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Characterization of Royal Jelly Proteins in both Africanized and European Honeybees (*Apis mellifera*) by Two-Dimensional Gel Electrophoresis

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In this study, the proteins contained in royal jelly (RJ) produced by Africanized honeybees and European honeybees (*Apis mellifera*) haven been analyzed in detail and compared using two-dimensional gel electrophoresis, and the N-terminal amino acid sequence of each spot has been determined. Most spots were assigned to major royal jelly proteins (MRJPs). Remarkable differences were found in the heterogeneity of the MRJPs, in particular MRJP3, in terms of molecular weights and isoelectric points between the two species of RJ. Furthermore, during the determination of the N-terminal amino acid sequence of each spot, for the first time, MRJP4 protein has been identified, the existence of which had been only implied by cloning of its cDNA sequence. The presence of heterogeneous bands of glucose oxidase was also identified. Thus, the results suggest that two-dimensional gel electrophoresis provides a suitable method for the qualitative analysis of the proteins contained in RJ derived from different honeybee species.

KEYWORDS: Royal jelly (RJ); major RJ protein (MRJP); two-dimensional gel electrophoresis

INTRODUCTION

Royal jelly (RJ) is part of the diet of honeybee larvae and is believed to play a role in the development of the queen honeybee (*1*). RJ collected from European honeybees (*Apis mellifera*) has been used as a dietary supplement in most countries of the world. In addition, although minor with regard to consumption, RJ derived from Africanized honeybees has been used in Brazil. The species of honeybees found in Brazil were mainly of European origin prior to the mid-1950s. In 1956, African bees (*Apis mellifera scutellate*) were introduced into southeastern Brazil. Because of the subsequent accidental escape of African queen bees, a process of Africanization occurred in the bees present in Brazil (*2*).

The biological functions of some components present in RJ have been described. Thus, *trans*-10-hydroxy-2-decenoic acid found in RJ has been demonstrated to exhibit antibacterial activity (*3*). However, proteins are major components, which correspond to ∼50% of the dry mass of RJ (*4*, *5*). The biological functions of some of the constituent proteins have been reported previously. Royalisin, a 5.5 kDa protein, was found to have potent antibacterial activity against Gram-negative bacteria (*6*). The proteins collectively labeled as the major RJ proteins (MRJP) belong to a large protein family. In this family, five species of proteins (MRJP1−5) with molecular masses in the range of 49−87 kDa have been identified by cDNA cloning and sequencing (*7*−*9*). MRJP3 can be further divided into five subspecies of proteins (*9*). MRJP1 is likely to promote liver regeneration and may have a cytoprotective action on hepatocytes (*10*). We have shown that MRJP3 can exhibit potent immunoregulatory effects in vitro and in vivo (*11*).

Thus, although a number of interesting biological functions associated with MRJPs have been identified, characterization of the MRJPs has not been satisfactorily performed. The only available relevant data indicate the relative contents of the MRJP1−3 and MRJP5 proteins as determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) analysis (*7*, *9*). Furthermore, to date, the existence of MRJP4 protein has not been confirmed by SDS-PAGE analysis (*7*, *9*).

In this study, RJ proteins produced by Africanized honeybees in Brazil and those produced by European honeybees in China were analyzed in detail by two-dimensional gel electrophoresis, and the N-terminal amino acid sequence of each spot has been determined.

MATERIALS AND METHODS

Preparation of Royal Jelly Samples. Nine lots from nine beehive colonies of European honeybee-derived RJ were randomly collected from the three provinces of Shandong, Henan, and Anhui, in China,

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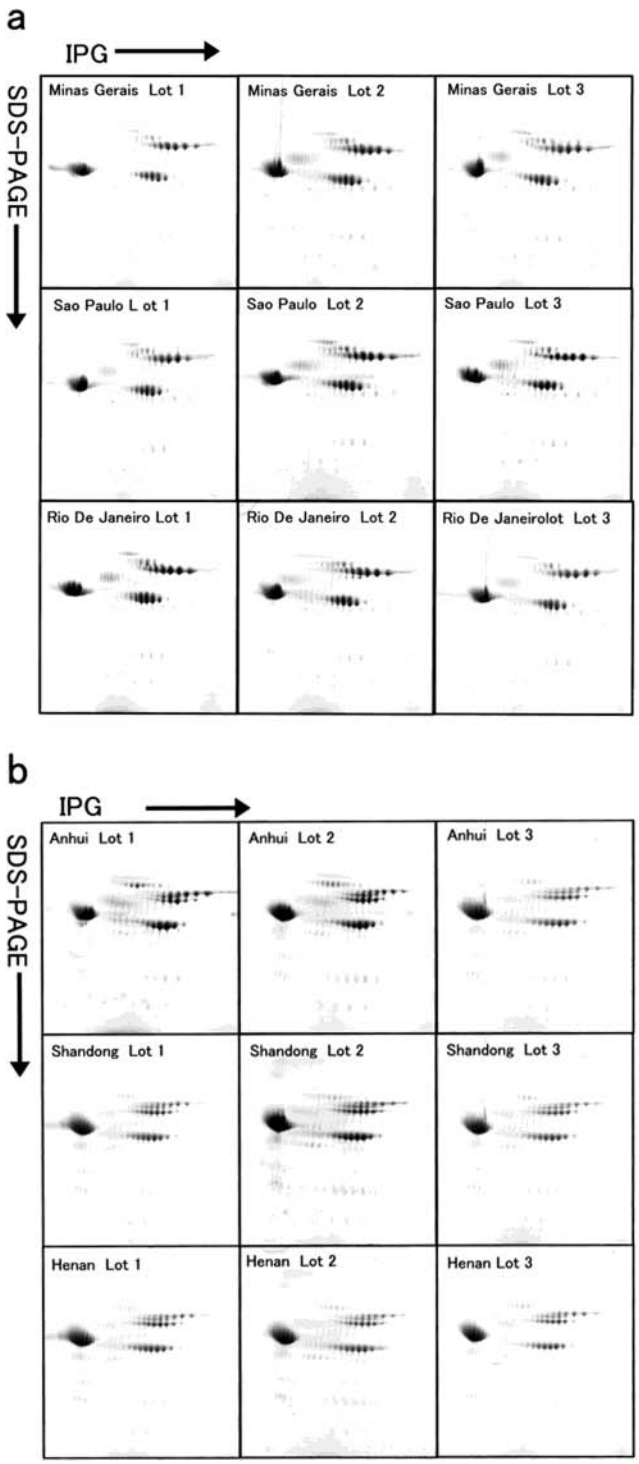

Figure 1. Two-dimensional gel electrophoretic analysis of nine lots of Africanized honeybee RJs (**a**) and European honeybee RJs (**b**). One hundred and fifty micrograms of each was subjected to two-dimensional gel electrophoresis followed by staining with CBB.

and nine lots from nine beehive colonies of Africanized honeybee-derived RJ were randomly collected from the three states of Minas Gerais, Sao Paulo, and Rio De Janeiro in Brazil. For qualitative analysis by two-dimensional gel electrophoresis, 100 mg wet weight of each RJ sample was suspended in 1 mL of distilled water.

Protein Determination. Protein concentrations were determined according to the method developed by Lowry et al. (*12*) using bovine serum albumin as a standard.

Two-Dimensional Gel Electrophoresis. Each of the 18 RJ suspensions was collected in a micro test tube and dried in a vacuum. Dried RJ was dissolved in 100 μL of isoelectric focusing (IEF) sample solution containing 8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid (CHAPS), and 0.5% Pharmarite (Amersham Pharmacia Biotech) and was mixed with 150 μL of rehydration buffer containing 8 M urea, 2% CHAPS, 50 mM dithiothreitol (DTT), and 1% immobilized pH gradient (IPG) buffer (Amersham Pharmacia Biotech). The mixtures were loaded onto an Immobiline strip tray (Amersham Pharmacia Biotech), and then an IPG gel strip and an Immobiline DryStrip, pH 3−10, 13 cm in length (Amersham Pharmacia Biotech) were loaded onto the Immobiline strip tray. IPG gel strips were rehydrated for 16 h at room temperature. IEF was carried out at 100 V for 2 h, 300 V for 5 h, and finally at 3500 V for 12 h. The IPG strips were first equilibrated for 15 min with 0.125 M Tris-HCl buffer (pH 6.8) containing 30% glycerol, 2.5% SDS, and 0.8% DTT, followed by equilibration with 0.125 M Tris-HCl buffer (pH 6.8) containing 30% glycerol, 2.5% SDS, and 4% iodoactamide for 15 min. The second dimension SDS-PAGE was performed at 10 °C and 10 mA/gel for 15 min followed by 20 mA/gel for 5 h using a 10% polyacrylamide gel, 20 cm in length.

Protein spots were visualized by staining the gels with 0.025% Coomassie Brilliant Blue (CBB) R250 in distilled water containing 40% methanol and 7% acetic acid for 16 h. Then the gels were destained with distilled water containing 40% methanol and 7% acetic acid for 30 min, followed by incubation with distilled water containing 5% methanol and 7% acetic acid for 5 h at room temperature.

Regarding the reproducibility of two-dimensional gel electrophoresis, we carried out two-dimensional gel electrophoresis of one lot from Africanized and European honeybee RJs three times. As indicated in the instructions from the manufacturer (Amersham Pharmacia Biotech), we obtained results with high reproducibility in terms of p*I* values and molecular weights of the RJ proteins (data not shown).

N-Terminal Sequence Analysis of Spots Generated by Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis-resolved proteins were electrotransferred onto poly(vinylidene difluoride) (PVDF) membranes ProBlott (Applied Biosystems), using a Bio-Rad Transblot apparatus at 100 V for 1 h with 0.01 M *N*-cyclohexyl-3-aminopropanesulfonic acid buffer (pH 11) containing 10% methanol. Protein spots were visualized by staining the membranes with 0.1% CBB R250 in distilled water containing 40% methanol and 1% acetic acid for 2 min and were destained with a solution of 50% methanol in water. Stained spots were cut off and subjected to N-terminal sequence analysis with automated Edman degradation on a model 492 protein sequencer (Applied Biosystems,).

Anti-MRJP3 Monoclonal Antibody. MRJP3 protein was purified to homogeneity by a combination of DEAE-5PW column (Tosoh, Tokyo, Japan), Resorce Q column (Amersham Pharmacia Biotech), Heparin-5PW column (Tosoh), and Superdex 200 gel filtration column chromatography (Amersham Pharmacia Biotech) as we have shown previously (*11*). Purities of the MRJP3 were >97%, as determined by N-terminal amino acid sequence analysis and SDS-PAGE followed by CBB staining.

Anti-MRJP3 mAb (no. 9), which reacts specifically with MRJP3, was obtained from a hybridoma cell line established by a cell fusion technique using SP2/O mouse myeloma cells and spleen cells from BALB/c mice hyperimmunized with MRJP3. Hybridoma colonies producing anti-MRJP3 antibodies were screened by enzyme-linked immunosorbent assay against MRJP3, and hybridoma cells were subjected to cloning twice. Anti-MRJP3 mAb (no. 9) was purified from the ascitic fluid using Protein G Sepharose (Amersham Pharmacia Biotech) (*11*).

Immunoblot Analysis. Two-dimensional gel electrophoresis-resolved proteins were electrotransferred onto nitrocellulose membranes (*13*), NitroPure (Osmonics) using a Bio-Rad Transblot apparatus at 100 V for 2 h at 4 °C in 25 mM Tris and 192 mM glycine buffer (pH 6.8), containing 20% methanol. The membranes were then incubated with 10% Block Ace (Dainippon Phamaceutical, Osaka, Japan) in TBS buffer (50 mM Tris-HCl buffer containing 0.15 M NaCl) at 4 °C for 16 h to block nonspecific binding of the detecting mAb. The membranes were washed three times with TBS buffer containing 0.05% Tween 20 for 10 min and were incubated with 0.5 μg/mL of anti-MRJP3 mAb (no. 9), in TBS buffer containing 1% Block Ace for 2 h. After the membranes had been washed three times with the TBS buffer containing

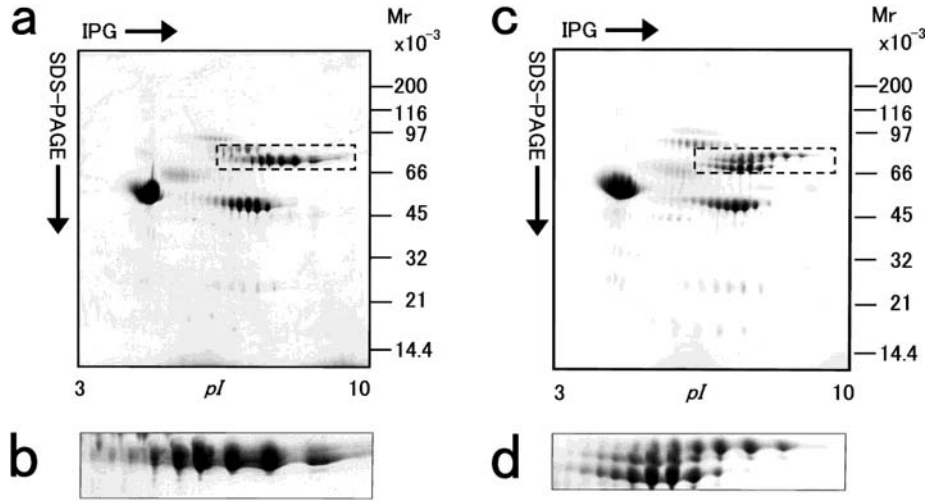

Figure 2. Different two-dimensional gel electrophoresis patterns between Africanized and European honeybee RJs. One hundred and fifty micrograms of Africanized honeybee RJ (**a**) and European honeybee RJ (**c**) was subjected to two-dimensional gel electrophoresis followed by staining with CBB. Data shown are representative of nine lots of Africanized honeybee and European honeybee RJs. The area circled with a dotted line in panels **a** and **c** was expanded and shown as panels **b** and **d**, respectively.

0.05% Tween 20 as described above, the membranes were incubated with horseradish peroxidase (HRPO)-conjugated goat antibodies to mouse IgG (Dako; 1:2000 dilution) for 2 h, washed three times, and then developed using 0.3 mg/mL 3,3′-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan) and 0.005% hydrogen peroxide.

RESULTS AND DISCUSSION

Protein Analysis of European Honeybee RJs and Africanized Honeybee RJs by Two-Dimensional Gel Electrophoresis. For the qualitative analysis of proteins contained in RJ, nine lots of Africanized honeybee RJs were collected in Brazil and nine lots of European honeybee RJs were collected in China. These RJs were analyzed by two-dimensional gel electrophoresis. Panels a and b of **Figure 1** show the results of nine lots of Africanized honeybee RJs and European honeybee RJs, respectively. The nine lots of Africanized honeybee RJs exhibited almost the same pattern (**Figure 1a**). Consistency of the pattern from lot to lot was also observed in European honeybee RJs (**Figure 1b**). Panels a and c of **Figure 2** show one representative of Africanized honeybee RJ and European honeybee RJ, respectively. Interestingly, two-dimensional gel electrophoresis patterns were substantially different between the two honeybee species. In particular, marked differences were observed in an area of p*I* range from 6 to 9 and a molecular weight range from 60 to 80 kDa on the gels (**Figure 2b**,**d**). These results suggest that two-dimensional gel electrophoresis of RJ provides more qualitative information about the RJ proteins than does single-dimension SDS-PAGE analysis. On the basis of the consistency of results across different lots of RJ, our results further imply that by using two-dimensional gel electrophoresis, we can determine whether the RJ is derived from European or Africanized honeybees.

To date, limited numbers of RJ-derived proteins have been reported. However, as shown in **Figures 1** and **2** and contrary to prior expectations, many protein spots were observed. Therefore, we decided to determine the N-terminal amino acid sequence of these two-dimensional gel electrophoresis spots.

N-Terminal Amino Acid Sequences of the Two-Dimensional Gel Electrophoresis Spots. To analyze the N-terminal amino acid sequences of the protein spots obtained from two-dimensional gel electrophoresis, proteins were electrotransferred onto PVDF membranes. Protein spots were first visualized by staining the membranes with CBB, and numbers were given to each spot. CBB-stained spots were cut off and subjected to N-terminal amino acid sequence analysis. **Figure 3a** shows the analyzed spots (HBRJ-A1 to -A52) of RJ proteins from the Africanized honeybee RJ. **Figure 3b** shows the analyzed spots (HBRJ-E1 to -E69) of RJ proteins from European honeybee RJ. The results of the N-terminal amino acid sequence analysis of each spot from Africanized honeybee RJ (HBRJ-A) and European honeybee RJ (HBRJ-E) are shown in **Tables 1** and **2**, respectively. The analyzed N-terminal amino acid sequences were compared with the cDNA sequences reported previously (*7*−*9*, *14*−*16*).

The spots HBRJ-A1 to -A5 observed in Africanized honeybee RJ were confirmed to be glucose oxidase (*16*) by N-terminal amino acid analysis. The observed molecular weights of the glucose oxidase were ∼85 kDa, which were compatible with that of glucose oxidase purified from hypopharyngeal glands of worker honeybees (*A. mellifera*) (*16*). In European honeybee RJ, spots HBRJ-E1 to -E5 corresponding to the glucose oxidase from the Africanized honeybee RJ were faint (**Figure 3b**). However, the N-terminal amino acid sequences of these spots were not determined by virtue of deficiency of protein quantity (**Table 2**).

A major spot (HBRJ-A29, HBRJ-E40, p*I* 5, MW 55 kDa), observed in both Africanized honeybee RJ and European honeybee RJ, had the N-terminal amino acid sequence of NILRGESLXKS, which was almost identical to that of MRJP1 deduced from the published cDNA sequence (*7*, *8*, *14*, *15*). Although the amino acid labeled X was not identified in our analysis, it was assumed to be Asn by comparison with the reported cDNA sequence. Because the Asn residue of an NKS sequence is known to be an N-glycosylation site, MRJP1 is considered to be a glycoprotein. A minor spot (HBRJ-A49) with lower molecular weight than that of spot HBRJ-A29 was also identified as MRJP1. No other spots attributable to MRJP1 were observed. These results suggest that MRJP1 is a fairly homogeneous protein.

The spots HBRJ-A12 to -A14, -A32 to -A38, -A43, and -A45 to -A48 and spots HBRJ -E43 to -E51 and -E65 to -E67 were confirmed to be MRJP2 (*7*, *8*, *14*, *15*) by N-terminal amino acid analysis. Although the seven spots HBRJ-A32 to -A38 had almost the same molecular weight, the N-terminal amino acid

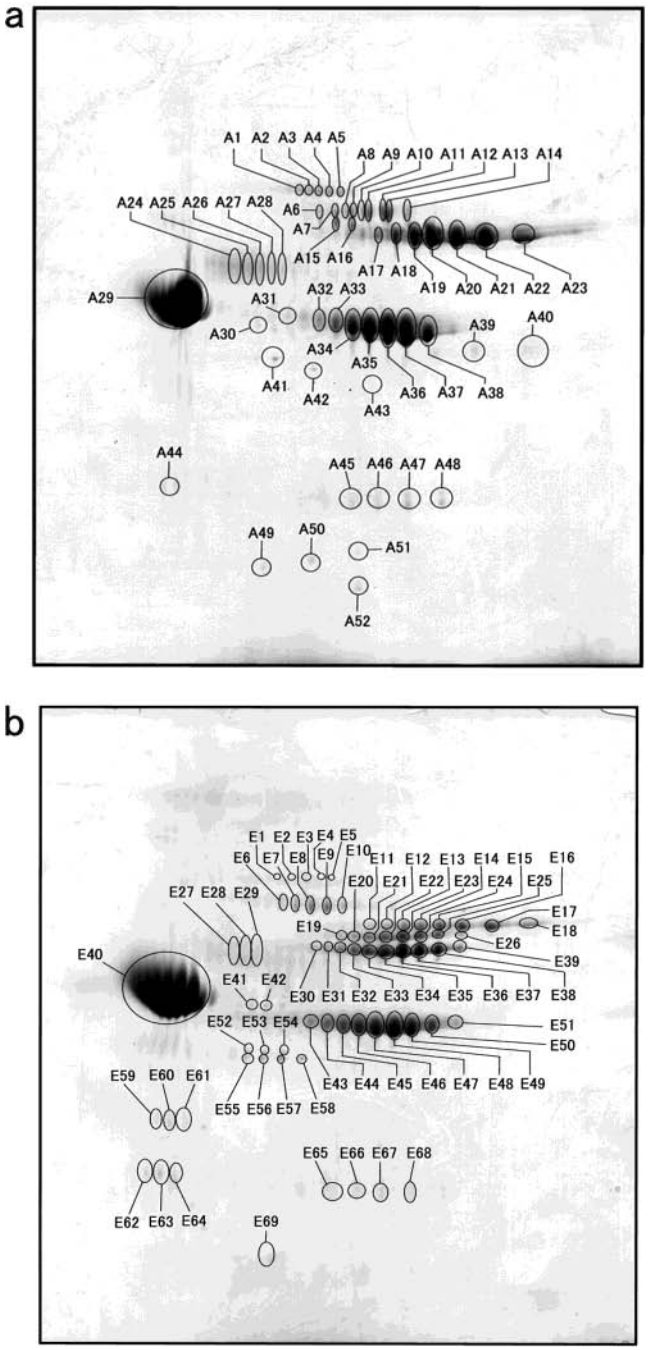

Figure 3. Spots of two-dimensional gel electrophoresis-resolved Africanized honeybee RJ proteins and European honeybee RJ proteins that were subjected to N-terminal sequence analysis. Two-dimensional gel electrophoresis-resolved proteins of Africanized honeybee RJ (**a**) and European honeybee RJ (**b**) were electrotransferred onto PVDF membranes. Protein spots were visualized by staining with CBB. The circled spots were cut off and subjected to N-terminal sequence analysis by automated Edman degradation on a model 492 protein sequencer.

sequence of spot HBRJ-A35 was shorter than the rest of spots HBRJ-A32, -A33, -A34, -A36, -A37, and -A38. Furthermore, it is not known why the spot HBRJ-A12 to -A14 appeared at a position corresponding to a higher molecular weight than that of HBRJ-A32 to -A38, despite these three spots having the same N-terminal amino acid sequence.

HBRJ-A17 to -A23 and HBRJ-E12 to -E25 and -E32 to -E39, which appeared with molecular weights of ~70 kDa and range of p*I* from 7 to 9, were assigned to MRJP3. Two amino acids,

Table 1. Summary of N-Terminal Amino Acid Sequence Analysis of HBRJ-A on Two-Dimensional Gel Electrophoresis-Resolved Proteins from Africanized Honeybee RJ

spot	N-terminal amino acid sequence[a]	protein identification	spot	N-terminal amino acid sequence[a]	protein identification
HBRJ-A1	AILNSMYNNV	glucose oxidase	HBRJ-A27	AVVRENSSRK	MRJP4
-A2	AILNSMYNNV	glucose oxidase	-A28	AVVRENSSRK	MRJP4
-A3	AILNSMYNNV	glucose oxidase	-A29	NILRGESLXKS[b]	MRJP1
-A4	AILNSMYNNV	glucose oxidase	-A30	AAVNHQRKSA	MRJP3
-A5	AILNSMYNNV	glucose oxidase	-A31	nd	
-A6	nd[c]		-A32	AIVRENSPRN	MRJP2
-A7	nd		-A33	AIVRENSPRN	MRJP2
-A8	nd		-A34	AIVRENSPRN	MRJP2
-A9	VTVRENSPRK	MRJP5	-A35	ENSPXXLEK	MRJP2
-A10	VTVRENSPRK	MRJP5	-A36	AIVRENSPRN	MRJP2
-A11	VTVRENSPRK	MRJP5	-A37	AIVRENSPRN	MRJP2
-A12	AIVRENSPRN	MRJP2	-A38	AIVRENSPRN	MRJP2
-A13	AIVRENSPRN	MRJP2	-A39	VTVRENSPRK	MRJP5
-A14	AIVRENSPRN	MRJP2	-A40	VTVRENSPRK	MRJP5
-A15	VTVRENSPRK	MRJP5	-A41	AAVNHTRKSA	MRJP3
-A16	VTVRENSPRK	MRJP5	-A42	AAVNHTRKSA	MRJP3
-A17	AAVNHQ(R/K)KSA	MRJP3	-A43	NLEKSSLNVIH	MRJP2
-A18	AAVNHQ(R/K)KSA	MRJP3	-A44	RVNRMDRMDR	MRJP5
-A19	AAVNHQ(R/K)KSA	MRJP3	-A45	AIVRENSPRN	MRJP2
-A20	AAVNHQ(R/K)KSA	MRJP3	-A46	AIVRENSPRN	MRJP2
-A21	AAVNHQ(R/K)KSA	MRJP3	-A47	AIVRENSPRN	MRJP2
-A22	AAVNHQ(R/K)KSA	MRJP3	-A48	AIVRENSPRN	MRJP2
-A23	AAVNHQ(R/K)KSA	MRJP3	-A49	PKFTKMTIDG	MRJP1
-A24	AVVRENSSRK	MRJP4	-A50	nd	
-A25	AVVRENSSRK	MRJP4	-A51	nd	
-A26	AVVRENSSRK	MRJP4	-A52	nd	

[a] Sequence is depicted by one-letter on notation of the corresponding amino acid. [b] X = amino acid residue that was not determined by this analysis. [c] Not determined by virtue of deficiency of protein quantity.

R and K, were identified at the seventh amino acid residue from the N termini in the spots of HBRJ-A17 to -A23 from the Africanized honeybees RJ. This finding has also been reported previously by Schmitzova et al. (*7*). Furthermore, the sixth amino acid residue of both HBRJ-A41 and -A42, which were confirmed to be MRJP3, was identified to be T. This intramolecular heterogeneity in the N-terminal regions of MRJP3 was not observed in the European honeybees RJ. In turn, intermolecular heterogeneity in the molecular weights of MRJP3 was observed in European honeybees RJ. Two spots (HBRJ-E56 and -E57) from the European honeybees RJ were also confirmed to be MRJP3. These spots were considered to result from degradation of the C-terminal region.

The C-terminal region of the MRJP3 protein has extensive repetitive regions consisting of XQNXX pentapeptides, as confirmed by internal amino acid sequence analysis (*7*, *8*, *14*, *15*). It has recently been reported that the structural features of N-glycans linked to royal jelly glycoprotein are divided into high-mannose type, hybrid type, and bianntennary type (*17*, *18*). These results suggest that the heterogeneity in both molecular weights and p*I* of the spots corresponding to MRJP3 protein may be caused by the extensive repetitive regions in the C-terminal region and by various sugar chains attached to the protein.

To date MRJP4's existence in RJ was only inferred from the isolation of its cDNA, and the presence of MRJP4 protein, therefore, had not been confirmed by SDS-PAGE analysis (*7*). In our two-dimensional gel electrophoresis, five spots, HBRJ-A24 to -A28, in Africanized honeybee RJ and two spots, HBRJ-E27 and -E28, in European honeybee RJ, although minor in quantity, were identified as MRJP4. The N-terminal amino acid sequence (GVVRENSSRK) of the spots in European honeybee RJ was identical to that of the reported MRJP4 cDNA (*7*). The

Table 2. Summary of N-Terminal Amino Acid Sequence Analysis of HBRJ-E on Two-Dimensional Gel Electrophoresis-Resolved Proteins from European Honeybee RJ

spot	N-terminal amino acid sequence[a]	protein identification	spot	N-terminal amino acid sequence[a]	protein identification
HBRJ-E1	nd[b]		HBRJ-E36	AAVNHQRKSA	MRJP3
-E2	nd		-E37	AAVNHQRKSA	MRJP3
-E3	nd		-E38	AAVNHQRKSA	MRJP3
-E4	nd		-E39	AAVNHQRKSA	MRJP3
-E5	nd		-E40	NILRGESLXK[c]	MRJP1
-E6	nd		-E41	nd	
-E7	VTVRENSPRK	MRJP5	-E42	nd	
-E8	VTVRENSPRK	MRJP5	-E43	AIVRENSPRN	MRJP2
-E9	VTVRENSPRK	MRJP5	-E44	AIVRENSPRN	MRJP2
-E10	VTVRENSPRK	MRJP5	-E45	AIVRENSPRN	MRJP2
-E11	n.d**		-E46	AIVRENSPRN	MRJP2
-E12	AAVNHQRKSA	MRJP3	-E47	AIVRENSPRN	MRJP2
-E13	AAVNHQRKSA	MRJP3	-E48	AIVRENSPRN	MRJP2
-E14	AAVNHQRKSA	MRJP3	-E49	AIVRENSPRN	MRJP2
-E15	AAVNHQRKSA	MRJP3	-E50	AIVRENSPRN	MRJP2
-E16	AAVNHQRKSA	MRJP3	-E51	AIVRENSPRN	MRJP2
-E17	AAVNHQRKSA	MRJP3	-E52	nd	
-E18	AAVNHQRKSA	MRJP3	-E53	nd	
-E19	AAVNHQRKSA	MRJP3	-E54	nd	
-E20	AAVNHQRKSA	MRJP3	-E55	nd	
-E21	AAVNHQRKSA	MRJP3	-E56	AAVNHQRKSA	MRJP3
-E22	AAVNHQRKSA	MRJP3	-E57	AAVNHQRKSA	MRJP3
-E23	AAVNHQRKSA	MRJP3	-E58	nd	
-E24	AAVNHQRKSA	MRJP3	-E59	nd	
-E25	AAVNHQRKSA	MRJP3	-E60	nd	
-E26	nd		-E61	nd	
-E27	GVVRENSSRK	MRJP4	-E62	nd	
-E28	GVVRENSSRK	MRJP4	-E63	nd	
-E29	nd		-E64	nd	
-E30	nd		-E65	AIVRENSPRN	MRJP2
-E31	nd		-E66	AIVRENSPRN	MRJP2
-E32	AAVNHQRKSA	MRJP3	-E67	AIVRENSPRN	MRJP2
-E33	AAVNHQRKSA	MRJP3	-E68	nd	
-E34	AAVNHQRKSA	MRJP3	-E69	nd	
-E35	AAVNHQRKSA	MRJP3			

[a] Sequence is depicted by one-letter on notation of the corresponding amino acid. [b] Not determined by virtue of deficiency of protein quantity. [c] X = amino acid residue that was not determined by this analysis.

N-terminal amino acid sequence (AVVRENSSRK) of the spot in Africanized honeybee was almost identical to the sequence (GVVRENSSRK) deduced from the reported cDNA sequence (*7*). It seems likely that this difference in one N-terminal amino acid residue may originate from the species differences between Africanized honeybees and European honeybees.

The observed molecular weight average (60 kDa) and p*I* (5−6) of MRJP4 that were determined by the two-dimensional gel electrophoresis were comparable between Africanized honeybee RJ and European honeybee RJ. However, the average molecular weight of MRJP4 was higher than that predicted from the cDNA sequence reported previously (*7*). Because the cDNA sequence of MRJP4 indicates eight possible N-glycosylation sites, the higher molecular weight of MRJP4 determined in our study may be ascribed to the attachment of sugar chains to the protein.

Although the spots attributed to MRJP5 protein (HBRJ-A9 to -A11, -A15, -A16, -A39, -A40, -A44 and HBRJ -E7 to -E10) were detected in both the Africanized honeybee RJ and the European honeybee RJ, the molecular weights and the p*I* values were different between the two RJs derived from different species of honeybees.

Immunoblot Analysis. The results of the N-terminal amino acid sequence analysis suggest that there exist remarkable differences in the heterogeneity of the MRJP3 between the two species of RJ. To visualize the differences clearly, immunostaining of the two-dimensional gel electrophoresis-resolved MRJP3 proteins was carried out with mAb against MRJP3.

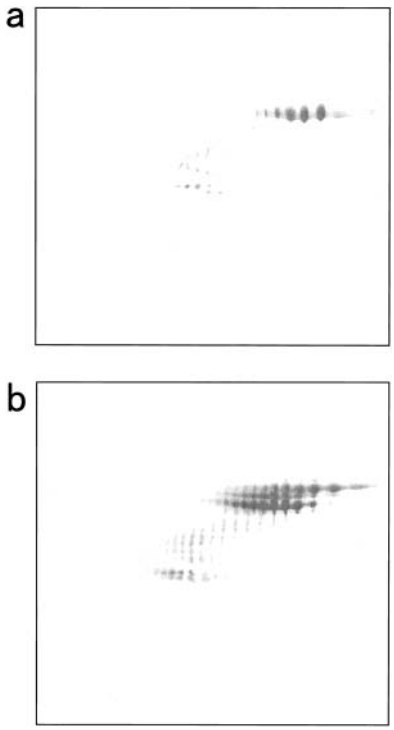

Figure 4. Western blotting analysis of two-dimensional gel electrophoresis-resolved Africanized honeybee RJ proteins and European honeybee RJ proteins. One hundred and fifty micrograms of Africanized honeybee RJ (**a**) and European honeybee RJ (**b**) was subjected to two-dimensional gel electrophoresis. Protein spots were then electrotransferred onto nitrocellulose membranes followed by immunostaining with anti-MRJP3 mAb and collectively with HRPO-conjugated goat antibodies to mouse IgG. Data shown are representative of nine lots of Africanized honeybee and European honeybee RJs.

Panels a and b of **Figure 4** show the results of the immunostaining with anti-MRJP3 mAb (no. 9) of the Africanized honeybee RJ and the European honeybees RJ, respectively. As expected from the results of N-terminal amino acid sequence analysis, the heterogeneity of the MRJP3 protein was remarkable, and the MRJP3 from European honeybees was more heterogeneous than that from Africanized honeybees. Furthermore, spots HBRJ-E52 to -E55 and -E58, which were not identified by the N-terminal amino acid analysis by virtue of deficiency of protein quantity, were assigned to MRJP3 by immunostaining with anti-MRJP3 mAb (mo. 9).

In summary, European honeybee RJ and Africanized honeybee RJ were analyzed by two-dimensional gel electrophoresis. Using N-terminal amino acid analysis, we observed considerable heterogeneity within each protein of the glucose oxidase and the MRJP family. The reason why the RJ proteins produced by Africanized and the European honeybees exhibited remarkable heterogeneity in two-dimensional gel electrophoresis remains unclear. In particular, the MRJP3 protein derived from European honeybee RJ exhibited considerable heterogeneity in terms of molecular size as well as p*I* values. In agreement with our findings, Albert et al. reported that MRJP3 from European honeybee RJ exhibits a size polymorphism as detected by SDS-PAGE (*9*). They have shown that polymorphism of the MRJP3 protein is a consequence of the polymorphism of a region with a variable number of tandem repeats located at the C-terminal part of the MRJP3 coding region. In contrast, our results showed that the MRJP3 protein derived from Africanized honeybee RJ was less polymorphic in size than that from European honeybee RJ. These results prompt us to speculate that the DNA sequence

of the MRJP3 coding region may also be less polymorphic in Africanized honeybees compared with that of European honeybees. These results further imply that the differences between the RJ proteins produced by Africanized and European honeybees may be ascribed to genetic differences in addition to posttranslational modifications. Further studies are necessary to confirm these possibilities.In this study, proteins with a molecular weight of <10 kDa, such as royalisin (5.5 kDa), were not detected, because the second-dimension SDS-PAGE was performed using 10% polyacrylamide gel. Identification of the proteins with low molecular weights would be possible by using polyacrylamide at concentrations >10%. Thus, two-dimensional gel electrophoresis provides a suitable method for the detailed analysis of the major RJ proteins.

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