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Research Article

A family of major royal jelly proteins of the honeybee *Apis mellifera* L.

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Abstract. The characterization of major proteins of honeybee larval jelly (49–87 kDa) was performed by the sequencing of new complementary DNAs (cDNAs) obtained from a honeybee head cDNA library, by the determination of N-terminal sequences of the proteins, and by analyses of the newly obtained and known sequence data concerning the proteins. It was found that royal jelly (RJ) and worker jelly (WJ) contain identical major proteins and that all the proteins belong to one protein family designated MRJP (from major royal jelly proteins). The family consists of five main members (MRJP1, MRJP2, MRJP3, MRJP4, MRJP5). The proteins MRJP3 and MRJP5 are polymorphic. MRJPs account for 82 to 90% of total larval jelly protein, and they contain a relatively high amount of essential amino acids. These findings support the idea that MRJPs play an important role in honeybee nutrition.

Key words. Honeybee; royal jelly; proteins; protein family; gene family; repetitive region; cDNA; nutrition.

Introduction

The development of a honeybee (*Apis mellifera* L.) larva depends on a complex proteinaceous secretion of the cephalic glandular system of nurse bees, the so-called larval jelly or bee milk. The quantity and composition of larval jelly which is provided to individual larvae by the nurse bees differs according to sex, caste and age of the larvae [1–5]. The larval jellies – royal (RJ), worker (WJ) and drone jellies (DJ) – differ in the ratio of fructose to glucose and in the content of the vitamins and proteins [5–10].

The attention of apidologists has been directed mainly to the study of RJ. It determines the development of young larvae hatched from a fertilized egg into a queen. It is also responsible for the high reproductive ability of the honeybee queen [3, 11–13]. The composition of RJ

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varies with seasonal and regional conditions. The average moisture content of RJ is 60–70%, crude protein 12–15%, total sugar 10–16%, lipids 3–6%, vitamins, salts and free amino acids [14–16]. The crude protein of RJ consists of water-soluble and water-insoluble proteins. The water-soluble proteins (WSPs) make up 46–89% of the total proteins in RJ [16–18].

Most investigations of WSPs of RJ were based on chromatographic and electrophoretic analyses. They focused on the content of WSPs in larval jelly, in the nurse bee glands, on their immunological properties and comparative analyses among the honeybee species [10, 17–28]. Only a few studies dealt with the characterization of individual RJ proteins. The activities of some enzymes in RJ were determined: invertase species [29–31], amylase [9, 30], ascorbinoxidase [32], catalase [30], acid phosphatase [21] and insulin-like peptide [33, 34]. The first royal jelly protein for which the complete amino acid sequence was determined was the antibacterial peptide royalizin [35]. Further proteins of RJ were characterized by cloning and sequencing their complementary DNAs (cDNAs): RJP57-1 and RJP57-2 [36, 37], α-glucosidase [38] and the dominant 56-kDa protein [39].

The WSPs fraction of RJ contains several major proteins (M_r's of 47–80 kDa) that are produced in the cephalic glands of nurse honeybees [22, 23, 27, 28, 40]. It is presumed that they have a nutritional function [20]. Recently, it was found that one of the major glycoproteins with an M_r of 55 kDa maintains a high viability of primary cultured rat liver cells [41]. From a medical point of view the significant finding is that the major RJ proteins (MRJPs) with an M_r of 47 and 55 kDa are the main allergenic RJ proteins. They give positive reactions with sera from RJ-sensitive patients suffering from asthma, rhinitis and/or eczema [42], and could be useful diagnostic markers for detecting the allergic sensitivity against RJ.

In our previous studies we started with the characterization of MRJPs by cloning and sequencing of their cDNAs. The cDNA clones encoding MRJPs were obtained from a cDNA library which was prepared from the heads of nurse honeybees (*A. mellifera carnica*). Three cDNAs corresponding to messenger RNAs (mRNAs) which are expressed at high levels in heads of 8-day-old nurse honeybees [40] were sequenced. Two of them, RJP57-1 and RJP57-2 cDNAs, were found to encode homologous proteins [36, 37]. The third cDNA – designated RJPX, which corresponds to the mRNA with the highest expression level – was found to be incomplete [40]. In this work we have identified and sequenced two cDNAs that encode major proteins of larval jelly. One of them was a full-length analogue of RJPX cDNA. Electrophoretic analyses and N-terminal sequencing of RJ and WJ proteins as well as the analysis of all known sequences of major proteins of larval jelly deduced from cDNAs were used in study of the proteins.

Materials and methods

Sources and preparation of biological materials. Honeybees (*A. mellifera carnica*), RJ and WJ were obtained from the Institute of Apicultural Research, Liptovsky Hrádok, and from the private apiary of J. Šimúth, Sebechleby, Slovakia.

For the quantitative and qualitative analysis by SDS-polyacrylamide gel electrophoresis (PAGE), the samples of RJ and WJ were collected from queen and worker larvae cells (at the age of 3 days) located in an area of ~ 1 dm^2 on the same comb. WJ was removed from the brood cell after its homogenization in 30 µl of phosphate buffer (50 mM NaH_2PO_4/Na_2HPO_4, pH 7.0; 100 mM NaCl; 20 mM EDTA, pH 8.0). Aliquots of WJ from 10 brood cells were pooled as one WJ sample.

Preparation of WSPs of RJ. RJ was homogenized at concentrations of 50 to 100 mg/ml in phosphate buffer (see above). The extraction of the proteins was performed for 30 min in an ice-water bath by repeated vortexing each 5 min. The homogenate was centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was dialysed at 4 °C for 2.5 to 4 days against 0.1 mM EDTA until the ratio of A280/A260 reached 1.8 and then against deionized water at 4 °C for 24 h. The precipitate formed was removed by centrifugation. The concentration of proteins was determined by the Bradford method [43] using bovine serum albumin (BSA) as standard. The value was multiplied by 1.5, which empirically corresponds better to the actual concentration of RJ proteins.

Preparation of polyclonal antiserum against WSPs of RJ. Two rabbits were each injected subcutaneously with 500 µg of WSPs suspended in Freund's adjuvant. Boosts followed 3 and 7 weeks after the primary injection. The polyclonal antiserum was prepared from blood collected 2 weeks after the third injection.

Fractionation of the MRJPs on DEAE cellulose DE-52. Preparation of WSPs of RJ (see above) was started by homogenization of 10 g of RJ (stored at −20 °C) in 180 ml of phosphate buffer. After dialysis the preparation was adjusted with 1 M Tris-HCl (pH 7.5) and 0.5 M EDTA (pH 8.0) to 20 mM Tris-HCl (pH 7.5) and 1 mM EDTA and subsequently loaded on a preequilibrated column of DEAE-cellulose DE-52 (25×230 mm). The proteins were eluted with a linear 0–0.3 M gradient of NaCl in the same buffer. Obtained peaks A (0.11–0.16 M NaCl), B (0.16–0.22 M NaCl) and C (0.22–0.3 M NaCl) were dialysed against deionized water for 24 h at 4 °C and further purified by rechromatography on DEAE cellulose DE-52.

SDS-PAGE method. SDS-PAGEs were carried out according to Laemmli [44]. Samples were denatured by boiling for 8 min in sample buffer containing dithiothreitol (DTT). After electrophoresis the gels were stained with Coomassie Brilliant Blue R-250 or by the silver-staining method [45]. The molecular masses of the MRJPs were determined from the plot of log M_r vs. relative mobility using protein molecular mass standards from Serva (Mix 4) or Gibco BRL (High Range). The gels for quantitative analyses were dried between cellophane sheets and scanned by a laser scanner, and the content of the proteins was determined using computer protram NIH IMAGE 1.61.

N-terminal sequencing of proteins. RJ and WJ proteins were separated by SDS-PAGE (8, 10 or 12% gels) and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore) [46] using a semidry transfer cell (model Trans-Blot SD, Bio-Rad). Transferred protein bands were visualized with 0.1% amidoblack solution in 25% methanol, excised and sequenced by automated Edman degradation on an Applied Biosystems gas-phase sequencer (model 473A).

Isolation of cDNA clones coding for RJ proteins. The Uni-ZAP XR expression cDNA library prepared from the heads of 8-day-old nurse honeybees (*A. mellifera carnica*) was screened with a polyclonal antiserum according to the procedure which we have described previosly [36]. Plasmid clones containing the cDNA inserts were derived from immunopositive phage clones by an in vivo excision procedure (Stratagene manual).

Dot-blot analysis. A modified method of DNA binding to a positively charged membrane at a high pH was used [47]. Analysed cDNA inserts in sizes of 1350 to 1600 bp were obtained by Xho I and Eco RI restriction of the recombinant plasmid DNAs and purified using the QIAEX gel extraction kit (Qiagen). They were diluted with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to a concentration of 0.5 mM. Two microlitres of each cDNA solution was added to 28 µl of denaturation solution (0.4 M NaOH and 10 mM EDTA, pH 8.2), incubated at 96 °C for 10 min and transferred onto GeneScreen Plus membrane (DuPont NEN) in a dot-blotting apparatus. Hybridization with ^{32}P-labelled RJPX cDNA ($\sim$1000 bp) was performed as we have described previosly [36].

Northern blot analysis. Total RNA was isolated from the heads of nurse honeybees [36, 48]. One microgram of total RNA was electrophoresed on a 1% agarose gel containing formaldehyde and transferred to GeneScreen membranes (DuPont NEN) employing the method of capillary blotting [49]. RNAs were fixed to the membrane by exposure to UV light. The blot was hybridized with ^{32}P-labelled cDNA probes (Megaprime DNA-labelled system, Amersham) at 65 °C for 24 h [50], and washed following the protocol of Khandijan [51]. Autoradiography was performed using a Kodak X-OMAT film with exposition at 4 °C for 30 min.

DNA sequence analysis. The isolation of recombinant plasmid DNA was done according to Lee and Rasheed [52]. The cDNA inserts were sequenced by the dideoxy termination method with radioactively labelled [^{35}S] dATP (2′-deoxyadenosine 5′-triphosphate) using the T7 sequencing kit (Pharmacia) or by the cycle-sequencing method employing the Prism Ready Reaction Dyedeoxy Terminator Kit (Perkin Elmer) on 373A DNA-sequencing device according to the manufacturer's instructions. DNA and protein sequence analyses were performed using computer programs of the University of Wisconsin Genetic Computer Group [53], PC GENE (6.70), and the Heidelberg UNIX Sequence Analysis Resources.

Results

Characterization of cDNAs encoding two MRJPs. For identification of cDNA clones encoding MRJPs, a cDNA library from the heads of nurse honeybees was immunoscreened with a polyclonal antiserum raised against WSPs of RJ. To find a clone containing the full-length analogue of the incomplete RJPX cDNA, 130 plasmid clones derived from immunopositive phage clones were analysed by restriction with Xho I and Eco RI. Forty-six percent carried cDNA inserts that ranged in size from 1350 to 1600 bp. Twenty-five of the cDNA inserts were analysed by dot-blot hybridization with radioactively labelled RJPX cDNA. Two cDNAs yielded a radioactive signal of the same intensity as the control RJPX cDNA. Sequencing of their 3′ ends showed that they are identical to RJPX cDNA. The longest cDNA insert, obtained from the pRJP120 clone, was completely sequenced. The length of the pRJP120 cDNA insert was 1444 bp, including 19 nucleotides of a poly(A) tail. The nucleotide sequence of RJP120 cDNA is identical with that of p56kP-4 encoding the major 56-kDa RJ protein published recently [39]. The protein encoded by RJP120 cDNA is the dominant protein of larval jelly, which we designated MRJP1 (fig. 3). The processed MRJP1 is rich in 10 essential amino acids (48%; table 1). Three potential N-glycosylation sites were predicted in its amino acid sequence (table 2).

Dot-blot analysis revealed that several other cDNA inserts hybridize with different intensities with RJPX cDNA. These cDNAs were partially sequenced from both ends. Seven of them were identical to RJP120 cDNA, but shorter. Three other cDNAs corresponded to one cDNA species with homology to RJP120 cDNA. The longest cDNA of the pRJP95 clone was completely sequenced. The cDNA sequence is 1579 bp long, [including 18 nucleotides of a poly(A) tail] and encodes a protein of 452 amino acids designated MRJP2 [open

Table 1. Amino acid composition of MRJPs.

	MRJP1	MRJP2	MRJP3	MRJP4	MRJP5
Ala	3.9	6.2	4.9	4.3	3.8
Arg	**3.4**	**3.8**	**4.9**	**4.1**	**9.0**
Asn	6.9	11.3	15.9	13.8	8.7
Asp	8.6	7.1	7.5	7.5	12.0
Cys	2.5	1.5	1.1	1.3	1.0
Gln	3.9	5.1	7.1	6.3	3.8
Glu	3.9	3.8	3.8	3.9	2.5
Gly	5.6	6.0	6.4	4.1	4.0
His	**2.3**	**2.4**	**2.2**	**3.9**	**1.8**
Ile	**6.0**	**5.1**	**4.0**	**3.2**	**4.8**
Leu	**9.5**	**8.2**	**6.8**	**9.7**	**5.2**
Lys	**5.1**	**6.9**	**5.8**	**5.0**	**4.3**
Met	**3.5**	**2.4**	**2.2**	**2.4**	**11.4**
Phe	**4.2**	**4.4**	**1.7**	**2.2**	**2.6**
Pro	3.7	3.1	2.5	2.2	2.6
Ser	8.1	5.8	5.9	8.4	6.2
Thr	**6.3**	**4.6**	**4.0**	**4.7**	**5.6**
Trp	**1.2**	**1.3**	**0.9**	**1.3**	**1.1**
Tyr	4.4	3.5	3.1	3.9	3.3
Val	**6.5**	**7.5**	**6.8**	**8.0**	**5.6**
Ess. aa.	**48%**	**47%**	**39.3%**	**44.5%**	**51.4%**

Percent content of amino acids in native protein was obtained by computer analysis of its sequence employing the program PC GENE 6.7. Essential amino acids for honeybee larvae [57] are marked in boldface.

reading frame (ORF): 67–1422 bp] (fig. 1). The protein is also rich in essential amino acids (47%; table 1) and contains two potential N-glycosylation sites (fig. 1, table 2).

Northern analysis. The analysis of total RNA from heads of nurse honeybees (*A. mellifera carnica*) employing cDNA probes derived from the clones pRJP57-1 [36], pRJP120 and pRJP95 showed that the sizes of pRJP120 and pRJP95 cDNA inserts correlate with the determined lengths of mRNAs (1500 and 1600 nucleotides, respectively). It also demonstrated a high abundance of the mRNAs in the heads of nurse honeybees. Molar ratios of RJP57-1 mRNA, RJP120 mRNA and RJP95 mRNA were determined to be approximately 1:3:1 (fig. 2).

Identification of individual major proteins in RJ and WJ by N-terminal sequencing. The proteins of RJ and WJ were separated by 8, 10 or 12% SDS-PAGE (fig. 3 and unpublished observations). The comparison of patterns of major RJ and WJ proteins showed that they are almost identical. Quantitative differences were seen in the proteins between 60 and 70 kDa. All major proteins of RJ and WJ were micro-sequenced from their N-termini. The major proteins of RJ and WJ with the same electrophoretic mobility on SDS-PAGE had the same N-terminal sequences, which indicates that these proteins are identical. By comparing the determined N-terminal sequences with amino acid sequences derived from five cDNAs corresponding to abundant nurse honeybee head mRNAs (sequences deduced from RJP95 and RJP120 cDNAs and [36, 37, Š. Albert et al., unpublished observations]), we could identify the individual major proteins on SDS-PAGE (fig. 3).

The protein of the dominant band (55 kDa), designated MRJP1, has the N-terminal amino acid sequence NILRGESLNKS, identical to the protein derived from RJP120 cDNA. The N-terminus of the native protein begins with Asn_{20} (fig. 5).

The protein with an M_r of 49 kDa, designated MRJP2, corresponds to the protein deduced from the nucleotide sequence of RJP95 cDNA. The determined N-terminus of the native protein was AIVRENSPRNLEK. The cleavage site of signal peptidase is localized between Gly_{17} and Ala_{18} (figs 1 and 5).

Four proteins with M_r's in the range between 60 and 70 kDa have almost identical N-terminal amino acid sequences: AAVNHQ(R/K)KSANNLAHS. These proteins, designated MRJP3s, differ in the presence of R or K in the position of the seventh amino acid residue (aar). The obtained N-terminal amino acid sequences are identical with the amino acid sequence of RJP57-1 protein determined previously [36, 37]. The RJP57-1 protein sequence has arginine in the seventh position. Partial sequencing of other cDNAs identified by immunoscreening of a cDNA library turned up a cDNA clone that encodes the protein with lysine in the seventh position (not shown). The signal peptidase cleavage sites are localized between Ser_{20} and Ala_{21}.

The 77-kDa and the 87-kDa proteins, designated MRJP5s, have identical N-terminal sequences: VTV(R/N)E(N/Q)SPR. They correspond to the amino acid sequence of the protein MRJP5 which was deduced from the cDNA sequence of a pRJP40 clone (Š. Albert et al., unpublished observations, fig. 5).

The protein, corresponding to the previously characterized RJP57-2 protein [36], renamed here as MRJP4, was not identified by SDS-PAGE. We assumed that the protein might be overlaid by some of the more abundant MRJPs in electrophoretic separation, so another separation method for RJ proteins was used. The WSPs of RJ were fractionated by ion-exchange column chromatography using a linear NaCl gradient. Three protein peaks – A, B, C – were collected and further purified by rechromatography. The proteins of the obtained preparations

Table 2. Characteristics of MRJPs.

	MRJP1	MRJP2	MRJP3	MRJP4	MRJP5
M_r	46.8	48.9	59.5	50.9	68.0
aar	416	435	528	449	581
N-glycos.	3	2	1	8	4

The characteristics were predicted by computer analysis (PC GENE 6.7) of protein sequences without their signal peptides. M_r, apparent molecular mass in kDa; aar, amino acid residues; N-glycos., consensus sequence for N-glycosylation.

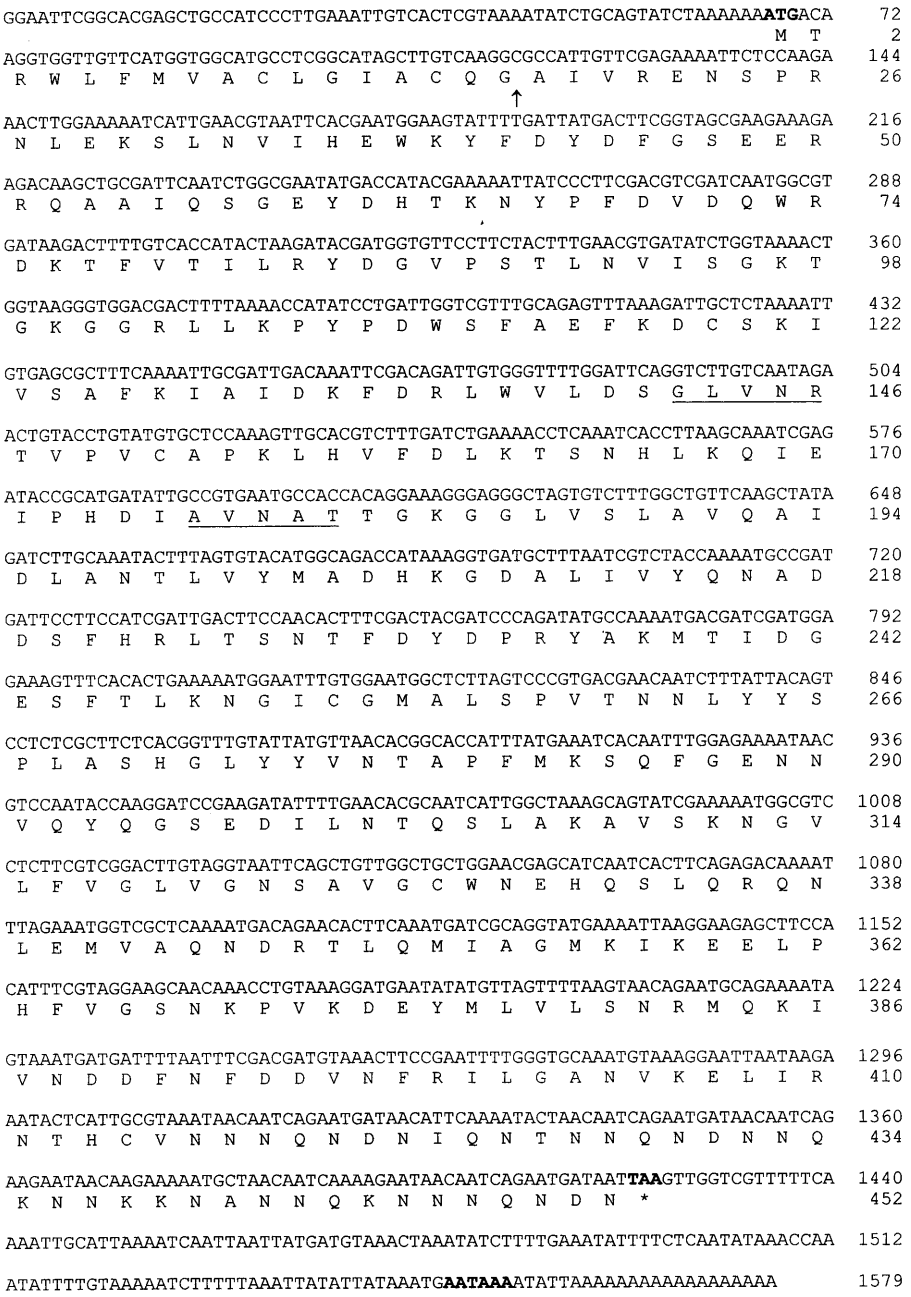

Figure 1. Nucleotide sequence of cDNA of pRJP95 and the deduced amino acid sequence of protein MRJP2. Translation initiation, termination codons and putative polyadenylation signal site are indicated by bold letters. The arrow shows the cleavage site for the signal peptidase determined by N-terminal sequencing of the protein. The consensus sequences for N-glycosylation are underscored.

were separated by SDS-PAGE (fig. 4) and micro-sequenced. Again, only N-terminal sequences of the proteins MRJP1 (lanes 1, 2), MRJP2 (lanes 1, 3, 4) and MRJP3 (lane 3), and none corresponding to MRJP4, were found.

A protein family of MRJPs. The comparison of nucleotide sequences of cDNAs as well as of the amino acid sequences of proteins MRJP1, MRJP2, MRJP3, MRJP4 and MRJP5 deduced from them showed high sequence identities among cDNAs and protein se-

quences. The homologies are interrupted by the regions containing repetitive motifs in MRJP3 (aar 424–523) and MRJP5 (aar 367–540) (fig. 5). From the sequence homologies it is possible to conclude that the MRJPs are members of one protein family.

The MRJPs are secretory proteins with N-terminal hydrophobic regions common to eukaryotic signal peptides (fig. 5). Some characteristics of the members of the MRJP protein family, such as the calculated M_r, the number of amino acid residues and potential N-glycosylation sites were predicted from their sequences (table 2).

Comparison of the protein sequences of individual MRJPs with the proteins deposited in the Swiss-Prot database gave the highest identity (23.4–27.2%) and similarity values of around 23% (program BESTFIT) with yellow protein from *Drosophila melanogaster*. Yellow protein is implicated in the processes of melanin pigmentation in insect cuticle [54–56]. The degree of homology between yellow protein and MRJPs indicates that their genes probably evolved from a common ancestral gene.

Three MRJPs contain high amounts of the 10 essential amino acids in honeybee [57]: MRJP5 (51.4%), MRJP1 (48%) and MRJP2 (47%). MRJP5 is rich in Arg and Met (9% and 11.4%). MRJP3 and MRJP4 have a lower overall content of essential amino acids, but they possess relatively higher amounts of some of them; MRJP3 Arg (4.9%), Lys (5.8%) and MRJP4 Leu (9.7%), Val (8%) (table 1).

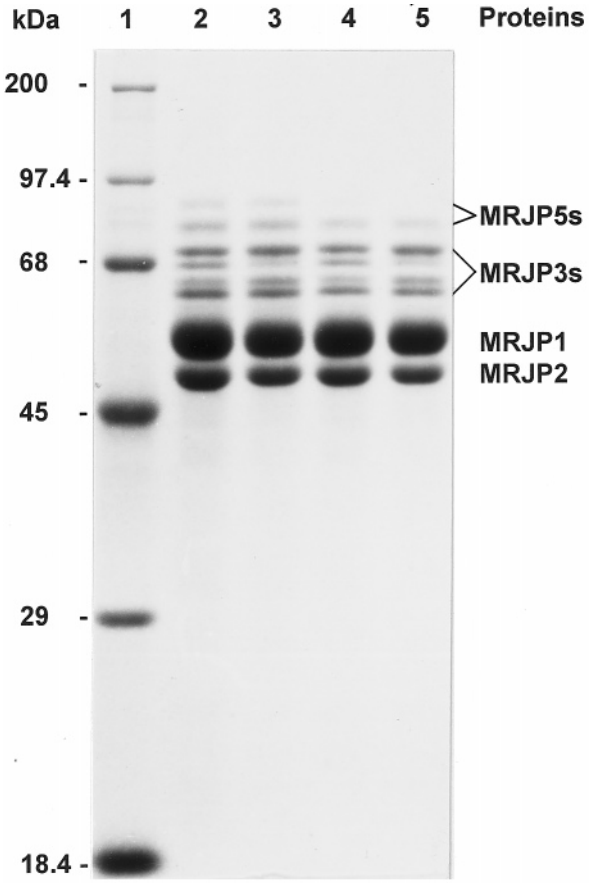

Figure 3. Analysis of the proteins of RJ and WJ by SDS-PAGE (10% gel). RJ and WJ were obtained from cells of the same hive comb. WJ represents the pool of jellies from 10 worker larvae cells (see 'Materials and methods'). Lane 1, protein molecular weight marker; lane 2, homogenate of WJ in phosphate buffer; lane 3, homogenate of RJ in phosphate buffer (70 mg/ml); lane 4, supernatant after centrifugation of WJ homogenate (9 µg of protein); lane 5, supernatant after centrifugation of RJ homogenate (8 µg of protein). Gel was stained with Coomassie Brilliant Blue R-250.

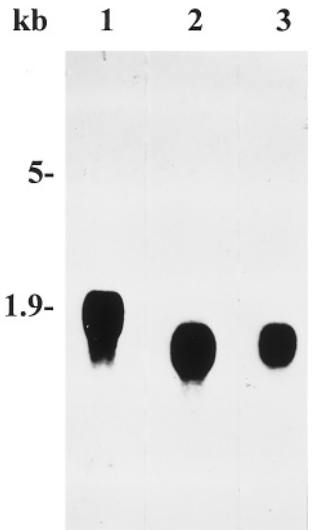

Figure 2. Northern analysis of total RNA, isolated from heads of nurse honeybeeds (*A. mellifera carnica*). Each lane contains 1 µg of total RNA. Hybridization was performed with a ^{32}P-labelled cDNA insert from pRJP57-1 [36] (lane 1) and cDNA fragments (1000 bp) of pRJP120 (lane 2) and pRJP95 (lane 3).

The electrophoretic and quantitative analyses of MRJPs in RJ and WJ. Varying data on content of WSPs (including MRJPs) in total RJ protein (46–89%), have been reported [16–18]. Electrophoresis (fig. 3) showed that the protein patterns of RJ homogenate in phosphate buffer and soluble RJ fraction (supernatant) differ mainly in the amount of MRJP5 proteins. This indicated that the insoluble RJ fraction contains the same proteins as soluble fraction. The SDS-PAGE analysis of RJ homogenate and distinct fractions of RJ obtained during the preparation of RJ WSPs (soluble, insoluble or those precipitated during dialysis against water) confirmed that they consist of the same MRJPs. However, they

differed partially in the content of individual MRJPs (fig. 6). This result revealed that individual MRJPs differ in their solubility in water and in phosphate buffer. The least soluble are MRJP5s in phosphate buffer and MRJP1 in water. The highest solubility in both these solvents exhibits MRJP2.

The amount of individual MRJPs in total RJ and WJ proteins was determined after SDS-PAGE separation of RJ and WJ homogenates (fig. 3, lanes 2 and 3; fig. 6, lane 2). The relative content of the individual MRJPs in the tested samples of RJ and WJ was determined as follows: MRJP1: 31%, 35%; MRJP2: 16%, 18%; MRJP3s together: 26%, 24%; MRJP5s together: 9%, 8%.

Together, MRJPs represented 82% of total RJ proteins and 85% of total WJ proteins. Only negligible amounts of other proteins were detected in the regions above and below MRJPs on the gel. Immunoblot analyses with antisera against MRJP1 and MRJP3 proteins revealed that some of low-M_r minor proteins are degradation products of MRJPs (unpublished observations). This indicates that the real percentage of MRJPs in total protein of larval jellies is higher then we determined. We suggest that it may reach a value of around 90%.

SDS-PAGE analyses of RJ proteins were used to determine the M_r of individual MRJPs. M_r's of proteins MRJP1 and MRJP2 were determined in the samples containing a lower amount of RJ proteins (fig. 4, lanes 2 and 4; fig. 6, lane 5). The M_r's of MRJPs determined are MRJP1: 55 kDa; MRJP2: 49 kDa; MRJP3s: 60, 63, 66 and 70 kDa, MRJP5s: 77 and 87 kDa.

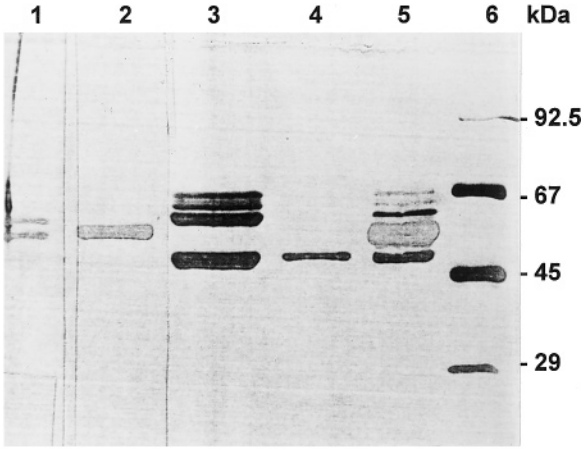

Figure 4. Ten percent SDS-PAGE of WSPs of RJ fractionated by chromatography on DEAE-cellulose DE-52 (see 'Materials and methods'). Lane 1, proteins of peak B after rechromatography; lane 2, proteins of peak C after rechromatography; lane 3, proteins of peak A – one cycle of chromatography; lane 4, proteins of peak A after rechromatography; lane 5, proteins of RJ before application on the column; lane 6, protein molecular weight markers. Proteins were stained by silver staining.

Discussion

We previously characterized cDNAs encoding two related major proteins of RJP57-1 (now MRJP3) and RJP57-2 (now MRJP4) [36, 37]. During preparation of this publication, Ohashi et al. [39] published the sequence of a cDNA that encodes a dominant major RJ protein, which is identical to MRJP1, and reported that these three proteins form a structurally related protein family. In this paper we show that not only these three proteins but all MRJPs of larval jellies (RJ, WJ) are members of the same protein family (fig. 3, fig. 5). Furthermore, N-terminal microsequencing provide evidence that RJ and WJ contain identical major proteins. This had been assumed from electrophoretic (10) and immunological (22) analyses.

The dominant 55-kDa larval jelly protein MRJP1 corresponds to the previously partially characterized glycoprotein MRJP, which was separated by isoelectric focusing into eight protein bands with similar isoelectric points (4.5–5.0) [27]. We propose that the proteins obtained by isoelectric focusing represent MRJP1 polypeptides which are posttranslationally modified to different extents and/or are products of polymorphic alleles with some amino acid substitutions affecting the pI of the polypeptides. The differences in posttranslational modifications may be caused by genetic variability of honeybee individuals in the hive as well as among distinct honeybee colonies and lines.

Proteins MRJP3 and MRJP5 contain repetitive regions with different lengths, sequences and localizations in the protein sequence (fig. 5). The repetitive region in MRJP3 is composed of 20 repetitions of the pentapeptide motif XQNXX located near the C-terminus (aar 424–523). In MRJP5, the repetitive region consists of a 58-fold repeated tripeptide motif with dominance of DRM sequence motifs. It is located further upstream (aar 376–540) than that of MRJP3. Investigations of molecular differences of four MRJP3 and two MRJP5 protein forms showed that the polymorphism of the proteins is connected with the genetically determined length variability of both repetitive regions among honeybee individuals in the bee colony. Immunoblot analyses of royal jellies as well as polymerase chain reaction (PCR) analyses of genomic DNA of honeybees from different *A. mellifera carnica* colonies and of some *A. mellifera* subspecies revealed a high variability of MRJP3s (Š. Albert and J. Klaudiny, unpublished observations). Thus MRJP3s and especially their repetitive regions may be good markers for different genetic studies of honeybee colonies [58] and *A. mellifera* subspecies.

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MRJP1       MTR-LFMLVCLGIVCQGTTGNILR-----GESLNKSLPILHEWKFFDYDFGSDERRQDAI     54
MRJP2       MTRWLFMVACLGIACQGAIV-----RENSPRN.E...NVI....Y.......E....A..     55
MRJP3       MTKWLLLVVCLGIACQDVTSAAVNHQRKSANN.AH.MKVIY...HI.F........DA..     60
MRJP4       MTKWLLLMVCLGIACQNIRGGVV-RENSS.KN.TNT.NVI.K..YL....DN.....A..     59
MRJP5       MTTWLLLVVCLGIACQGITSVTV--RENSPRK.AN.MNVI....YL..........A.M     58

MRJP1       LSGEYDYKNNYPSDIDQWHDKIFVTMLRYNGVPSSLNVISKKVGDGGPLLQPYPDWSFAK    114
MRJP2       Q.....HTK...F.V...R..T...I...D....T.....G.T.K..R..K........E    115
MRJP3       K...F.HTK...F.V.R.R..T...IE.N.........VTN.K.K.....R.........    120
MRJP4       Q.....RTK...L.V....N.T.LAVI...........V.D.T.N..R............    119
MRJP5       Q.....HTK...F.V...RGMT...VP..K..........E.I.N..R.........W.N    118

MRJP1       YDDCSGIVSASKLAIDKCDRLWVLDSGLVNNTQPMCSPKLLTFDLTTSQLLKQVEIPHDV    174
MRJP2       FK.C.K....F.I....F............R.V.VCA...HV...K..NH...I.....I    175
MRJP3       .E.C......F.I.V..F.........,...N...C.........K..K.V.......NI    180
MRJP4       .E.C......H.I...EYE..............C....FA...N..............      179
MRJP5       .K.C......Y.I....F....I....II......C....HV...N..HQ....VM...I    178

MRJP1       AVNATTGKGRLSSLAVQSLDCNTNSDTMVYIADEKGEGLIVYHNSDDSFHRLTSNTFDYD    234
MRJP2       .........G.V.....AI.---LAN.L..M..H..DA....Q.A..............      232
MRJP3       .......M.E.V.....AI.---RTN..............M.Q..............      237
MRJP4       .---.....E.V..T..AM.---STN....MV.N.-NT..I.Q.A.......S.H.LNHN    232
MRJP5       ....S..N.G.V..V..AM.---PVN.I..M..D..DA....Q...E..............    235

MRJP1       PKFTKMTIDGESYTAQDG---ISGMALSPMTNNLYYSPVASTSLYYVNTEQFRTSDYQQN    291
MRJP2       .RYA.........F.LKN.----.C......V.........L..HG......AP.MK.QFGE.    289
MRJP3       .RY..L.VA...F.VKN.---.Y.I....V.........LL.HG....D....SNPQ.EE.    294
MRJP4       S--D..SDQQ.NL.LKEVDNKVY......V.H....NSPS.EN.......SLMK.EN.G.    290
MRJP5       ..YI..MDA...F.....---.F..............LS.R.......KP.MK.E.GA.      292

MRJP1       DIHYEGVQNILDTQSSAKVVSKSGVLFFGLVGDSALGCWNEHRTLERHNIRTVAQSDETL    351
MRJP2       NVQ.Q.SED..N...L..A...N....V....N..V.C....QS.Q.Q.LEM...N.R..    349
MRJP3       NVQ...S.D..N...FG.....N....L....N.GIACV...QV.Q.ESFDV...NE...    354
MRJP4       .VQ..R..DVF.S.LTV.A...N ...L...ANNT.SC....QS.D.Q..DV..RNED..    349
MRJP5       NVQ.Q...D.FN.E.I..IM..N.......MNN..I.C....QP.Q.E.MDM...NE...    352

MRJP1       QMIASMKIKEALPHVPIF---DRY--------INR-------------------------    375
MRJP2       ....G.....E...---.VGS------------.KPVK----------------------    372
MRJP3       ...V....M.N..Q------SG.----------..DPEG----------------------    377
MRJP4       ..VV.....QNV.Q------SG.---------V.-------------------------      368
MRJP5       .TVVA..MM-H..Q------SNKMNRMHRMNRV..VNRMDRMDRIDRMDRMDRMDTMDTM    405

MRJP1       ------------------------------------------------------------    375
MRJP2       ------------------------------------------------------------    372
MRJP3       ------------------------------------------------------------    377
MRJP4       ------------------------------------------------------------    368
MRJP5       DRIDRMDRMDRIDRIDRMHTMDTMDTMDRTDKMSSMDRMDRMDRVDRMDTMDRTDKMSSM    465

MRJP1       ------------------------------------------------------------    375
MRJP2       ------------------------------------------------------------    372
MRJP3       ------------------------------------------------------------    377
MRJP4       ------------------------------------------------------------    368
MRJP5       DRMDRMDRVDTMDTMDTMDRMDRMDRMDRMDRMDRMDTMDRTDKMSRIDRMDKIDRMDRM    525

MRJP1       -----------------EYILVLSNKMQKMVNNDFNFDDVNFRIMNANVNELILNTRCEN    418
MRJP2       ----------------D..M.....R...I..D...........LG...K...R..HCV.    416
MRJP3       ----------------N..M.A...R...II......N......LG...DD.MR...CGR    421
MRJP4       ------------NTQRN..L.A..DRN.NVL...L.LEH...Q.LG....D..R.S.CA.    416
MRJP5       DRTNRMDRMNRMNRQMN..MMA..M.L..FI...Y..NE.....LG....D..M...CA.    585

MRJP1       ------------------------------------------------------PDN--    421
MRJP2       ---------------------NNQNDNIQNTNNQNDNNQKNNKKNANNQKNNNQ-N..--    452
MRJP3       YHNQNAGNQNADNQNADNQNANNQNADNQNANKQNGNRQNDNRQNDNKQNGNRQ-N..KQ    480
MRJP4       FDNQ----------------DNNHYNHNHNQARHSSKSDNQ---------NNNQHN.--Q    449
MRJP5       -----------------------------------SDNQ---------NNNQ------      593

MRJP1       ----------------------------------------------------DRTPFKI--S    429
MRJP2       ------------------------------------------------------------    452
MRJP3       NGNRQNDNKQNGNRQNGNKQNDNKQNGNRQNDNKRNGNRQNDNQNNQNDNNRNDNQVHHS    540
MRJP4       A-----------------------------------------------------HHS      453
MRJP5       ------------------------------------------------------------    593

MRJP1       IHL---------                                                    432
MRJP2       ------------                                                    452
MRJP3       SKLH--------                                                    544
MRJP4       SKS-NNRHNNND                                                    464
MRJP5       -----NKHNN--                                                    598
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Figure 5. Comparison of the protein sequences of the members of MRJP family. Alignment of amino acid residues was done employing the program CLUSTAL V [63]. Points indicate positions in the alignment which are perfectly conserved. The sequences of signal peptides (putative for MRJP4) and conserved cysteines are given in bold letters. The regions of MRJP3 and MRJP5 with repetitive motifs are underlined. Amino acid sequences were deduced from cDNA sequences characterized in this work: MRJP1 from RJP120 cDNA; MRJP2 from RJP95 cDNA, or previously: MRJP3 from RJP57-1 cDNA [37]; MRJP4 from RJP57-2 cDNA [36]; and MRJP5 from RJP40 cDNA [Š. Albert et al., unpublished observations].

In addition to a high degree of sequence identity (fig. 5), the members of the MRJPs family possess some common structural features: the conserved position of four cysteines; the presence of several blocks of almost completely conserved amino acids; and a highly hydrophilic character, mainly their C-termini. This indicates similarity in tertiary structures and the possibility of similar biological function. Structural difference could be expected for MRJP5 because of the long repetitive region located deeper inside the protein molecule.

It is presumed that MRJPs have nutritional functions in honeybee larval food [20, 59]. The analysis of their amino acid composition showed that three of them – MRJP1, MRJP2 and MRJP5 – contain a high amount of essential amino acids, comparable with other nutritional proteins (casein, 49.1% [60]; chicken ovalbumin, 51.6% [61]; quail ovalbumin, 48.8% [62]) and MRJP3 and MRJP4 possess a higher amount of some essential amino acids (table 1). The amino acid composition of MRJPs, and also their dominant content in larval jelly, indicate that they together represent a balanced mixture of the amino acids essential for nourishing both honeybee larvae and the queen. However, the nutritional function of MRJPs does not exclude other roles of some individual MRJPs in honeybee physiology, especially MRJP3s and MRJP4, which contain lower amounts of essential amino acids.

We have identified nine members of the MRJP family. Five of them represent the main members of the protein family, and the remaining members are polymorphic variants of MRJP3 and MRJP5 proteins. It was not possible to identify MRJP4 either by SDS-PAGE of RJ or WJ proteins or by chromatographic fractionation of RJ proteins. MRJP4 mRNA is expressed to the lowest extent compared with other MRJP mRNAs in the heads of nurse honeybees [40]. We presume that the content of MRJP4 in larval jelly is low, which complicates its identification among other abundant MRJPs. It is possible that larval jelly contains other protein(s) which belong to the MRJP family. More extensive cDNA screening and sequencing as well as the use of better protein separation methods could reveal new members of the family present in lower amounts in larval jelly.

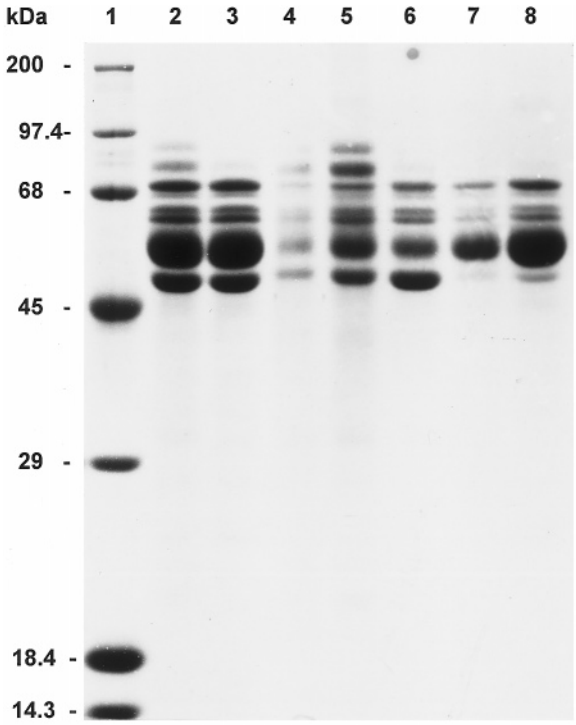

Figure 6. Analysis of homogenate of RJ and different fractions obtained in the preparation of WSP of RJ by SDS-PAGE (10% gel). The loaded samples correspond to comparable aliquots (except for lanes 5 and 7). Lane 1, protein molecular weight marker; lane 2, homogenate of RJ (100 mg of RJ/ml of phosphate buffer); lane 3, supernatant after centrifugation of RJ homogenate (14 μg of protein); lane 4, pellet after centrifugation of RJ homogenate; lane 5, as lane 4 but three times more pellet; lane 6, supernatant after centrifugation of water dialysate (6 μg of protein); lane 7, pellet after centrifugation of water dialysate; lane 8, as lane 7 but three times more pellet.

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