



Hypoallergenicity and Immunological Characterization of Enzyme-Treated Royal Jelly from *Apis mellifera*

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Royal jelly (RJ), the exclusive food for queen bees, is taken as a dietary supplement because it is highly rich in nutrients. However, RJ is known to induce an anaphylactic response in some individuals. We evaluated in the present study the hypoallergenicity of alkaline protease-treated RJ *in vitro* and *in vivo*. We first confirmed that this treated RJ contained the same levels of vitamins, minerals and specific fatty acid as in untreated RJ. We then showed that the IgE-binding capacity of the treated RJ was very significantly reduced by conducting *in vitro* assays of the blood from RJ-sensitive patients. An *in vivo* skin-prick test on the RJ-sensitive patients also showed that, in the majority of the patients (3 out of 4 tested), the treated RJ did not evoke any allergenic response. It is thus advantageous to prepare hypoallergenic RJ by a protease enzyme treatment for its safe consumption.

Key words: royal jelly; allergenic protein; major royal jelly protein; enzyme-treated royal jelly; hypoallergenic royal jelly

Royal jelly (RJ), which is widely consumed as a dietary supplement, contains abundant nutrients. RJ is composed of water, proteins, carbohydrates, lipids, mineral salts and vitamins.^{1,2)} (E)-10-Hydroxy-2-decenoic acid (10-HDA), which is the major fatty acid in RJ, has been used as an indicator of the quality of RJ.²⁾ Dried RJ is largely made of proteins, mainly belonging to one protein family and designated as major royal jelly protein (MRJP).³⁻⁶⁾ MRJPs account for 82–90% of total RJ proteins. MRJP1 (also known as royalactin) is the most abundant RJ protein (31%), followed by MRJP3 (26%), MRJP2 (16%), and MRJP5 (9%).⁷⁾ An important role of MRJP1 is to differentiate honeybee larvae into

queens through an EGFR (epidermal growth factor receptor)-mediated signaling pathway.⁸⁾

RJ has been shown to prevent osteoporosis in an ovariectomized rat model.⁹⁾ It has also improved tinnitus in Japanese patients.¹⁰⁾ Enzymatic hydrolysis of RJ proteins produces free amino acids and small peptides, including some bioactive peptides. It has previously been demonstrated that enzyme-treated RJ was more potent than untreated RJ in preventing osteoporosis, hypertension and insulin resistance in animal disease models.^{9,11,12)}

Human safety is an important factor to consider, because RJ is consumed as a dietary supplement. In this regard, it is noteworthy that untreated RJ has been reported to cause such adverse reactions as urticaria, eczema, acute asthma and anaphylaxis,¹³⁻²¹⁾ although such adverse reactions rarely happen.^{18,22)} Rosmilah *et al.*¹⁷⁾ and Leung *et al.*¹⁹⁾ have reported that RJ-sensitive subjects possessed IgE antibodies against a number of RJ proteins. In fact, the IgE antibodies found in Malaysian patients with RJ allergy were found to recognize MRJP1 and MRJP2, suggesting that MRJP1 and MRJP2 might be allergenic to humans.

It is generally known that a protease treatment of the allergenic proteins in foods produces smaller molecules and reduces the allergenicity.^{23,24)} However, hydrolysis of the proteins by acid or protease could also increase the allergenicity and antigenic epitopes; it has been reported that inadequate hydrolysis of wheat proteins increased the antigenicity of the serum antibodies obtained from patients with wheat allergy.²⁵⁻²⁷⁾

The purpose of this present study is to demonstrate that the antigenicity does not increase in protease-treated RJ. We therefore hydrolyzed RJ proteins by using

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Abbreviations: 10-HDA, (E)-10-hydroxy-2-decenoic acid; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; RJ, royal jelly; MRJP, major royal jelly protein; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GF-HPLC, gel-filtration high-performance liquid chromatography; IgE, immunoglobulin E; CBB, Coomassie brilliant blue; SD, standard deviation

bacterial alkaline proteases to reduce the size of the allergenic proteins. We then performed *in vitro* and *in vivo* assays and tests to examine whether the protease treatment reduced the RJ-associated allergenicity. The antigenicity of the enzyme-treated RJ sample was evaluated *in vitro* by immunoblotting, IgE-ELISA, competitive inhibition ELISA and a histamine-release test, and the *in vivo* allergenicity was evaluated by using a human skin-prick test. Our results clearly show that protease-treated RJ did not evoke any adverse response in the majority of RJ-sensitive patients as the test subjects.

Materials and Methods

Preparation of enzyme-treated RJ. RJ (Zhejiang Sheng, China) was digested for 4 h with bacterial alkaline proteases, which were purchased from Kaken Pharmaceutical Co. (Japan), at a temperature of 40–50 °C and in a pH range of 7.8–9.0. After the enzymatic hydrolysis, the proteases were denatured by heating the reaction mixture at 80 °C for 15 min and then lyophilizing the mixture. Untreated RJ was prepared by processing RJ in the same manner as that just described in the absence of any added proteases and then lyophilized as before.

Analysis of (E)-10-hydroxy-2-decenoic acid, vitamins and minerals in untreated and enzyme-treated RJ. Lyophilized powder (0.3 g) of untreated RJ or enzyme-treated RJ was dissolved in 30 mL of a solvent (ethanol/water, 2/1, v/v) and then sonicated for 10 min. To this solution, 1 mL of 1 M NaOH was added to dissolve the precipitate. The mixture was shaken for 10 min and then diluted with water to 0.5 mg/mL.

The amount of 10-HDA in this solution was analyzed by a Prominence HPLC system (Shimadzu, Japan) fitted with a YMC-Pack ODS-AM column (4.6 × 150 mm; YMC Co., Japan), the temperature of which was maintained at 40 °C, using an isocratic solvent composed of methanol/10 mM phosphoric acid (2/3) at a flow rate of 1.0 mL/min, the elution being monitored at 210 nm. We purchased 10-HDA from Matsuura Pharmaceutical Co. (Japan) and used it as a standard. Vitamin B₁ and vitamin B₂ were analyzed by HPLC, using a reverse-phase C₁₈ column (Shimadzu, Japan) according to the standard procedure. Vitamin E was also analyzed by HPLC, using a normal-phase silica column (Shimadzu, Japan). Minerals (calcium, magnesium, copper, zinc and manganese) were analyzed by atomic absorption spectrometry, using an AA-890 atomic absorption spectrometer (Nippon Jarrell-Ash, Japan) and following the manufacturer's instructions. Potassium was measured by using an AA-220 atomic absorption spectrometer (Varian, Australia).

Subjects. Four Japanese patients (4 adult females) with RJ anaphylaxis were used as subjects to test the allergenic response of RJ. A healthy subject who showed no anaphylactic response to RJ was included as a control subject. Blood samples were taken from the control subject and patients to obtain serum antibodies. Informed consent was obtained from all subjects, including the normal control subject. This project was approved by the local ethics committee (approval number 23-5 of the Institutional Review Board) and the study was performed in accordance with the Declaration of Helsinki.

Electrophoresis and immunoblotting. RJ proteins were separated by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method of Laemmli,²⁸⁾ using 10% polyacrylamide gel.

The western immunoblotting analysis was conducted on the RJ proteins separated by SDS-PAGE which were transferred on to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, USA). The transferred membrane was incubated for 1 h in PBS containing 0.1% Tween-20 and 5% skim milk for blocking. The membrane was then incubated overnight at 4 °C with the patient's serum diluted in the blocking solution. Bound serum antibodies were detected by using HRP-conjugated goat anti-human IgE (Kirkegaard & Perry Laboratories, MD, USA) and an enhanced chemiluminescence

(ECL)-Western blotting kit (GE Healthcare UK). RJ proteins on the SDS-PAGE gel were also stained with Coomassie brilliant blue (CBB) R-350 by following the standard procedure.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Immobiline DryStrip pH 3–10 (GE Healthcare UK) was immersed for the first-dimension isoelectric focusing gel, and the sample lysate applied to the gel according to the manufacturer's instructions. SDS-PAGE was performed after isoelectric focusing, and after 2D-PAGE, the gel was stained with CBB R-350 or used for the immunoblotting analysis as before.

Identification of the allergenic proteins by 2D-PAGE and an N-terminal amino acid sequence analysis. The RJ proteins were separated by 2D-PAGE as just described, and the separated proteins were transferred on to a PVDF membrane which was then stained with 0.1% Ponceau S (Sigma-Aldrich, USA) containing 1% acetic acid. Protein spots corresponding to the patients' IgE-binding proteins were selected, and these were subjected to an N-terminal amino acid sequence analysis using an Applied Biosystems 492 protein sequencer (Perkin Elmer, USA).

Fractionation of untreated RJ and enzyme-treated RJ by gel-filtration HPLC (GF-HPLC). Approximately 1 mg of each sample was dissolved in a 0.05 M phosphate buffer (pH 7.0) containing 0.3 M NaCl and then passed through 0.45-μm syringe filter. Each sample solution (400 μL) was loaded into a GF-HPLC column (TSKgel G3000SW, 6.5 × 600 mm (Tosoh, Japan)) at the flow rate of 0.4 mL/min. The same 0.3 M NaCl-containing phosphate buffer was used for elution, fractions being collected at 2-min intervals. The fractions containing the eluted proteins were identified by measuring the absorbance at 280 nm.

IgE-ELISA assay of the fractions eluted from the GF-HPLC column. Aliquots of the collected fractions obtained from the TSKgel G3000SW gel filtration column were added into the wells (100 μL/well) of an ELISA plate (Iwaki, Japan), and the plate was incubated at 37 °C for 3 h to coat each well. After blocking with 1% BSA in PBS, mixed sera from the RJ-sensitive patients, diluted (×50) in CanGetSignal™ solution 1 (Toyobo, Japan), were added to each well, and the plate was incubated overnight at 4 °C. The wells were subsequently washed with PBS containing 0.1% Tween-20 (PBS-T), and incubated at 37 °C for 2 h with a solution diluted in CanGetSignal™ solution 2 (Toyobo, Japan) of HRP-labeled goat anti-human IgE (Kirkegaard & Perry Laboratories). After washing with PBS-T (PBS containing 0.1% Tween-20), the amount of IgE bound to the protein in each GF-HPLC separated fraction was detected by using HRP substrate TMB (Kirkegaard & Perry Laboratories). Once the desired color had been achieved, the reaction was stopped by adding 1 M phosphoric acid, and the absorbance at 450 nm was recorded for each well with a plate reader (Wallac ARVO multi-label counter).

Competitive inhibition ELISA. ELISA plates (Iwaki, Japan) were coated with 30 μg/mL of each RJ solution in 100 μL of PBS per well at 37 °C for 1 h. After blocking with Blocking One-P (Nacalai Tesque, Japan) to prevent non-specific binding to the coated ELISA plates, untreated human serum or RJ-treated human serum was added to each well and incubated at room temperature for 1 h. The concentration of RJ (untreated or enzyme-treated) was varied from 0.686 × 10⁻³ mg/mL to 0.167 mg/mL for pre-incubating with the serum. The wells were subsequently washed with PBS containing 0.1% Tween-20 (PBS-T), and incubated at 37 °C for 1 h with a solution containing biotin-labeled goat anti-human IgE (Kirkegaard & Perry Laboratories) diluted in CanGetSignal™ solution 2 (Toyobo). After washing with PBS-T, the amount of bound IgE was detected at 450 nm, after adding HRP-conjugated streptavidin (GE Healthcare UK), the TMB substrate (Kirkegaard & Perry Laboratories, MD, US) and 1 M phosphoric acid to each well.

Histamine release test. Whole human blood diluted 4:1 in a B-CAST-DS dextran solution (Bühlmann, Switzerland) was incubated for 90 min at room temperature. The upper phase was then transferred into another tube and centrifuged for 15 min at 130 × g at room

temperature. The supernatant was discarded, and the basophil cells and leukocytes were resuspended in an equal volume of a B-CAST-DS stimulation buffer (Bühlmann, Switzerland). Next, the cells were challenged for 40 min at 37 °C with various dilutions of the allergen (untreated or enzyme-treated RJ). The reaction was stopped by centrifugation at $1000 \times g$ and 4 °C for 3 min.

Total histamine released in the supernatant was measured after acylation by using a Histamine EIA kit (Immuno Biological Laboratories, Germany). The anti-IgE receptor was used as a positive control.

Human skin-prick test. RJ or enzyme-treated RJ was diluted in PBS and used at concentrations 0.025, 0.25 and 2.5 mg/mL. The RJ samples were tested on patients who had an anaphylactic reaction to RJ. These patients were selected for this investigation from the outpatients at the Department of Dermatology, Ogaki Municipal Hospital and Minami-Okayama Medical Center. The skin surface of the right and left forearm of each patient was pricked by a needle at 3-cm intervals. Diluted untreated RJ or enzyme-treated RJ was then placed on the right or left forearm of the patients, with a 1% histamine solution used as a positive control and PBS as a negative control. Erythema and wheals caused by the histamine solution and RJ samples were measured at 5-min intervals after applying the samples.

Results

Measurement of (E)-10-hydroxy-2-decenoic acid, vitamins and minerals in untreated and enzyme-treated RJ

To prepare enzyme-treated RJ, RJ was digested by bacterial alkaline proteases which had been selected as the most efficient food-additive enzyme for digestion. Table 1 summarizes that proteolysis (enzymatic hydrolysis) had no effect on the 10-HDA content of RJ. In addition, such other components of RJ as minerals, vitamins, iron, calcium, potassium, magnesium, copper, zinc, and manganese also remained almost unchanged in enzyme-treated RJ (Table 1). As expected, the contents of peptides and free amino acids increased after the enzymatic hydrolysis of RJ.

Identification of allergenic proteins in RJ by using 2D-PAGE, immunoblotting analysis and N-terminal amino acid sequencing

Figure 1A shows the protein profile of RJ proteins obtained from 2D-PAGE and subsequent CBB staining. Figure 1B shows an immunoblot of the 2D-PAGE-separated RJ proteins, using the serum of a patient who was sensitive to RJ. Figure 1B shows that there were two major immunoreactive spots (A and B). N-Terminal

amino acid sequencing of the proteins isolated from these two spots revealed two peptide sequences: NILR-GESE and AIVRENSPR (Table 2); these two peptide sequences respectively matched the N-terminal sequences of MRJP1 and MRJP2. The protein in spot A (MW of 55 kDa, pI of 5.4 on 2D-PAGE gel) was therefore confirmed as MRJP1 (Genbank ID, AF000633; Uniprot ID, Q548D6; alternative name, royalactin), and the protein in spot B (MW of 49 kDa, pI of 6.7 on 2D-PAGE gel) was confirmed as MRJP2 (Genbank ID, AF000632).

GF-HPLC and SDS-PAGE analysis of RJ proteins in untreated RJ and enzyme-treated RJ

Figure 2A and B respectively show the GF-HPLC profiles of untreated RJ and enzyme-treated RJ. The eluted peak fractions of the molecular mass standards used were as follow: blue dextran (2000 kDa, void volume), fraction no. 1; bovine serum albumin (BSA, 66 kDa), fraction no. 12; cytochrome *c* (12.4 kDa), fraction no. 20. The absorbance at 280 nm of fractions no. 33–50 of untreated RJ and enzyme-treated RJ was around zero (data not shown), suggesting that there were no proteins in these eluted fractions. This was also supported by the data shown in Fig. 2C and D. The HPLC elution profile of untreated RJ showed two major peaks, one of which (corresponding to the high molecular mass component) disappeared upon the protease treatment (Fig. 2B). Figure 2C and D respectively show CBB-stained SDS-PAGE gels of the GF-HPLC fractions of untreated RJ and enzyme-treated RJ samples. It is evident that the protein components of RJ, most of which were in the molecular mass range of 25 kDa and above, had completely disappeared following proteolysis (Fig. 2D). Some faint bands around 50–75 kDa were detected in fractions no. 32 and later of the untreated RJ

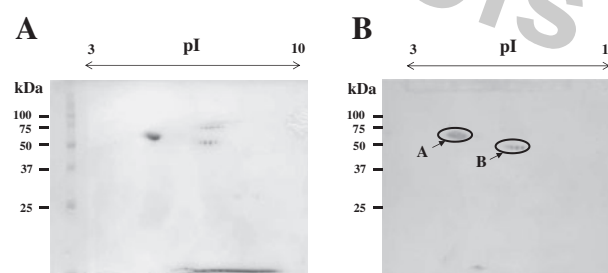


Fig. 1. Detection of the Allergenic Proteins in RJ by 2-D Gel Electrophoresis and an Immunoblot Assay.

A, CBB stained 2D-PAGE of the RJ proteins. B, Identification of allergenic proteins in untreated RJ by immunoblotting. The proteins in RJ were separated by 2D-PAGE, transferred to a PVDF membrane, and then the membrane was used in an immunoblot assay with the serum of an RJ-sensitive patient or a healthy subject (control). Proteins reacting with the indicated serum were detected by using chemiluminescent reagents as described in the Materials and Methods section. The arrowheads indicate protein spots which were subjected to an N-terminal amino acid sequence analysis.

Table 2. N-Terminal Amino Acid Sequences, Molecular Masses and Isoelectric Points of Proteins in Spots A and B (see arrows in Fig. 1B)

Spot	N-Terminal sequence	Molecular mass	pI	Protein name
A	NILRGESE	55 kDa	5.4	MRJP1
B	AIVRENSPR	49 kDa	6.7	MRJP2

Table 1. Analysis of Fatty Acids, Minerals and Vitamins in Untreated and Enzyme-Treated RJ

Category	Component	Weight (%)	
		Untreated RJ	Enzyme-treated RJ
Fatty acid	10-HDA	1.6	1.6
Mineral	Copper	1.3×10^{-3}	1.3×10^{-3}
	Manganese	2.6×10^{-4}	2.2×10^{-4}
	Zinc	6.5×10^{-3}	6.6×10^{-3}
	Potassium	8.1×10^{-1}	8.1×10^{-1}
	Magnesium	8.9×10^{-2}	9.1×10^{-2}
	Calcium	4.1×10^{-2}	4.1×10^{-2}
Vitamin	Vitamin B1	1.0×10^{-3}	1.0×10^{-3}
	Vitamin B2	1.9×10^{-3}	2.0×10^{-3}
	Vitamin E	1.0×10^{-3}	1.0×10^{-3}

The experiment was performed by using the same lot of royal jelly.

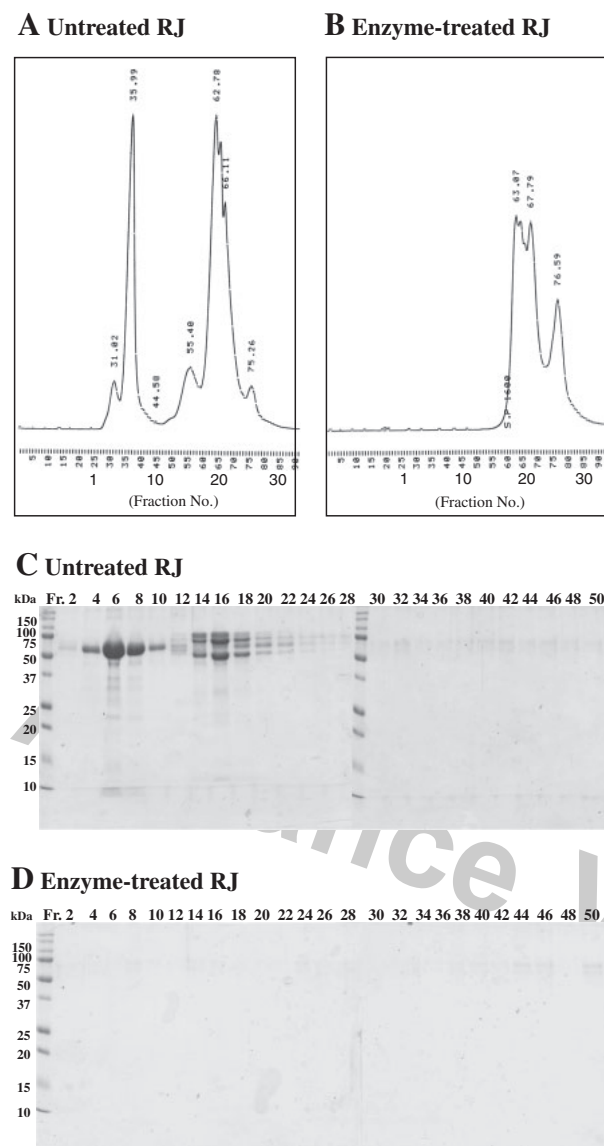


Fig. 2. Fractionation of the Untreated and Enzyme-Treated RJ Samples by GF-HPLC and an SDS-PAGE Analysis of the Collected Fractions.

Panels A and C, untreated RJ (control); panels B and D, enzyme-treated RJ. A and B, Four hundred micrograms of the indicated RJ sample was applied to the HPLC gel filtration column. The mobile phase was a 0.05M phosphate buffer containing 0.3M NaCl (pH 7.0). The eluted proteins were monitored by measuring the absorbance of the eluate at 280 nm. The flow rate was 0.4 mL/min, and fractions were collected at 2-min intervals. CBB stained SDS-PAGE of the HPLC fractions obtained from untreated RJ (C) and enzyme-treated RJ (D).

and enzyme-treated RJ samples. Similar bands were detected (data not shown) when no fraction (only water) was treated with the SDS sample buffer and applied to SDS-PAGE, suggesting that these faint bands were derived from non-specific contaminated proteins.

Immunological reactivity of serum IgE antibodies with the untreated and enzyme-treated RJ samples

The binding of IgE antibodies to RJ proteins was evaluated by an immunoblot analysis (typical results are shown in Fig. 3, middle and right-hand panels). It is clear that sera from the patients recognized a protein with a molecular mass of 55 kDa in the untreated RJ sample (Fig. 3), this being one of the major protein

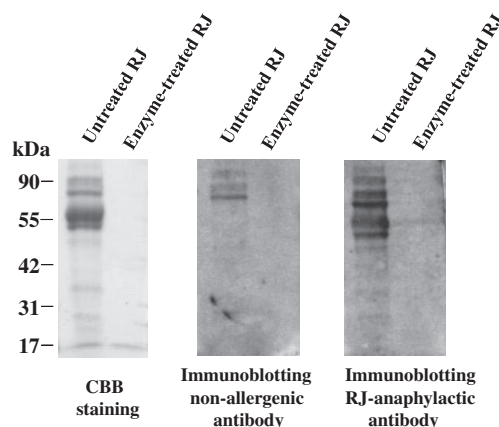


Fig. 3. Immunological Reactivity of Serum IgE Antibodies with the Untreated and Enzyme-Treated RJ Samples.

The untreated and enzyme-treated RJ samples were separated by SDS-PAGE and then used for an immunoblot analysis with sera from the RJ-sensitive patients and healthy control. CBB staining is shown of SDS-PAGE of the untreated and enzyme-treated RJ samples (left panel). Immunoblots of the untreated and enzyme-treated RJ samples are shown using the serum obtained from a healthy subject (middle panel) and the serum obtained from an RJ-sensitive patient (right panel).

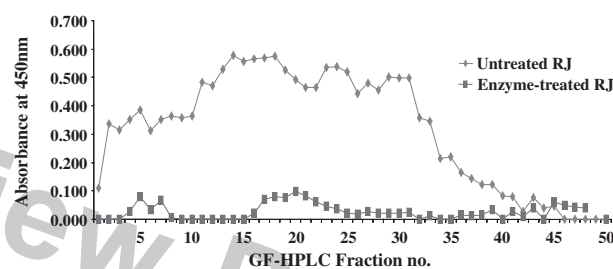


Fig. 4. IgE-ELISA Assay of the Eluted Fractions from GF-HPLC.

The GF-HPLC fractions of untreated RJ (closed diamonds) and enzyme-treated RJ (closed squares) were analyzed by ELISA, using the sera of RJ-sensitive patients. The wells of ELISA plates were coated by adding aliquots of the GF-HPLC fractions (100 μ L) and incubating at 37 $^{\circ}$ C for 3 h. ELISA was performed as described in the Materials and Methods section, using the serum of an RJ-sensitive patient. The amount of IgE bound to the separated epitopes was detected by using the horseradish peroxidase-conjugated secondary antibody and a TMB substrate.

bands found by CBB-stained SDS-PAGE of RJ (see Fig. 2C and Fig. 3 left-hand panel). This 55-kDa protein band was absent in the immunoblot carried out with the control serum (Fig. 3, middle panel). There were also additional protein bands of higher molecular mass (60–90 kDa) on both immunoblots (Fig. 3, middle and right-hand panels). These bands might result from non-specific binding of antibodies to proteins which may have nothing to do with allergy. To further confirm the binding, IgE-ELISA was carried out using the GF-HPLC fractions and sera of patients. Figure 4 shows that the GF-HPLC fractions obtained from enzyme-treated RJ had significantly less IgE binding than those of untreated RJ. Only fractions 5–8 and 17–24 of enzyme-treated RJ showed slight binding to the IgE antibodies of RJ-sensitive patients (Fig. 4).

Competitive inhibition ELISA

We performed an ELISA-based competitive inhibition assay to assess the affinity of serum antibodies of the patients for the untreated and enzyme-treated RJ

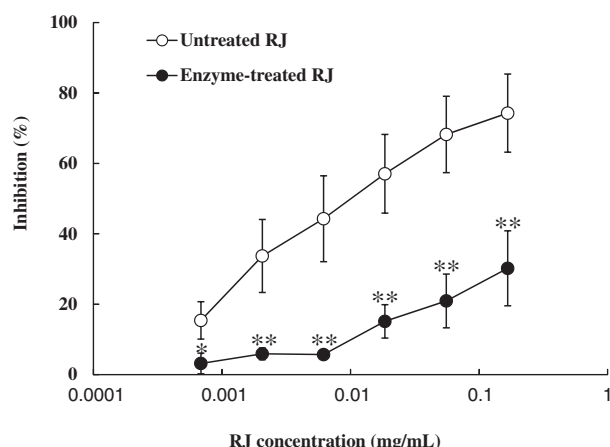


Fig. 5. Competitive Inhibition ELISA.

Human serum obtained from the RJ-sensitive patient was pre-incubated with untreated RJ (open circles) or enzyme-treated RJ (closed circles). A competitive inhibition assay was performed as described in Materials and Methods section, using different concentrations (0.000686, 0.00206, 0.00617, 0.0185, 0.0556 and 0.167 mg/mL) of the indicated RJ sample. All experiments were carried out in duplicate. Data shown are presented as the mean $\pm$ SD values. The statistical significance of the data was evaluated by an analysis of variance and Dunnett's test (* p < 0.05, ** p < 0.01, n = 4, vs. untreated RJ).

samples. The results shown in Fig. 5 reveal that untreated RJ bound more strongly to sera antibodies than the enzyme-treated RJ; we observed significant differences in IgE-binding for the entire concentration range of RJ used in this experiment (from 0.686×10^{-3} mg/mL to 0.167 mg/mL). The allergenicity was compared with the concentration of RJ at approximately 30% inhibition, that of treated RJ (0.167 mg/mL) being approximately 80-fold higher than that of untreated RJ (0.00206 mg/mL).

Histamine-release test

IgE-mediated allergic reactions involve the release of such chemical mediators as histamine from basophils or mast cells. The histamine-release test revealed that the untreated RJ-induced release of histamine from the basophil cells and leukocytes, which had been isolated from the RJ-sensitive patients, was more than that induced by enzyme-treated RJ (Fig. 6). At both test concentrations (0.01 and 0.1 mg/mL), there were significant differences between the amount of histamine released by untreated RJ and enzyme-treated RJ (t -test: * p < 0.05, n = 4).

Human skin-prick test

To evaluate allergenicity *in vivo*, we used various dilutions of the untreated and enzyme-treated RJ samples (diluted with PBS to 0.025, 0.25 and 2.5 mg/mL) for performing the human skin-prick test. The results after 15 min are shown in Fig. 7. Three patients did not exhibit erythema or wheals when challenged with enzyme-treated RJ. However, one patient did exhibit erythema and wheals when challenged with a high concentration (0.25 and 2.5 mg/mL), but not when challenged with 0.025 mg/mL of enzyme-treated RJ (Fig. 7, subject 2). It is possible that the protease treatment did not remove some of the allergenic epitopes from RJ.

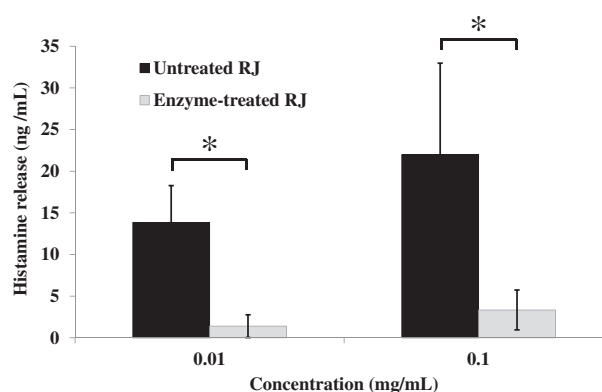


Fig. 6. Histamine Release Induced by the Untreated and Enzyme-Treated RJ Samples.

Basophils and leukocytes were treated with the indicated concentration of an RJ sample (untreated or enzyme-treated), and the histamine released from the basophils and leukocytes was measured by using a Histamine EIA kit (Immuno Biological Laboratories, Germany) as described in the Materials and Methods section. The anti-IgE receptor was used as a positive control (Histamine EIA kit, Immuno Biological Laboratories, Germany). Data are presented as the mean $\pm$ SD values. The statistical significance of the data was evaluated by an analysis of variance and Student's t -test (* p < 0.05, n = 4).

Subject	UntreatedRJ (mg/mL)			Enzyme-treated RJ (mg/mL)		
	0.025	0.25	2.5	0.025	0.25	2.5
1	2 x 2	3 x 3	4 x 5	n.d.	n.d.	n.d.
2	n.d.	3 x 3	18 x 17	2 x 2	13 x 15	19 x 22
3	n.d.	2 x 2	2 x 2	n.d.	n.d.	n.d.
4	9 x 9	5 x 5	20 x 16	n.d.	3 x 3	1 x 1

Fig. 7. Evaluation of the Allergenicity of Enzyme-Treated RJ.

The allergenicity of enzyme-treated RJ was evaluated by a human skin-prick test, using the indicated concentrations of untreated or enzyme-treated RJ. The recorded values for the right shoulder in each picture represent the size of erythema. n.d. means not detected.

Discussion

We have demonstrated in the present study, through *in vitro* and *in vivo* tests, that the enzymatic hydrolysis digested away most of the epitopes of the antigenic proteins in RJ, and significantly reduced the immunoreactivity and allergenicity of RJ.

We initially confirmed the results of Rosmilah *et al.*¹⁷⁾ and demonstrated by 2D-PAGE, an immunoblot assay and N-terminal amino acid sequence analysis that MRJP1 and MRJP2 were antigenic proteins of RJ (Fig. 1 and Table 2). Furthermore, by using GF-HPLC, SDS-PAGE and the immunoblot assay, we analyzed whether most of the antigenic proteins of RJ were digested by the alkaline proteases (Figs. 2 and 3). We also used ELISA-based IgE competitive inhibition assays to confirm that the IgE-binding affinity of enzyme-treated RJ had been significantly reduced (Figs. 4 and 5). We finally measured the histamine released from basophils and leukocytes isolated from the RJ-sensitive patients and also performed a skin-prick

test on RJ-sensitive patients to demonstrate that the allergenicity of RJ was less after enzymatic hydrolysis (Figs. 6 and 7).

The results obtained from GF-HPLC indicate that there were two main elution peaks, around fractions no. 4–8 and no. 14–20 of the untreated RJ sample (Fig. 2A and C). The estimated molecular mass of the first protein peak seemed to be larger than that of BSA (66 kDa), because the elution times were earlier than BSA. However, the molecular masses of the component polypeptides were around 55 kDa detected by SDS-PAGE in the first protein peak, suggesting that polypeptides around 55 kDa might have taken oligomeric forms in the native untreated RJ sample.

Fukutomi *et al.*²⁵⁾ have demonstrated in a previous study that inadequate hydrolysis of wheat proteins increased the reactivity to IgE antibodies obtained from Japanese women with wheat allergy. It has been demonstrated in another study that hydrolysis of wheat proteins by acid or protease in cosmetics could induce immediate hypersensitivity.²⁶⁾ These results suggest that intramolecular antigenic epitopes of intact wheat proteins had probably been exposed by the inadequate hydrolysis.

We therefore evaluated in the present study the difference between the binding affinity of enzyme-treated RJ and untreated RJ for serum IgE of the RJ-sensitive patients. The results described in this study suggest that most of the antigenic epitopes of RJ proteins had indeed been digested by the proteases, resulting in their affinity for IgE of the RJ-sensitive patients being significantly reduced.

We performed a histamine-release test and skin-prick test to assess the allergenicity of enzyme-treated RJ. Basophils and leukocytes isolated from all four patients released histamine to a much lower extent when incubated with enzyme-treated RJ than when incubated with untreated RJ (Fig. 6). This result suggests that enzymatic hydrolysis of RJ could reduce allergenicity in the patients. To further confirm this notion, we next performed the skin-prick test. We observed that the application of untreated RJ (0.025–2.5 mg/mL) to the skin induced erythema and wheals in all four RJ-sensitive patients (Fig. 7); in contrast, the application of enzyme-treated RJ did not induce erythema and wheals in 3 out of the 4 patients, even at 2.5 mg/mL. However, enzyme-treated RJ induced erythema and wheals in one patient at a 2.5 mg/mL concentration (Fig. 7, subject 2). This observed allergenicity of enzyme-treated RJ in one patient might have been due to the presence of some residual antigenic epitopes in the enzyme-treated RJ sample. Figure 4 shows that fractions no. 5–8 and no. 17–24 were significantly reactive with the serum antibodies obtained from the RJ-sensitive patients. This result suggests that enzymatic hydrolysis could not completely digest the antigenic epitopes in RJ. Consistent with this notion, the competitive inhibition results shown in Fig. 5 indicate that enzyme-treated RJ still had some affinity for serum IgE at 0.075 mg/mL.

The inhibition plot shown in Fig. 5 was actually generated by averaging the inhibition data obtained from four patients. The inhibition data for one of the patients, however, showed a lower decrease in the affinity for

serum IgE (results not shown). This patient was positive to enzyme-treated RJ in the skin-prick test (0.25–2.5 mg/mL, Fig. 7, subject 2).

These results suggest that a number of epitopes may have been present in the MRJP1 and MRJP2 proteins, and the enzymatic hydrolysis described in this study could not completely digest all the antigenic epitopes. The epitopes recognized by IgE of at least 3 patients appeared to have been digested by the protease treatment.

We thought that we had optimized the enzymatic hydrolysis of RJ in this study. However, a few antigenic epitopes may still have been present in the enzyme-treated RJ sample, and they may have induced the positive response in some patients. In Fig. 4, small peaks reactive to patients' IgE were detected around fractions no. 4–8 and no. 16–24 of enzyme-treated RJ. These fraction areas corresponded to the major protein peaks of untreated RJ, suggested that small amounts of the undigested intact RJ allergenic proteins might have contaminated the enzyme-treated RJ preparations. These non-digested protein residues in enzyme-treated RJ might have reacted with patients' IgE *in vitro* and *in vivo* (Figs. 5–7). A further investigation is therefore needed to find better conditions for completely digesting all the allergenic proteins and epitopes present in RJ.

In conclusion, we confirmed that MRJP1 (alternative name to royalactin) and MRJP2 were IgE-binding allergenic proteins in RJ, and that the protease treatment effectively degraded these allergenic proteins, reducing the IgE-binding capacity assessed by the *in vitro* assays. Furthermore, using the histamine-release test and human skin-prick test, we demonstrated that enzymatic hydrolysis markedly reduced the allergenicity of RJ in patients with RJ anaphylaxis. Our results also suggest that a further study is needed to make an epitope-free RJ preparation that would be completely allergen free.

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Advance View Proofs