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MRJP9, an ancient protein of the honeybee MRJP family with non-nutritional function

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Summary

Major royal jelly proteins (MRJPs) form a subfamily within a larger Yellow/MRJP family of proteins. Whereas multiple Yellow-like proteins have been found in all insect genomes including honeybee, MRJPs seem to be restricted to honeybees. Eight MRJP proteins, termed MRJP1-8, have been characterized so far. We found that proteomic analyses of bee hypopharyngeal glands and venom sac identified proteolytic fragments of a protein, which might be a novel member of the MRJP family. Using this information and availability of honeybee genome, we cloned the corresponding cDNA and confirmed by sequencing that it encodes an uncharacterized MRJP9 protein. Interestingly, a cDNA coding for a relative of MRJP9 was found in paper wasp EST library. By phylogenetic analyses we show that MRJP9 is an ancient member of the MRJP family, which diverged prior to occurrence of nutritionally valuable nitrogen-rich repeat regions in MRJPs. Identification of MRJP9 in several bee organs, its comparatively low expression levels there, together with the existence of a MRJP-like protein in wasp indicate that it performs function(s) different from larval nutrition. A novel hypothesis on possible role of MRJP9 found in bee venom as a possible immunosensitizing factor contributing to allergy against royal jelly is discussed.

Keywords: Royal jelly, bee venom, *Apis mellifera*, cDNA, nutrition, allergy, phylogeny, MRJP, yellow.

Introduction

Major royal jelly protein (MRJP) family is a group of highly homologous proteins found in honeybees, which shares common origin with yellow-like proteins (Drapeau, *et al.*, 2006). In contrast to MRJPs found so far only in honeybees (Klaudiny, *et al.*, 1994; Ohashi, *et al.*, 1997; Schmitzova, *et al.*, 1998; Albert and Schmitz, 2002; Srisuparbh, *et al.*, 2003; Su, *et al.*, 2005), yellow-like proteins are present in all insects but there are no yellow proteins in vertebrates (Maleszka and Kucharski, 2000; Drapeau, *et al.*, 2006; Xia, *et al.*, 2006).

Five MRJP proteins were identified as major components of a secreted food provided by nurses to bee larvae and queen, termed royal jelly (RJ) (Schmitzova, *et al.*, 1998; Sano, *et al.*, 2004; Scarselli, *et al.*, 2005). The cDNAs encoding these proteins (termed MRJP1-5) were characterized as abundant cDNAs using the cDNA library prepared from nurse honeybee heads or hypopharyngeal glands (Klaudiny, *et al.*, 1994; Ohashi, *et al.*, 1997; Schmitzova, *et al.*, 1998; Albert, *et al.*, 1999a). The expression of

the MRJP1-5 varies from high levels in the heads (hypopharyngeal glands) of nurses to low levels in foragers (Ohashi, *et al.*, 2000). The abundant MRJPs seem to have nutritional function consistent with high percentage of essential amino acids found in MRJPs (Schmitzova, *et al.*, 1998) as well as with the appearance of extensive nitrogen-rich repetitive regions in the primary structure of several MRJPs (Albert, *et al.*, 1999a; Albert, *et al.*, 1999b; Albertova, *et al.*, 2005). MRJP1 seems to have additional roles in honeybee physiology. Its expression was documented in mushroom bodies of bee brains (Kucharski, *et al.*, 1998) and its C-terminal proteolytic fragment was identified as an antimicrobial peptide jelleine in RJ (Fontana, *et al.*, 2004). Besides that, several biological activities in heterologous systems have been assigned predominantly to MRJP1 and MRJP3 (Kamakura, *et al.*, 2001; Matsui, *et al.*, 2002; Okamoto, *et al.*, 2003; Salazar-Olivo and Paz-Gonzalez, 2005; Majtan, *et al.*, 2006).

Three cDNAs encoding additional members of the MRJP family (MRJP6-8) were identified among less abundant cDNAs in the bee brain EST library (Whitfield, *et al.*, 2002; Albert and

Klaudiny, 2004). It was hypothesized that the amounts of MRJP6-8 are too low for their identification at the protein level. Indeed, initial proteomic analyses of RJ based on two-dimensional electrophoretic separation of proteins followed by N-terminal sequencing of protein spots (Sano, *et al.*, 2004), or by mass spectrometry sequencing of tryptic peptides derived from proteins in spots (Scarselli, *et al.*, 2005) did not identify the MRJP6-8 proteins. However, recent proteomic analyses of bee hypopharyngeal glands extracts led to identification of not only major (MRJP1-5) but also three minor MRJPs (Santos, *et al.*, 2005). Here we report on identification and characterization of a full cDNA encoding a novel MRJP9 protein that bears the peptide of a hypopharyngeal protein identified by Santos *et al.* (2005), as well as all peptides of an orphan protein identified by proteomic analysis of bee venom (Peiren, *et al.*, 2005)

Materials and Methods

Homology searches and assembly of DNA sequences, phylogenetic analyses

NCBI BLAST (Altschul, *et al.*, 1990) server was used for searches in the database of *A. mellifera* genomic sequences (<http://www.ncbi.nlm.nih.gov/blast/>). BLAST program was employed for searches at the honeybee brain EST server (http://titan.biotech.uiuc.edu/bee/honeybee_project.htm) and at the server containing the *Apis mellifera* genome (Amel_2.0) at Baylor College of Medicine (<http://www.hgsc.bcm.tmc.edu/projects/honeybee>). The sequence of the MRJP9 cDNA was assembled at a local computer using the EMBOSS program package (Rice, *et al.*, 2000). Phylogenetic analyses were done with aligned sequences using CLUSTAL X (Thompson, *et al.*, 1997) and Gonnet protein weight matrix. Am-yellow-f rooted phylogenetic tree was calculated with exclusion of positions with gaps and with correction for multiple substitutions. Branch stability has been tested by Bootstrap analysis (1000 repetitions).

PCR amplifications

The complete coding region of the MRJP9 cDNA and of 3' and 5' ends of the cDNA were determined by polymerase chain reaction (PCR) using a cDNA library (Lambda ZAP, Stratagene) from heads of nurse honeybees (Klaudiny *et al.*, 1994). The 5' end fragment of the cDNA was amplified with the T3 primer priming in the cloning vector: 5'-AAT TAA CCC TCA CTA AAG GG-3' and MRJP9 reverse primer (#32): 5'-ACA CTC GTA ATA CCA GAA CAG-3'. The 3' end fragment was amplified with MRJP9 forward primer (#33): 5'-AAG AAA AAT AGG AAG ATC AAA GG-3' and T7 primer in the vector: 5'-TAA TAC GAC TCA CTA TAG GG-3'. Polymerization reactions were done in 50 µl volume as follows: 5 µl of the cDNA library stock were used as a template in a modified hot start procedure. After initial denaturation for 5 min at 94°C, subsequent cooling to 85°C and addition of 2 U of Taq polymerase (AmpliTaq, Perkin Elmer), PCRs were performed in a standard PCR buffer containing 1.5 mM MgCl₂, 0.1 mM dNTP and 0.35 µM of each primer: 35 PCR cycles (40 s at 94°C, 50 s at 50°C and 120 s at 72°C) were run, followed by 10 min incubation at 72°C.

Cloning and DNA sequencing

PCR products were size-separated by gel electrophoresis (1.5% agarose), purified by QIAEX II kit (Qiagen) and ligated into PCR2.1 TA cloning vector (Invitrogen) or sequenced directly. DNA sequences were determined by the cycle sequencing method (Prism Ready Reaction BigDye Terminator kit, Perkin Elmer) on an ABI 373A automatic sequencer. Obtained DNA sequences were assembled and analyzed using program packages mentioned above.

Results

Detailed analysis of peptide sequences published by Santos *et al.* (2005) revealed that one of the identified peptides, identified as MRJP8, differs from the sequence inferred from the MRJP8 cDNA (Albert and Klaudiny, 2004) in a way which can hardly be assigned to genetic variability or experimental error: 9 out of 26 amino acids differ and one amino acid is deleted (Fig. 1).

MRJP8	LWVLDSGLINNVR	SVCPPQLLVFDLN
spot #42	LWVLDSGLINNVR	SVCPPQLLVFDLN

Fig. 1. A. Amino acid sequence alignment of a tryptic peptide isolated from a protein spot number 42 of 2-D-separated honeybee hypopharyngeal glands extract (Santos *et al.* 2005) with the fragment of MRJP8 protein. Although initial 5 amino acids are identical in both sequences, further differences prompt that these do not belong to the same protein.

We searched the early editions of honeybee genome for a homology with the peptide identified by Santos *et al.* (2005) and found a genomic contig termed Un1029, which encodes a protein with 100% homology to this peptide. The contig Un1029 is 17,349 bp long and harbors a gene with a structure similar to other *mrjp* genes (Drapeau, *et al.*, 2006). The ends of the mRNA could not be unequivocally determined. Therefore, the primers for 5' and 3' RACE were designed and obtained PCR products sequenced. The assembled MRJP9 cDNA is 1859 base pairs long and contains a complete open reading frame coding for a new member the MRJP family (Fig. 2), which we termed MRJP9. The encoded MRJP9 protein consists of 423 amino acid residues. In its primary structure it contains the peptide identified in hypopharyngeal glands by Santos *et al.* (2005) and all six peptides of the protein identified in venom sac proteome (Peiren, *et al.*, 2005), each preceded by arginine or lysine residue, consistent with tryptic digestion of the proteins for analyses (Fig. 2, underlined). Of note, no tryptic peptides derived from the MRJPs highly expressed in RJ were found in venom sac confirming that identification of MRJP9 is not due to sample contamination but an inherent property of the protein. In addition, a fragment of MRJP9 cDNA was also identified in the EST library prepared from honeybee antennae (GenBank Accession number BE844327). Further search in databases for the homologs of MRJPs revealed an MRJP-like EST (GenBank accession number DT319131), which is differentially expressed among young, worker and queen castes of a paper wasp *Polistes canadensis* (Sumner, *et al.*, 2006).

1	GAGAAAGCCAATATGTCTTTCAATATCTGGTGGTTGATACTGTATTTTAGTATAGTTTGT	60
1	M S F N I W W L I L Y F S I V C	16
61	CAAGCAAAAGCCCATTTCTTTGAGAGATTTCAAGGCAAATATCTTCCAAGTTAAATAT	120
17	Q A K A ↑ H Y S L R D F K A N I F Q V K Y	36
121	CAATGGAAATACTTCGATTATAATTTTGGTAGTGATGAAAAGAGACAAGCTGCAATTCAA	180
37	Q W K Y F D Y N F G S D E K R Q A A I Q	56
181	TCTGGCGAATACAATTATAAGAATAATGTTCCAATAGACGTCGATCGATGGAATGGTaaa	240
57	S G E Y N Y K N N V P I D V D R W N G K	76
241	acttttgtcaccatattaagaaatgatgggtgtgccttcgtcttgaacgtgatatctaac	300
77	T F V T I L R N D G V P S S L N V I S N	96
301	aaaattggcaatgggtggaccacttttgaaccatatccaaattggctcgtgggcaaaaaat	360
97	K I G N G G P L L E P Y P N W S W A K N	116
361	caaaactgttctggtattacagagtgtttacagaattgcgattgacgaatgggatagatta	420
117	Q N C S G I T S V Y R I A I D E W D R L	136
421	tgggttttagacaatgggtattagcggtagacatctgtatgcccttcgcagattgtcgtc	480
137	W V L D N G I S G E T S V C P S Q I V V	156
481	tttgatctaataaaattcaaaattgttaaaacaagttaaaataccgcacgatattgcgata	540
157	F D L K N S K L L K Q V K I P H D I A I	176
541	aactctactactggaaaaagaaatgtagtaactccaatcgttcaaagttttgattataat	600
177	N S T T G K R N V V T P I V Q S F D Y N	196
601	aatacctgggtgtatatagcagacgtcgaaggttatgcattgattatttataacaatgcc	660
197	N T W V Y I A D V E G Y A L I I Y N N A	216
661	gacgattctttccaacgattgacttcgagcactttcgtatacgatcccagatacacaaa	720
217	D D S F Q R L T S S T F V Y D P R Y T K	236
721	tacactatcaatgacgaaagtctctcgttgcaagatggaattttgggtatggcgctaagt	780
237	Y T I N D E S F S L Q D G I L G M A L S	256
781	cataaaacgcaaaatctttattacagtgtatgtcctctcataatttgaattatgtcaac	840
257	H K T Q N L Y Y S A M S S H N L N Y V N	276
841	acaaaacaattttacacaagggaaatttcaggctaataatgatatacaatatcaaggAGCCTCA	900
277	T K Q F T Q G K F Q A N D I Q Y Q G A S	296
901	GATATCTTATGGACTCAAGCGAGCGCTAAAGCAATATCGGAAACTGGTGCTCTCTTTT	960
297	D I L W T Q A S A K A I S E T G A L F F	316
961	GGACTCGTGAGTGACACAGCACTTGGCTGCTGGAACGAGAATCGGCCACTAAAAAGAAGG	1020
317	G L V S D T A L G C W N E N R P L K R R	336
1021	AATATTGAAATAGTCGCCAAAAATAACGACACTCTTCAATTCATCAGTGGTATAAAATT	1080
337	N I E I V A K N N D T L Q F I S G I K I	356
1081	ATCAAGCAAATATCTTCAAATATTTATGAACGTCAAATAACGAGTATATATGGATTGTA	1140
357	I K Q I S S N I Y E R Q N N E Y I W I V	376
1141	AGTAACAAATATCAAAAATAGCAAATGGAGATTTAAATTTTAAATGAAGTGAATTTTCGA	1200
377	S N K Y Q K I A N G D L N F N E V N F R	396
1201	ATTTTGAATGCGCCTGTAAATCAATTAATAAGGTACACTCGTTGCGAAAATCCTAAAACA	1260
397	I L N A P V N Q L I R Y T R C E N P K T	416
1261	AATTTTTTCTCAATTTTCCTTTGATCTTCCTATTTTTCTTTCTATTGTAATGTCCAAATT	1320
417	N F F S I F L *	424
1321	AAATTATATGTTTATCAAAGATTTCTAGTTTTTTCTTTAAAAGTATGGAATAAATATTAT	1380
1381	ATAATATTATTTTCCATTTTAAATTTTATTTTAAAGAATAATTCTTTGTAGTTTCTTG	1440
1441	TTAGCGAGATTTTATAACTTTATGCGCAAATGTAGTATTTTAAAAAATATTTTCTTTATA	1500
1501	TTGCGAGATGAATAACATTTATCAGTTGTAAATTCTAATAACAATTTCTGATTTTAC	1560
1561	TGCTACTTAATTGCCGTCTTGCGTTTCCGAATAGAGGTTAAGATATTGCTAGAAATTTCA	1620
1621	ATGAGTCAGTGCTATTGAAAATATAATATTATGTTATAAAAAATTATTTTTCATCAGAG	1680
1681	TTATTTTATCTCTTTATAAATCTTATTGTTAAGTAATATTTATTATAGCTTATTCGTGA	1740
1741	TTTTTTCAAATTTATAAATTTGTTAAAAATCTTCGAAATATCATATTGAAAGATTAAAT	1800
1801	ATTGTGTAATTTTATCTTCAAAAAGAATTA AATA AAATTTTCAATATTCATTTATTAG	1859

..... peptides determined by Peiren et al. (2005)
 — peptide determined by Santos et al. (2005)

Fig. 2. Nucleotide sequence of MRJP9 cDNA and its conceptual translation. The encoded MRJP9 protein contains a region with 100% identity with the peptide identified by proteomic approach in the protein spot #42 of hypopharyngeal glands extract (Santos et al. 2005, underlined by solid line). Regions corresponding to the peptide sequences determined by sequencing of the bee venom protein spot #4 (Peiren et al., 2005) are underlined by a dotted line. Vertical arrow indicates the predicted signal protease cleavage site. Polyadenylation signal is bold. The sequence has been deposited in GenBank under accession number DQ000307.

DT319131	335	LVTPLVIARGKECQHITVYMADIEGYGLVVWRG-DDKFQRFESPAFAKNPKA
		+VTP+V + + + VY+AD+EGY L+++ DD FQR S F +P+
MRJP9	185	VVTPIV--QSFYNNNTWVYIADVEGYALIIYNNADDSFQRLTSSTFVYDPRY

DT319131	182	SNFDIDNDKFTLDDGVVGLAMHIGEPSLKCKTLFCNSLYFSSLTSYNMYHVS
		+ + I+++ F+L DG++G+A L KT +LY+S+++S+N+ +V+
MRJP9	234	TKYTINDESFSLQDGILGMA-----LSHKT---QNLYYSAMSSSHNLNYV

Identities = 36/104 (34%), Positives = 66/104 (63%), Gaps = 13/104 (12%)

Fig. 3. Comparison of honeybee the MRJP9 with its wasp homolog. The wasp EST sequence DT319131 was identified among ESTs that are differentially expressed among wasp castes. The complementary strand of DT319131 (nucleotides 27-335) was translated in the 2nd translational frame and compared with honeybee MRJP9. The alignment was done using TBLAST program.

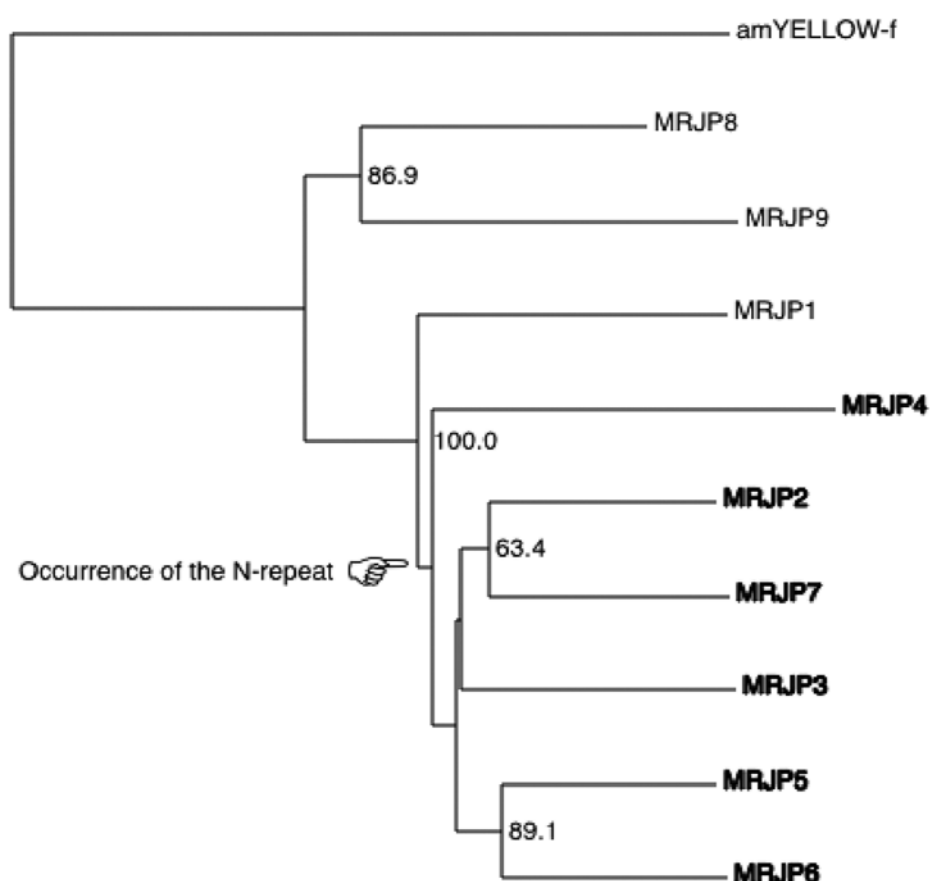


Fig. 4. MRJP9 is an ancient MRJP. Protein sequences of nine MRJPs were aligned using CLUSTAL-X and the tree was calculated by neighbor-joining method. Relevant bootstrap values (in %) are shown on the right side of the nodes. The analysis puts MRJP9 near the basis of the MRJP tree. Repeat-containing MRJPs are bold, and presumable appearance of the repetitive region in the common precursor of MRJP2-7 is indicated.

The protein fragment encoded by the wasp EST is related to MRJP9 (BLAST E-value 3×10^{-10} , see Fig. 3) and more distantly related to all yellow-like proteins in the databank (E-values $\geq 1 \times 10^{-5}$).

The sequence-based phylogenetic analysis (Fig. 4) puts MRJP9 together with MRJP8 close to the basis of the yellow-rooted MRJP tree (86.9% Bootstrap value). This can be interpreted that MRJP8 and MRJP9 are the most ancient members of the MRJP family. In line with stated above, MRJP9 is one of the three MRJP proteins (MRJP1, 8, 9) that do not contain any repetitive nitrogen-rich regions, supposed to contribute to the nutritional value of the proteins (Albertova, et al., 2005). All three repeat-free MRJPs segregate near the basis of the tree, strongly supporting a single origin of the nitrogen-rich C-terminal repeats in the predecessor of *mrjp2-7* genes (Fig. 4, bold).

Discussion

Several lines of evidence support the notion on non-nutritional function of MRJP9. The MRJP9 polypeptide or cDNA encoding it have been identified independently in three functionally divergent honeybee organs: antennae, hypopharyngeal glands (Santos, et al. 2005) and venom sac (Peiren, et al., 2005). In contrast to abundant MRJPs, expression of MRJP9 in food-producing hypopharyngeal glands is very low. The paper wasp *MRJP* gene is expressed in queen and young castes but not in worker caste, members of which forage and care for brood (Sumner, et al., 2006). Moreover, to our best knowledge no wasp caste secretes any larval food. Finally, MRJP9 represents an ancient member of the MRJP family (Fig. 4), which does not contain nutritionally interesting repetitive regions present in several MRJPs (Albert, et al., 1999a). Initial suggestion for additional non-nutritional function of MRJP proteins in honeybee physiology came from the observation that MRJP1, the most abundant protein of RJ, is expressed also in a subset of Kenyon cells (intrinsic neurons) of mushroom bodies – presumable centers of learning and memory (Kucharski, et al., 1998). The extremely high number of MRJP2 singlets in honeybee brain EST library (Albert and Klaudiny, 2004) suggests that MRJP2 can also be expressed in bee brain.

It has been reported that IgE antibodies recognizing MRJP1 are present in sera of RJ-allergic patients but also in sera of individuals allergic to bee venom (38% of tested patients), and in 52% of patients with respiratory and/or food allergies (Thien, et al., 1996). The authors concluded that IgE antibodies in the latter patients could arise in response to common inhalant allergens that show cross-reactivity with RJ proteins. Unequivocal identification of MRJP9 in bee venom by Peiren et al., (2005) led us to hypothesize that the presence of this protein in bee stings could be one of the possible factors responsible for development of allergy against RJ, at least in some sensitive individuals. Accordingly, some of the people allergic against RJ (without previous contact with RJ) could have been sensitized by MRJP9 present in bee stings. Sequence similarity between MRJP1 and MRJP9 (and other MRJPs) is emphatic (74%). It is highly probable that some immunological epitopes are shared among MRJP proteins. It has been reported that antibodies raised against one

of the MRJPs cross-react with other members of the family (Hanes and Simuth, 1992). It cannot be ruled out that venoms of other hymenoptera (wasps or bumblebees) also contain MRJP9-like protein, which, depending on degree of similarity, could have a similar effect. More detailed studies will be necessary to test the relevance of the outlined immunological and allergic effects. Nevertheless, until full clarification of this issue it is advisable for people overreacting against bee (or wasp and bumblebee) stings to be careful with consumption of RJ and RJ-containing products. Preventive tests for MRJP-reactive antibodies in these patients could be effective in preventing life-threatening allergic reactions (Leung, et al., 1997).

In summary, we have characterized MRJP9 as a novel MRJP family member having non-nutritional function(s) in bee physiology. We also present here the first evidence for the existence of MRJP-like gene in non-*Apis* hymenopteran. It is probable that the MRJP9 function in venom and other organs is more ancient than the nutritional function of evolutionarily younger MRJP1-5, which are highly expressed in royal jelly. The latter function presumably evolved with the occurrence of highly social siblings feeding behavior in honeybees. Further efforts will be necessary to unveil the physiological function of MRJP9 as well as other members of the MRJP family expressed in tissues others than hypopharyngeal glands.

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