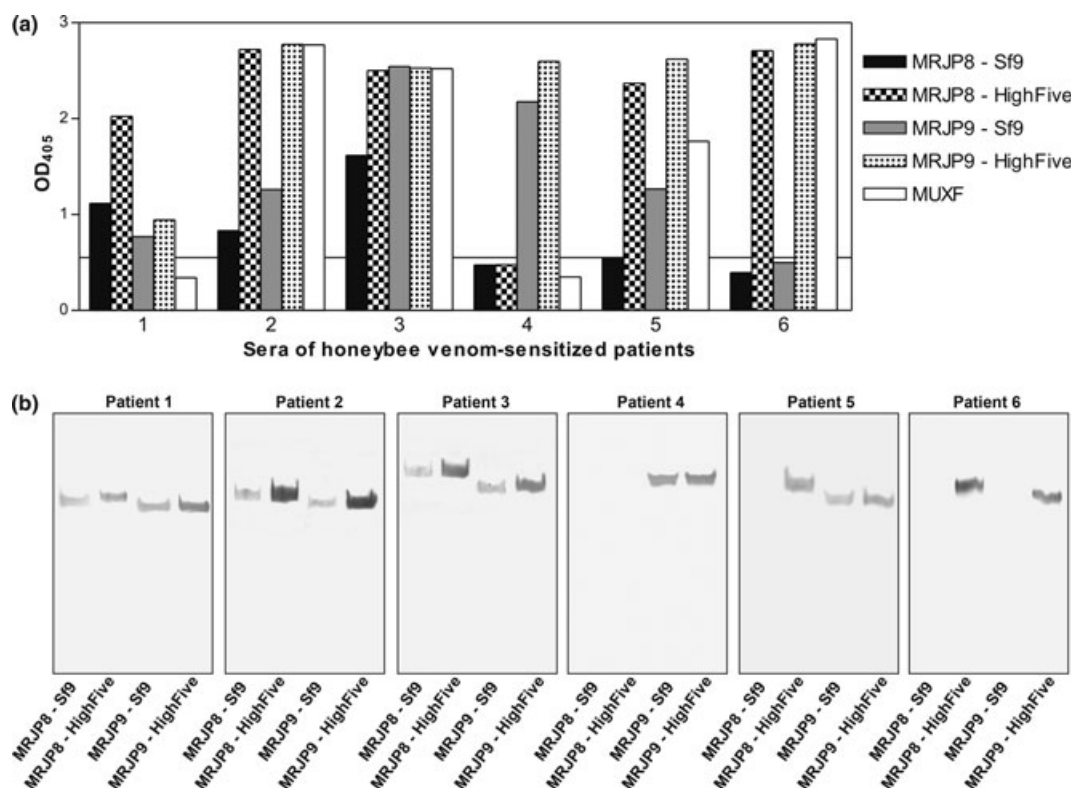


Fig. 4. IgE reactivity of selected representative patient sera with MRJP8 and MRJP9, produced in Sf9 and HighFive insect cells. (A) The IgE reactivity of individual sera from honeybee venom-sensitized patients with MRJP8 and MRJP9 expressed in Sf9 and HighFive cells as well with the MUXF-HSA conjugate was assessed in ELISA. The lower end functional cut-off of the ELISA is represented by a solid line. (B) In parallel, the reactivity of the selected sera with the recombinant MRJPs was assessed in immunoblot. The patients 1 to 6 correspond to clinical patient data 37, 28, 21, 20, 27 and 31 in supplemental data table 1.

that even 52% of patients with inhalant or food allergies were reactive to RJ. The MRJPs 1–5 represent the majority of the proteins in RJ and the MRJPs 6–9 only minor components [15, 17, 37, 38]. In contrast, only MRJP8 and MRJP9 so far were identified as components of HBV underlining the multifunctional role of MRJPs in the whole honeybee organism beyond a mere nutritional function. MRJP8 and MRJP9 are the most ancient members of the MRJP family and have evolved prior to the occurrence of the nutritionally important nitrogen-rich repeats of other MRJPs. The function of MRJPs in the venom is unclear, but this also holds true for other venom allergens.

Despite low case numbers of RJ and honey allergy, and the role of MRJPs as allergens in the venom, possible allergic cross-reactivity of the venom and honeybee-derived nutritional products might be relevant in some cases and should be taken into account. Nevertheless, due to the different routes of exposure, most honey and RJ allergic individuals are atopic [29, 32, 33] due to sensitization through the intestine, in contrast to BV allergic patients sensitized through the skin.

To address the sensitization to both MRJPs in a group of HBV-allergic patients, however, we produced soluble

recombinant MRJP8 and MRJP9 applying the baculovirus-based expression in Sf9 and HighFive insect cells. The differences between the predicted molecular weights and the apparent molecular weights in SDS-PAGE, which is more pronounced in the case of MRJP8, can be contributed to extensive posttranslational modifications. Actually, MRJP8 and MRJP9 carry six and three putative N-glycosylation sites, respectively, from which at least some must be occupied.

IgE may be directed against xenobiotic CCDs provided by a broad panel of proteins in food, pollen and hymenoptera venom [9]. The hallmark of classical CCDs comprises carbohydrates carrying α 1,3-linked core L-fucose residues on insect venom allergens and an additional β 1,2-linked D-xylose on plant-derived glycans.

The role of CCDs for occurrence of allergic symptoms still is controversial [39]. Recently, a novel type of CCD, Gal- α -1,3-Gal structures, has entered the field of CCDs. IgE-mediated carbohydrate specific anaphylaxis via Gal- α -1,3-Gal structures as present on the chimeric antibody cetuximab provided final evidence for the detrimental potential of glycan epitopes [40].

The exact epitopes of CCD-specific IgE remain enigmatic, although we could recently define the α -Gal

epitope using NMR [41]. For xylose and fucose CCDs, structural analyses suggest a specific and independent binding [42, 43]. Such analyses suggest that core fucose, core xylose or 1,3-galactose represent well defined IgE epitopes.

With regard to the CCD-reactivity, the use of Sf9 insect cell lines constitutes a strategy recently reported by us to circumvent the establishment of CCDs [5, 23], while maintaining the advantages of an autologous eukaryotic expression system. In addition to these obvious advantages in using insect cell lines for solving the CCD issue, we and others have demonstrated that production of insect venom allergens in insect cells is generally superior in terms of functionality, epitope authenticity and folding [4, 8, 23]. That this also holds true for MRJPs is underlined by the only other report concerning the recombinant production of MRJPs, MRJP1 and MRJP2 from *Apis cerana*, which could be produced in *Escherichia coli* only in insoluble form [44].

Applying the differentially glycosylated MRJPs, approximately 15% of HBV-allergic patients showed sensitization to MRJP8 and approximately 34% to MRJP9, respectively in ELISA beyond CCD-reactivity. These percentages were even higher with the HighFive cell-derived MRJPs displaying CCDs. In the group of patients recognizing proteinic epitopes, 19.1% showed reactivity with MRJP9, but not with MRJP8, sharing 62% identity on protein level. The remaining 14.9% showed reactivity to both MRJPs, but none of the patients showed reactivity with MRJP8 exclusively. If this is based on cross-reactive epitopes, it should be addressed in further studies by competition experiments. Would this be the case one might speculate that MRJP9 is the primary sensitizing allergen and that reactivities to MRJP8 might be attributed to cross-reactivity? One might further speculate that the extenuated reactivity on protein level of MRJP8 compared to MRJP9 can be due to the higher extent of glycosylation

of MRJP8 and a kind of masking of proteinic epitopes. This, however, was not observed in the case of the highly glycosylated allergen Api m 10 as described previously [5].

The obtained data suggest that MRJP8 and MRJP9 have to be considered as relevant allergens of HBV with IgE-sensitizing potential in a fraction of HBV-allergic patients. Due to their allergenic properties, MRJP8 and MRJP9 have been designated as isoallergens Api m 11.0101 and Api m 11.0201, respectively, by the International Union of Immunology Societies.

In summary, in this study the MRJPs 8 and 9 from HBV for the first time were cloned, produced by the baculovirus infection of insect cells and assessed for their allergenic potential. The obtained results clearly demonstrate an IgE-sensitizing potential beyond carbohydrate-based cross-reactivity, which is more pronounced in the case of the less abundantly glycosylated MRJP9. Moreover, the data suggest a role as relevant allergens for MRJP8 and MRJP9 in HBV allergy. The recombinant, CCD-free MRJPs present valuable tools to clarify molecular cross-reactivities with other allergen sources and to elucidate individual component-resolved reactivity profiles of venom-allergic patients, and to provide insights into the role of particular venom components which might help to elucidate the needs and pitfalls of successful VIT.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Serological data of patients assessed in IgE reactivity analysis.

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Supplemental data table I: Serological data of patients assessed in IgE reactivity analysis

The sIgE levels for honeybee venom (HBV) (i1) and yellow jacket venom (YJV) (i3) were determined with the Immulite 2000 (Siemens Healthcare Diagnostics, Los Angeles, Ca.) or ImmunoCap 250 (Phadia, Uppsala, Sweden), and for MRJP8, MRJP9, and MUXF-HSA as described for Fig. 3 (lower end functional cutoff: OD₄₀₅ = 0,6). For intradermal testing of patients with suspected insect venom allergies serial 10-fold dilutions of venom extracts with concentrations ranging from 0.0001 to 0.1 mg/L were performed. Histamine hydrochloride and physiologic saline were used as positive and negative control solutions, respectively. Intradermal tests were rated positive when the wheal size was >5 mm in diameter with a surrounding erythema.

Patient I.D.	Skin test ¹		sIgE – Extract ²		sIgE – Allergens ³				
	HBV (µg/ml)	VV (µg/ml)	i1 (kU/L)	i3 (kU/L)	MRJP8 Sf9 (OD _{405nm})	MRJP8 HighFive (OD _{405nm})	MRJP9 Sf9 (OD _{405nm})	MRJP9 HighFive (OD _{405nm})	MUXF-HSA (OD _{405nm})
1	0,001	negative	13,4	1,58	0,3677	0,9104	0,4784	1,0134	0,6100
2	0.001	0.01	0,436	0,191	0,3209	0,3013	0,3417	0,3627	0,2903
3	0,01	negative	0.62	0.63	0,2875	0,2781	0,2632	0,2918	0,2650
4	0,0001	negative	60.8	0.521	0,3078	0,2841	0,2838	0,3685	0,3296
5	0,001	negative	0,76	0,15	0,5547	0,4236	0,5372	0,5904	0,5088
6	0,001	negative	27.6	<0.1	0,5220	0,4443	0,5878	0,4430	0,3838
7	0,01	negative	6.59	0.224	0,4755	0,4775	1,2266	1,4543	0,4140
8	0.1	negative	2.33	<0.1	0,4189	0,5120	2,2902	2,3947	0,3952
9	0,001	0,1	10,2	14,5	0,3381	0,7667	0,3576	0,7955	0,7527
10	0,0001	negative	23,8	2,19	0,4020	2,6714	0,5312	2,7947	2,7769
11	0,0001	negative	72,7	0,124	0,3656	0,8150	1,0828	1,4936	0,7186
12	0,01	0,1	3,21	2,17	0,6488	2,0046	0,7005	2,7694	2,4933
13	0,001	negative	35,9	0,889	0,3650	0,4257	0,3940	0,4058	0,3379
14	0,01	negative	81,4	3,31	0,4631	2,7499	0,6511	2,8348	2,7876
15	0,0001	0,01	1,89	0,19	0,4055	0,4649	0,4163	0,5053	0,3323
16	0,01	0,01	4,32	8,31	0,5213	1,1628	0,5468	0,9801	0,6690
17	0,0001	negative	3,26	0,11	0,4718	0,4660	0,7162	0,7121	0,4038
18	0,001	0,001	1.33	0.467	0,3870	0,3819	0,3239	0,3640	0,2863
19	0,01	0,01	3,88	0,4	0,4700	0,7828	0,9425	0,8697	0,6201

20 ⁴	nd	nd	20,5	1,42	0,4748	0,4760	2,1767	2,5967	0,3484
21 ⁴	nd	nd	22,2	2,82	1,6155	2,5012	2,5425	2,5280	2,5220
22	0,01	0,001	3,15	4,46	0,2714	0,3276	0,3260	0,3642	0,3266
23	0,01	0,001	32,9	14,4	0,3449	2,6739	0,4574	2,7462	2,7578
24 ⁵	nd	nd	3,54	6,75	0,6148	1,6337	0,6705	1,9634	1,3261
25	0,1	0,0001	1,76	16,4	0,2629	1,0876	0,2941	1,3894	1,3843
26	0,01	0,01	5,46	3,64	0,5501	2,3679	1,2669	2,6215	1,7649
27	0,1	0,0001	14,1	20	0,8311	2,7211	1,2616	2,7731	2,7678
28	0,0001	0,001	13,8	17,8	0,4079	1,4324	0,4469	1,9295	1,7274
29	0,01	negative	0,62	0,63	0,2963	0,3498	0,3142	0,3402	0,2929
30	0,1	0,01	5,94	2,14	0,3934	2,7093	0,4988	2,7798	2,8325
31	0,001	0,0001	1,44	6,04	0,3881	1,7250	0,4650	1,7627	1,1886
32	0,01	0,001	25,9	>100	0,7551	2,7056	1,8505	2,7458	2,7347
33	0,1	0,01	0,163	4,34	0,3488	0,4330	0,3392	0,4685	0,4221
34	0,001	negative	>100	<0,1	0,2880	0,3688	0,3139	0,4255	0,4004
35	0,001	negative	27,2	1,57	0,4973	0,6724	0,4115	0,8925	0,7779
36	0,0001	negative	5,44	1,43	1,1175	2,0242	0,7686	0,9445	0,3424
37	0,0001	0,01	1,19	4,86	0,2797	0,9209	0,3049	1,2801	1,1329
38	0,001	0,001	5,2	0,77	0,2719	0,4216	0,3007	0,4436	0,4344
39	0,0001	0,01	4,01	0,266	0,3732	0,4656	0,4017	0,5417	0,5012
40	0,1	negative	3,8	0,25	0,5696	1,2020	1,5306	2,2599	0,7960
41	0,01	0,01	3,1	5,51	0,4103	0,4545	0,4201	0,4931	0,2979
42	0,0001	0,1	6,28	1,18	0,3835	0,7457	0,4551	1,0600	0,9814
43	0,001	0,1	16,1	0,361	0,3617	0,4274	0,3082	0,4070	0,3513
44	0,01	negative	>100	6,84	1,6470	2,7165	2,1054	2,8133	2,7965
45	0,01	negative	5,24	0,508	0,3740	1,2015	0,3785	1,0282	0,7726
46	0,0001	0,001	1,01	0,362	0,4436	0,3069	0,3759	0,3878	0,3139
47	0,01	negative	1,55	<0,1	0,3470	0,2620	0,2970	0,3316	0,2609

¹ Shown is the lowest venom extract concentration that gave a positive result in intradermal skin testing applying a ten-fold concentration range from 0.1 to 0.0001 µg/ml for each of the venoms.

² As tested in either UniCAP250 or Immulite2000 sIgE assays using a lower end cut-off of 0.1 kU/L.

³ As tested in an IgE ELISA using a lower end cut-off of 0.6 as described in the methods section.

⁴ No skin test was performed, but these patients were medicated in the casualty department due to a clear clinical reaction after a honeybee sting.

⁵ Due to a clinical history of Urticaria factitia for this patient the skin test is not evaluable.

nd, not determined