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Isolation and Characterization of Major Royal Jelly cDNAs and Proteins of the Honey Bee (*Apis cerana*)

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An expressed sequence tag (EST) library was established from the hypopharyngeal glands of *Apis cerana*. Sixty-six recombinant clones, possessing inserts >500 bp, were randomly selected and unidirectional sequenced. Forty-two of these (63.6%) were identified as homologues of Major Royal Jelly Proteins families 1, 2, 3, and 4 of *A. mellifera* (AmMRJP) for which MRJP1 was the most abundant family. The open-reading frame of the MRJP1 homologue (AcMRJP1) was 1299 nucleotides that encoded 433 deduced amino acids with three predicted *N*-linked glycosylation sites. The AcMRJP1 sequence showed 93% and 90% homologies with nucleotide and deduced amino acid sequences of AmMRJP1, respectively. Two complete transcripts of apisimin, and one and two partial transcripts of α-glucosidase and glucose oxidase, were also isolated. In addition, the royal jelly proteins of *A. cerana* were purified and characterized using Q-Sepharose and Sephadex G-200 column chromatography. The native forms of protein peaks A1, A2, B1, and C1 were 115, 55, 50, and 300 kDa, respectively. SDS-PAGE analysis indicated that A1 and C1 were dimeric and oligomeric forms of the 80 kDa and 50 kDa subunits, respectively. The ratio of the total protein quantities of A1 : A2 : B1 : C1 were 2.52 : 4.72 : 1 : 12.21. Further characterization of each protein, using *N*-terminal and internal peptide sequencing, revealed that the respective proteins were homologues of MRJP3, MRJP2, MRJP1, and MRJP1 of *A. mellifera*.

Keywords: *Apis cerana*, cDNA, Honey bee, MRJPs, Royal jelly

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Introduction

Royal jelly is the primary food that is secreted from the hypopharyngeal and mandibular glands of nurse bees, mainly between 5-15 days of their life (Lensky and Rakover, 1983; Knecht and Kaatz, 1990; Kubo *et al.*, 1996). It plays a major role in honeybee queen development as larvae are non-selectively fed for three days, but only the queen larvae are continually fed royal jelly throughout their lives (Schmitzova *et al.*, 1998).

The compositions of *A. mellifera* royal jelly were 65-70% moisture content, 12-17% crude proteins, 3-4% crude lipid, and 10-12% total sugar (Howe *et al.*, 1985; Karaali *et al.*, 1988; Palma, 1992). The main part of the proteins was represented by major royal jelly proteins (Brouwers, 1982; Huang *et al.*, 1989; Knecht and Kaatz, 1990).

The classification of major royal jelly proteins (MRJPs) of *A. mellifera* has been reported, based on the *N*-terminal sequences of purified proteins and cDNA sequences of a honey bee head cDNA library. Five families of MRJPs (MRJP1, MRJP2, MRJP3, MRJP4, and MRJP5) with molecular weights of 49-87 kDa were found (Schmitzova *et al.*, 1998). Based on SDS-PAGE analysis, an apparent molecular weight of MRJP1 and MRJP2 was 55 and 49 kDa, respectively. Nevertheless, MRJP3 and MRJP5 displayed size polymorphism with molecular weight ranges between 60-70 and 77-87 kDa, respectively. MRJP4 could not be identified at the protein level, neither by SDS-PAGE nor by column chromatography, but was available at the transcriptional level. MRJP1 was the most abundant protein (31%) followed by MRJP3 (26%), MRJP2 (16%), and MRJP5 (9%), respectively.

Recently, Simuth (2001) separated royal jelly of *A. mellifera* by ultracentrifugation and reported the existence of different forms of MRJP1. These included a monomer (55 kDa), oligomers (approximately 420 kDa), and water insoluble aggregates after interaction with fatty acids. The oligomeric form of MRJP1 showed self-assemble ability in

water solutions.

Although royal jelly proteins of *A. mellifera* have been extensively studied and characterized, knowledge about MRJPs in other species (including *A. cerana)* is rather limited. Recently, Takenaka and Takenaka (1996) reported that cheminal compositions of *A. mellifera* and *A. cerana* royal jelly components (levels of proteins, 10-hydroxydecenoic acid, and glucose/fructose ratio) were different. An analysis of the water soluble proteins in royal jelly by electrophoresis revealed 21 protein bands in each species; fourteen protein bands were shared between the royal jelly of these bees. A highly aggregated protein was found in *A. cerana* but not in *A. mellifera*. Four (bands 6, 7, 12, and 16) of six major bands (bands 4, 6, 7, 12, 16, and 21) in the royal jelly of *A. mellifera* were more heavily stained than those of *A. cerana*. Additionally, two protein bands (bands 10 and 11 with the range of 42.7-66.2 kDa in size) were major and specific to *A. mellifera* royal jelly.

Currently, *A. cerana* is widely used for commercial beekeeping in Thailand, primarily due to its disease resistance against bee mites. Queen-rearing experiments supported the differences of *A. mellifera* and *A. cerana* royal jelly because *A. cerana* queens could not be sucessfully reared with *A. mellifera* royal jelly and *vice versa* (Pothichot and Wongsiri, 1993; Takenaka and Takenaka, 1996).

Next, we established an EST library from hypopharyngeal glands of *A. cerana* and identified cDNAs encoding MRJP1, MRJP2, MRJP3, and MRJP4 homologues. A full sequence of MRJP1 in *A. cerana* and other important cDNAs (apisimin, α-glucosidase, and glucose oxidase) were reported. In addition, major royal jelly proteins of *A. cerana* were chromatographically purified and further characterized by SDS-PAGE, isoelectric focusing, and amino acid sequencing.

Materials and Methods

Biological specimens Nurse bees (<10 days after eclosion) of *A. cerana* were collected when they were feeding their brood, then snapped frozen in liquid N_2 and kept at −80°C until required. The hypopharyngeal gland of each bee was dissected under a binocular microscope and pooled ($N = 50$) before being subjected to mRNA isolation. Royal jelly from one colony of *A. cerana* was collected from 1 d old larvae that was grafted from queen larval cells. It was then pooled, mixed, and stored at −80°C until used.

Construction of an EST library and DNA sequencing Fifty hypopharyngeal glands of *A. cerana* nurse bees were pooled and mRNA was directly extracted using a QuickPrep Micro mRNA Purification Kit (Amersham Biosciences, Uppsala, Sweden). Three microgrammes of purified mRNA were reverse-transcribed to cDNA using a SuperScript™ Plasmid System with Gateway™ Technology cDNA Synthesis and Cloning Kit (GibcoBRL, Grand Island, USA). Size-fractionated cDNA (>500 bp) was directionally ligated to a *Not* I/*Sal* I digested pSPORT 1 at 16°C for 16 h. Recombinant plasmids were transformed into *E.coli* DH5-α (Maniatis *et al.*, 1982). The insert sizes were verified by colony PCR using primers pUC1 (5'-TCC GGC TCG TAT GTT GTG TGG A-3') and pUC2 (5'-GTG CTG CAA GGC GAT TAA GTT GG-3').

Sixty-six positive colonies, having inserted cDNA greater than 500 bp, were randomly selected. Plasmid DNA was extracted using a GFX™ Micro Plasmid Prep Kit (Amersham Biosciences) and unidirectional sequenced using a Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit (Amersham Biosciences) with the M13 reverse primer on an automated DNA sequencer (LI-COR, Lincoln, USA). Nucleotide sequences were compared with those deposited in the GenBank using the BLASTN and BLASTX programs (http://www.ncbi.nlm.nih.gov). Significant probabilities were considered when the E values were $<10^{-4}$.

Isolation and characterization of major royal jelly proteins Royal jelly (0.3 g) was dissolved in 10 ml of a phosphate buffer (50 mM NaH_2PO_4/Na_2HPO_4 pH 7.5, 10 mM NaCl, 20 mM EDTA pH 8.0). The mixture was centrifuged at 10,000× *g* for 20 min at 4°C. The supernatant was dialysed against a Tris-HCl buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 1 mM DTT and 100 μg/ml PMSF) overnight at 4°C. The protein concentrations were examined according to Lowry *et al.* (1951).

Dialysed royal jelly was loaded onto a Q-Sepharose column (5 cm long and 2.5 cm in diameter). Fractionated proteins were eluted out using a Tris-HCl buffer containing a linear gredient of 0.0-0.3 M NaCl with a flow rate of 0.5 ml/min. Each protein peak was further purified using Sephadex G-200 (85 cm long and 2.5 cm in diameter column with V_o and V_t of 134 ml and 366 ml, respectively) with a flow rate of 0.2 ml/min. Aliquots of 2 ml eluent were collected. The molecular weight of the native proteins was compared with those of the standard protein markers (ferritin, 440 kDa; catalase, 233 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; chymotrypsinogen, 25 kDa).

SDS-PAGE, deglycosylation and isoelectric point analysis The purified proteins were concentrated and dissolved in a SDS loading buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% Bromophenol Blue), denatured by boiling for 5 min, and electrophoretically analyzed using 10% SDS-PAGE (Laemmli, 1970) at 5 V/cm. The BenchMark™ Protein Ladder (Invitrogen, Carlsbad, USA) was included as a molecular weight protein standard. The gels were stained with Coomassie Brilliant Blue R-250 and PAS reagents (Fukuda and Kobata, 1993). Five micrograms of purified proteins were treated with 1 unit of *N*-glycosidase F (PNGaseF) and incubated at 37°C for 3 h. The resulting product was analysed by SDS-PAGE.

IEF were carried out in 5% polyacrylamide gels containing ampholytes with a gradient of pH 3-10 following the conditions recommended by the manufacturer (BioRad, Hercules, USA). The IEF gel was pre-run at 100 V for 15 min, followed by 200 V for 15 min and 450 V for 60 min after application of the samples.

***N*-terminal amino acid and internal peptide sequencing** Each purified protein was resolved by 10% preparative SDS-PAGE and electroblotted onto a piece of PVDF membrane (Towbin *et al.*, 1979). The *N*-terminal amino acid sequences of the corresponding protein peaks A1 and B1 were analyzed using an automated amino

acid sequencer (Department of Biological Sciences, National University of Singapore, Singapore and Department of Bioresources Science, Faculty of Agriculture, Kochi University, Japan).

Internal peptide sequencing was carried out for proteins A2 and C1. Each purified protein was evaporated and disssolved in 20 µl of 8 M urea and incubated at 37°C for 1 h prior to the addition of 60 µl of 0.3 M Tris-HCl (pH 9.0) and 10 µl (33 pmol) of lysyl endopeptidase. The solution was mixed and further incubated at 37°C for 16 h and applied to a Shimadzu HPLC (C18 column). Each peptide peak was collected and sequenced. The *N*-terminal and internal peptide amino acid sequences of the royal jelly proteins were blasted against an amino acid database of the GenBank (http://www.ncbi.nlm.nih.gov).

Results and Discussion

EST analysis The EST library that was established from the hypopharyngeal glands of *A. cerana* nurse bees showed 98% recombinant. The insert sizes were generally greater than 500 bp in length, based on colony PCR of 500 randomly picked positive clones. Sixty-six clones were unidirectionally sequenced. Forty-two recombinant clones (63.6%) were significantly homologous with MRJPs of *A. mellifera* (hereafter called AmMRJPs) The most abundant MRJP homologues were MRJP1 (50.0%) followed by MRJP2 (6.06%), MPJR3 (6.06%), and MPJR4 (1.52%), respectively (Table 1). Nevertheless, MRJP5 was not found in this library.

A relatively high content of MRJPs1, 2 and 3 of *A. cerana* royal jelly (hereafter called AcMRJPs) at the transcriptional level was consistent with previously reported AmMRJPs at the protein level (Schmitzova *et al.*, 1998). Complete and partial sequences of AcMRJP 1 and AcMRJPs 2, 3, and 4 were first reported by this study.

AmMRJPs were previously characterized by cloning and sequencing of cDNAs that were established from the heads of *A. mellifera carnica* nurse bees. Two complete cDNAs, encoding for AmMRJP3 (RJP57-1) and AmMRJP4 (RJP57-2), were isolated and sequenced (Klaudiny *et al.*, 1994). Subsequently, complete sequences of highly expressed transcripts that encoded AmMRJP1 (Judova *et al.*, 1998; Schmitzova *et al.*, 1998) and AmMRJP2 (Bilkova *et al.*, 1999) were recently characterized and expressed *in vitro*.

The complete nucleotide sequence of AcMRJP1 was deduced from pCUAC322 (accession number 17086885), pCUAC147 (17086860), and pCUAC171 (17086866), which resulted in an ORF of 1299 nucleotides encoding 433 amino acids (Fig. 1). AcMRJP1 showed high homology to its homologue from *A. mellifera* at both the nucleotide (93%) and protein (90%) levels. The putative AcMRJP1 contained 44.57% hydrophobic, 27.94% neutral, and 27.48% hydrophilic amino acid residues, respectively. The essential amino acid content was 48.28%, which is as high as that previously reported in AmMRJP1 (48%) (Schmitzova *et al.*, 1998). The estimated pI value of AcMRJP1 was 5.5. Three potential *N*-linked glycosylation sites were found at nucleotides 124-132, 472-480, and 571-579. These are identical to those in AmMRJP1 (Klaudiny *et al.*, 1994).

Sequence divergence between different families of AmMRJPs and AcMRJP1 were estimated using a Kimura's two-parameter model (Kimura, 1980). The lowest protein sequence divergence was 0.102 (AmMRJP1-AcMRJP1), whereas the highest divergence was 0.634 (AmMRJP3-AmMRJP4). A bootstrapped parsimony tree indicated monophyletic-clading between the same protein family (AmMRJP1-AcMRJP1) of different species rather than between different AmMRJPs families (Fig. 2).

One α-glucosidase and two glucose oxidase ESTs were found in the hypopharyngeal gland library of *A. cerana* nurse bees. In contrast, Ohashi *et al.* (1997 and 1999) isolated the

Table 1. Gene homologues of ESTs from hypopharyngeal glands of *A. cerana* nurse bees

Gene homologues*	Closest species	Redundancy	% EST	Probability
MRJP1	*A. cerana*	33	50.00	1×10^{-117} - 0.0
MRJP2	*A. cerana*	4	6.06	9×10^{-16} - 0.0
MRJP RJP57-1 (MRJP3)	*A. mellifera*	4	6.06	6×10^{-79} - 0.0
MRJP RJP57-2 (MRJP4)	*A. mellifera*	1	1.52	1×10^{-150}
Apisimin	*A. mellifera*	2	3.03	1×10^{-139} - 1×10^{-144}
Glucose oxidase	*A. mellifera*	2	3.03	1×10^{-170} - 0.0
α-glucosidase	*A. mellifera*	1	1.52	0.0
Elongation factor-1αF2 gene	*A. mellifera*	1	1.52	0.0
Heat shock protein 86	*Mus musculus*	1	1.52	1×10^{-77}
Cytochrome oxidase II	*A. cerana*	1	1.52	1×10^{-151}
16S ribosomal DNA	*A. cerana*	1	1.52	4×10^{-46}
NADH 4L	*A. mellifera*	1	1.52	1×10^{-14}
60S ribosomal RNA gene	*Spodoptera frugiperda*	1	1.52	9×10^{-31}
Unknown	-	13	19.70	$>1 \times 10^{-4}$

*Accession numbers 17086850-17086911

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GTACAATATCCATTGCTTCGTTACTCGCAGCCTAGAAAAATGACAAGGTGGTTGTTTATG   60
                                    M  T  R  W  L  F  M

GTGGTATGCCTTGGCATAGTTTGTCAAGGTACGACAAGCAGCATTCTTCGAGGAGAATCT  120
 V  V  C  L  G  I  V  C  Q  G  T  T  S  S  I  L  R  G  E  S

TTAAACAAATCATTAAGCGTCCTTCACGAATGGAAATTCTTTGATTATGATTTCGATAGC  180
 L  N  K  S  L  S  V  L  H  E  W  K  F  F  D  Y  D  F  D  S

GATGAAAGAAGACAAGATGCAATTCTATCTGGCGAATACGACTACAGGAAAAATTATCCA  240
 D  E  R  R  Q  D  A  I  L  S  G  E  Y  D  Y  R  K  N  Y  P

TCCGACGTTGATCAATGGCATGGTAAGATTTTTGTCACCATGCTAAGATACAATGGCGTA  300
 S  D  V  D  Q  W  H  G  K  I  F  V  T  M  L  R  Y  N  G  V

CCTTCCTCTTTGAACGTGATATCTAAAAAGATCGGTGATGGTGGACCTCTTCTTCCACCT  360
 P  S  S  L  N  V  I  S  K  K  I  G  D  G  G  P  L  L  P  P

TATCCCGATTGGGCGTTTGCTAAATATGACGATTGCTCTGGGATCGTGAGCGCCACAAAA  420
 Y  P  D  W  A  F  A  K  Y  D  D  C  S  G  I  V  S  A  T  K

CTTGCGATCGACAAATGCGACAGATTGTGGGGTCTGGACTCAGGTCTTGTCAATAATACT  480
 L  A  I  D  K  C  D  R  L  W  G  L  D  S  G  L  V  N  N  T

CAACCCCTGTGTTCTCCCAAACTGCTCACCTTTGATCTGACTACCTCGCAATTGCTCAAG  540
 Q  P  L  C  S  P  K  L  L  T  F  D  L  T  T  S  Q  L  L  K

CAAGTCGAAATACCGCATGATGTTGCCGTAAATGCCACCACAGGGAAGGGGAGACTATCA  600
 Q  V  E  I  P  H  D  V  A  V  N  A  T  T  G  K  G  R  L  S

TCTCTAGCTGTTCAACCTTTAGATTGCAATATAAATGGTGATACTATGGTATACATAGCA  660
 S  L  A  V  Q  P  L  D  C  N  I  N  G  D  T  M  V  Y  I  A

GACGAGAAAGGTGAAGGTTTAATCGTGTATCATGATTCTGATAATTCTTTCCATCGATTG  720
 D  E  K  G  E  G  L  I  V  Y  H  D  S  D  N  S  F  H  R  L

ACTTCCAAAACTTTCGATTACGATCCTAAATTTACCAAAATGACGATCAATGGAGAAAGT  780
 T  S  K  T  F  D  Y  D  P  K  F  T  K  M  T  I  N  G  E  S

TTCACAACGCAAAGTGGAATTTCTGGAATGGCTCTTAGTCCTATGACTAACAATCTCTAT  840
 F  T  T  Q  S  G  I  S  G  M  A  L  S  P  M  T  N  N  L  Y

TACAGTCCTGTAGCTTCTACCAGTTTGTACTATGTTAACACGGAACAATTCAGAACATCC  900
 Y  S  P  V  A  S  T  S  L  Y  Y  V  N  T  E  Q  F  R  T  S

AATTATGAACAAAATGCCGTACATTATGAAGGAGTTCAAAATATTTTGGATACCCAATCG  960
 N  Y  E  Q  N  A  V  H  Y  E  G  V  Q  N  I  L  D  T  Q  S

TCTGCTAAAGTAGTATCGAAAAGTGGCGTCCTCTTCTTCGGACTGGTGGGCGATTCAGCT 1020
 S  A  K  V  V  S  K  S  G  V  L  F  F  G  L  V  G  D  S  A

CTTGGCTGCTGGAACGAACATCGATCACTTGAAAGACACAATATCCGTACCGTCGCTCAA 1080
 L  G  C  W  N  E  H  R  S  L  E  R  H  N  I  R  T  V  A  Q

AGTGATGAAACACTTCAAATGATCGTTGGCATGAAGATTAAGGAAGCCCTTCCACACGTG 1140
 S  D  E  T  L  Q  M  I  V  G  M  K  I  K  E  A  L  P  H  V

CCCATATTCGATAGATATATAAACCGTGAATACATATTGGTTTTAAGTAACAGAATGCAA 1200
 P  I  F  D  R  Y  I  N  R  E  Y  I  L  V  L  S  N  R  M  Q

AAAATGGCGAATAATGACTATAACTTCAACGATGTAAACTTCAGAATTATGGACGCTAAT 1260
 K  M  A  N  N  D  Y  N  F  N  D  V  N  F  R  I  M  D  A  N

GTAAATGACTTGATATTGAACACTCGTTGCGAAAATCCTAATAATGATAACACACCTTTC 1320
 V  N  D  L  I  L  N  T  R  C  E  N  P  N  N  D  N  T  P  F

AAAATTTCAATACATCTGTAAAATCTGTTTTTTTCGATATATATTAAATATTGTTCGAGA 1380
 K  I  S  I  H  L  *

TTTCTTATGAATGTATTATGAATGTATAAAATAAATATTGTTTTCGCATAAAAAAAAAAA 1440

AAAAAAAAAAAAAAAAAAAA 1461
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Fig. 1. Nucleotide and deduced amino acid sequences of a MRJP1 homologue in *A. cerana*. Initiation of translation, terminational codons, and putative polyadenylation signal sites are bold-faced. The potential *N*-linked glycosylation sites are boxed. The signal peptide sequence is underlined.

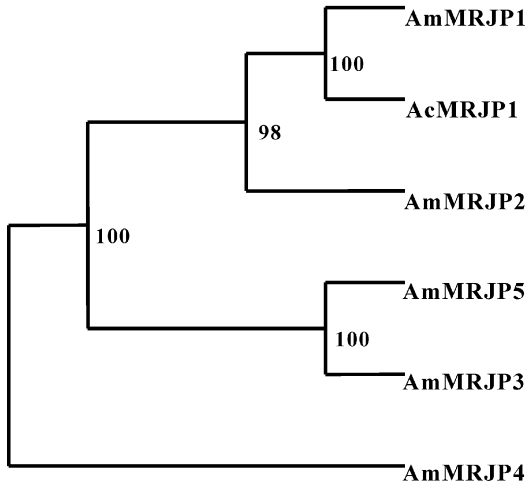

Fig. 2. A bootstrapped parsimony tree indicating relationships of AcMRJP1 in this study and MRJPs of *A. mellifera*. Values at the node represent the percentage of times that the particular note occurred in 1,000 trees that were generated by bootstrapping of the original protein sequences.

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GAACCGAGCTTTCTAGAAGCAATTCCAAACAACACACAAATCAAAATGAGCAAAATCATT   60
                                    M  S  K  I  I

GCTGTCGTCGTCCTAGCTGCCTTCTGCGTAGCCATGTTGGTCAGCGATGTATCCGCCAAA  120
 A  V  V  V  L  A  A  F  C  V  A  M  L  V  S  D  V  S  A  K

ACATCGATCAGTGCCAAGGCCGAGTCGAATGTAGATGTCGTTTCCCAAATCAACAGTTTG  180
 T  S  I  S  A  K  A  E  S  N  V  D  V  V  S  Q  I  N  S  L

GTTTCATCTATCGTAGCTGGTGCCAACGTGTCGGCAGTTCTTCTAGCTCAAACTTTAGTT  240
 V  S  S  I  V  A  G  A  N  V  S  A  V  L  L  A  Q  T  L  V

AATATCCTCCAAATTCTCATCGACGCTAATGTTTTTGCTTAATTTATATATTCTTTAGCT  300
 N  I  L  Q  I  L  I  D  A  N  V  F  A  *

TTGTATTGCGCGCATACAACGCATTCGAATAAAATAAGTAATAAAATTCAAAAAAAAAAA  360

AAAAAAAAAAAAA 373
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Fig. 3. Nucleotide and deduced amino acid sequences of an apisimin homologue in *A. cerana*. Initiation of translation, terminational codons, and the putative polyadenylation signal site are bold-faced. The potential *N*-linked glycosylation site is boxed. The signal peptide sequence is underlined.

complete cDNA coding for α-glucosidase (accession number D79208) and glucose oxidase (AB022908) in *A. mellifera.* It showed that both α-glucosidase and glucose oxidase were specifically expressed in the hypopharyngeal gland of forager bees, but not in nurse bees. Using homologous α-glucosidase primers, we found no expression of α-glucosidase in the emerged *A. cerana* bees, but moderate and high expression levels were observed in *A. cerana* nurse and forager bees, respectively (data not shown). This suggested an early expression of α-glucosidase in *A. cerana*.

Two full-length transcripts of apisimin (accession numbers 17086895 and 17086897), a serine-valine-rich peptide, were also isolated (Fig. 3). This acidic peptide was recently purified and characterized in royal jelly of *A. mellifera.* It was also proposed that it might play important physiological roles in honeybee colonies because of the relative high expression level of apisimin throughout the whole life span of the honeybees (Bilikova *et. al.*, 2002). The ORF of *A. cerana* apisimin was 234 bp in length, encoding 78 amino acid residues, as in *A. mellifera*. The deduced amino acid sequence possessed K-T-S-I-S-I-K, which was nearly identical to K-T-S-I-S-V-K that was found from the *N*-terminal sequencing of natural apisimin that was purified from royal jelly of *A. mellifera*. Like *A. mellifera* apisimin, Cys, Met, Pro, Arg, His, Tyr, and Trp residues were not found in that of *A. cerana*. The sequence showed a 92.7% and 94.9% similarity with the nucleotide and amino acid sequences of *A. mellifera* apisimin, respectively.

A single transcript of COII, NADH 4L, and 16S rDNA from mitochondrial genome was also found. Thirteen clones (19.70%) showed no significant homology to genes that were

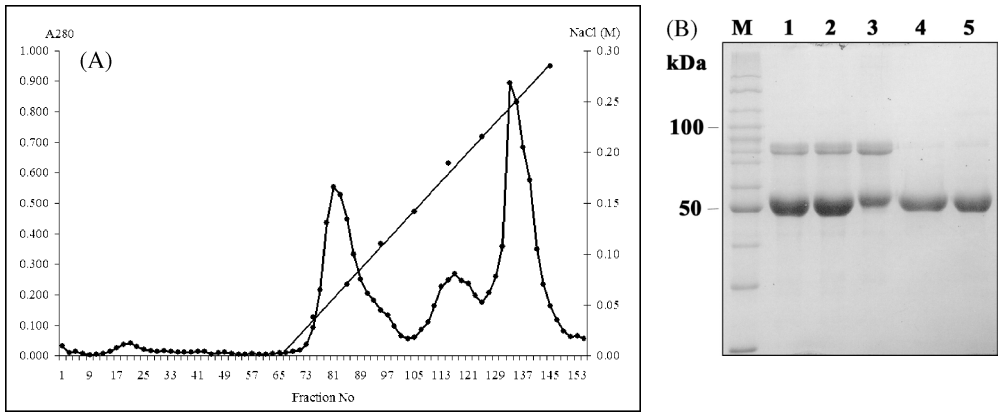

Fig. 4. (A) Elution profiles of crude royal jelly proteins on Q-Sepharose using a gradient of 0.0-0.3 M NaCl (B) 10% SDS-PAGE corresponded to crude royal jelly proteins (lane 1), dialysed royal jelly (lane 2), and protein peaks A, B1, and C1 (lanes 3-5). Lane M is a standard protein marker.

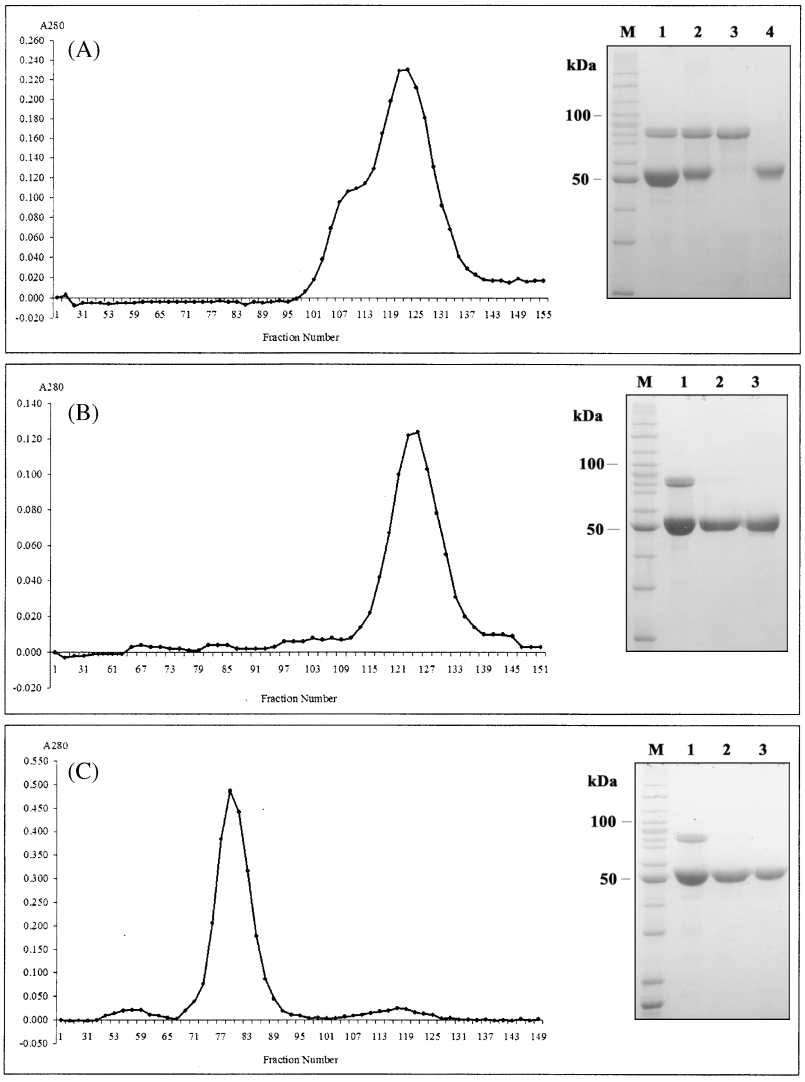

Fig. 5. Elution profiles of peak A (A), B1 (B), and C1 (C) from Q-Sepharose on Sephadex G-200 and corresponding 10% SDS-PAGE of protein peaks A1 (panel A, lane 3) and A2 (panel A, lane 4), B1 (panel B, lane 3) and C1 (panel C, lane 3). Lanes M, 1, and 2 of each panel are a standard protein marker, crude proteins, and Q-Sepharose purified proteins, respectively.

Table 2. *N*-terminal and/or internal peptide sequences of purified MRJPs of *A. cerana*

Peak	*N*-terminal and/or Internal peptide sequences	MRJP family*
A1	(G/A)AVNHQRKSA	MRJP3
A2	1: LHVFDLK	MRJP2
	2: GDALIVYQNSDDSFHR	MRJP2
	3: NLENSLNVIHEWK	MRJP2
B1	SILRGESLDK	MRJP1
C1	1: IGDGGPLLQPYPDXSFAK	MRJP1
	2: SAVLFFGLVGDSALGXXNEHNSLE	MRJP1
	3: MENNDYNENDVNFRIMDANVRDLI	MRJP1

*referred to MRJPs of *A. mellifera*

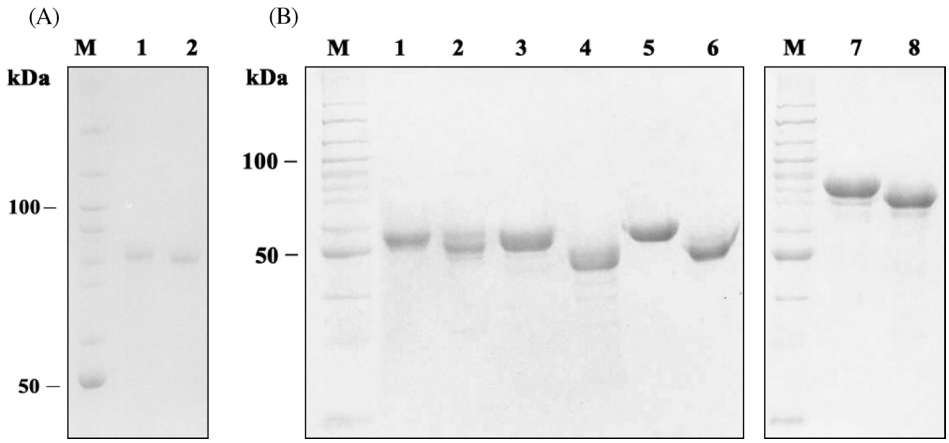

Fig. 6. Undigested and PNGaseF-digested AcMRJPs of peaks A1 (panel A, lanes 1-2), A2 (panel B, lanes 1-2), B1 (B, lanes 3-4) and C1 (B, lanes 5-6) were size-fractionated through 7.5% (panel A) and 10% (panel B) SDS-PAGE. Transferin (B, lanes 7-8) was included as a positive control. Lanes M is a standard protein marker.

previously deposited in the GenBank and regarded as unknown transcripts.

Isolation and purification of AcMRJPs AmMRJPs were successfully purified by DEAE ion-exchanged chromatography (Tomoda *et al.*, 1977; Schmizova *et al.*, 1998). However, only two protein peaks of AcMRJPs with an equal denatured molecular weight of 50 kDa were obtained when that approach was used.

Using Q-sepharose chromatography, three protein peaks (A, B1, and C1) were eluted at 0.075 M, 0.175 M, and 0.25 M NaCl, respectively (Fig. 4A). An SDS-PAGE analysis illustrated two proteins bands (55 kDa and 80 kDa) for the peak A protein, whereas only a 50 kDa band was found in the B1 and C1 peaks (Fig. 4B). Further purification of these proteins using Sephadex G-200 revealed that only the peak A protein could be further separated to subpeaks A1 (115 kDa and 80 kDa for native and denatured forms, respectively; fraction number 109) and A2 (55 kDa for both forms; fraction number 123), while a single peak of B1 (50 kDa for both forms; fraction number 125) and C1 (300 kDa and 50 kDa for multimeric and monomeric forms, respectively; fraction number 77) were obtained (Fig. 5).

N-terminal and internal peptide sequencing were used to clarify the proteins that were purified from the royal jelly of *A. cerana*. The B1 proteins contained S-I-L-R-G-E-S-L-D-K, which was nearly identical to S(N)-I-L-R-G-E-S-L-N-K that was deduced from AmMRJP1 (Schmitzova *et al.*, 1998), while internal sequencing of the C1 protein showed a high homology with the deduced amino acid sequence of AmMRJP1 (Table 2). As a result, the B1 and C1 proteins were clarified as monomeric (50 kDa) and oligomeric (300 kDa) forms of AcMRJP1, respectively.

Simuth (2001) isolated AmMRJP1 using ultracentrifugation and size exclusion chromatography and reported the native and denatured forms of 420 kDa and 55 kDa, repectively. A comparison of the deduced amino acids of MRJP1 of these species should have exhibited an identical molecular size accordingly. As a result, the size differences between AcMRJP1 and AmMRJP1 should have resulted from either subunit polymorphism between different bee species or different oligosaccharide chains at the glycosylated sites.

The *N*-termanal amino acid sequence of A1 was (G/A)-A-V-N-H-Q-R-K-S-A, which was slightly different from

RJP571 (AmMRJP3) at the first position (Klaudiny *et al.*, 1994). This may have resulted from polymorphism between the subunits of oligomeric AcMRJP3 because of substitutional mutations at the 2nd position from the C to G changes, alanine (A) to glycine (G). Internal peptide sequences of protein A2 revealed that these purified proteins were a homologue of AmMRJP2.

The post-translation modification was also found in AcMRJPs. Each purified protein in this study showed different isoelectric points. While the A1 (AcMRJP3) and A2 (AcMRJP2) proteins were basic proteins with isoelectric points of 8.3 and 7.0-8.0, the B1 (AcMRJP1) and C1 (AcMRJP1) proteins were acidic proteins that had isoelectric points of 5.2-5.7 and 5.7, respectively. Hanes and Simuth (1992) partially characterized a 57 kDa protein (AmMRJP1) and indicated that this protein exhibited isoelectric points of pH 4.5-5.0. Moreover, Bilikova *et al.* (1999) purified AmMRJP2 and illustrated that the single protein band on SDS-PAGE was composed of at least 8 different isoelectricfocusing variants of pH 7.5 to 8.5. All of the purified products in this study were glycoproteins that were revealed by positive staining with PAS. Digestion with PNGaseF shifted the mobility of these proteins (A1, A2, B1, and C1), which suggests the availability of *N*-linked oligosaccharides in those proteins (Fig. 6).

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