

Immunological Characterization of Honey Proteins and Identification of MRJP 1 as an IgE-Binding Protein

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We encountered a fourth case of honey allergy in Japan. We characterized and identified the IgE-binding proteins in honey using the serum of a honey-allergic patient. Immunoblot analysis revealed that IgE in the patient serum specifically bound to four proteins in each honey sample. At least three of these IgE-binding proteins were N-linked glycoproteins. To identify the 60-kDa IgE-binding protein in dandelion honey, the N-terminal sequences of the fragmented protein were analyzed, revealing the protein to be major royal jelly protein 1 (MRJP 1). Three IgE-binding proteins removed of N-linked oligosaccharide showed a large reduction in IgE-binding activity as compared with the intact protein. This suggests that the carbohydrates in the IgE-binding proteins are a major epitope for patient IgE.

Key words: honey allergy; IgE-binding protein; major royal jelly protein 1

Honey is composed mainly of carbohydrates (95–97%) and has a relatively low protein content (0.25%),¹ and is an uncommon cause of allergy.^{2,3} To our knowledge, only three cases of honey allergy have been reported in Japan to date.⁴ Recently we encountered a honey-allergic patient who was found to have a delayed hypersensitivity reaction to honey.

Several studies in the literature postulate that honey proteins are derived from plant pollen or the honeybee. Several allergens derived from bee proteins have been identified from study of related allergies, such as royal jelly (RJ) and bee venom allergies, but not honey allergy;^{5–7} the allergens are registered in the allergen database ALLERGEN NOMENCLATURE, approved by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) (<http://www.allergen.org/>). Several studies detected specific binding of honey proteins to IgE in the sera of honey-allergic patients in Europe and the USA. According to de la Torre *et al.*, IgE binding bands, mainly at 54, 46, 17, and 16 kDa, were recognized by the sera of the three honey-allergic patients.³ Bauer *et al.* reported protein

binding to IgE of 23 patients at molecular masses of 72, 60, 54, 33, and 30 kDa, with respect to sunflower honey proteins.² To our knowledge, however, no allergens have been subjected to sequence analysis in honey allergy.

In contrast to honey allergy, RJ allergens have been well investigated. RJ contains 12–15% protein, more than 80% of which are major royal jelly proteins (MRJPs).⁸ In previous reports, the MRJP family was categorized into nine members, MRJP 1–9.⁹ MRJP 1 is a weakly acidic glycoprotein (pI 4.9–6.3, 55 kDa) that forms an oligomer.^{10,11} MRJP 2, MRJP 3, MRJP 4, and MRJP 5 are estimated to be glycoproteins of 49 kDa, 60–70 kDa, 60 kDa, and 80 kDa respectively.^{12,13} Of these MRJP members, MRJP 1 and MRJP 2 are reported to be major allergens for RJ allergy.⁵

In this study, we characterized honey proteins and IgE-binding proteins using the serum of a honey-allergic patient. Also discussed here are the results for the identification of an IgE-binding protein, and the epitope of it.

Materials and Methods

Patient. The patient was a 4-year-old female with delayed hypersensitivity to honey. She had honey (*Acacia longifolia*)-specific IgE (100 UA/mL) as tested by IgE RAST (Phadia, Tokyo), prick tests and oral food challenge. Her mother gave written informed consent to participate in the study. Serum was obtained from the patient, and sera from two non-allergic subjects were used for control experiments.

Materials. Homemade honey (hH), which is a trigger for the onset of honey allergy, was produced by the patient's family. hH is composed of honeydew from several flowers. Dandelion (*Taraxacum officinale*) honey (dH) and Chinese milk vetch (*Astragalus sinicus*) honey (cH) were purchased from the Fujii Apiary (Fukuoka, Japan) and Kato Brothers Honey (Tokyo) respectively.

Extraction of total protein from the honey. The honey was mixed with an equal volume of distilled water and suspended overnight at 4°C. Samples were dialyzed (SnakeSkin Pleated Dialysis Tubing, molecular weight MW cutoff, 3,500; Thermo Fisher Scientific, Rockford, IL) against distilled water. The dialysates were lyophilized and stored at 4°C. Approximately 600 mg of honey proteins was extracted per 100 g of honey.

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Abbreviations: kDa, kilodalton; MRJP, major royal jelly protein; RJ, royal jelly; hH, homemade honey; dH, dandelion honey; cH, chinese milk vetch honey; MW, molecular weight; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; 2-DE, two-dimensional polyacrylamide gel; IEF, isoelectric focusing; PNGase F, peptidase N-glycosidase F

SDS-PAGE and 2-DE. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10 and on 15% acrylamide gels by the method of Laemmli.¹⁴ The protein band intensities on SDS-PAGE gels were quantified with Imagequant TL image analysis software (GE Healthcare UK, Buckinghamshire, England).

Two-dimensional polyacrylamide gel electrophoresis (2-DE) was conducted using the Bio-Rad PROTEAN IEF system (Hercules, CA). First-dimension separation of proteins (100 µg) was carried out in immobilized pH gradient IPG strips (7 cm, pH 4–7). The isoelectric focusing (IEF) program was 250 V for 20 min, followed by a linear ramp to 4000 V for 1 h, and 4000 V for a total 10,000 V-h with a rapid ramp. After first-dimension separation, the IEF strips were equilibrated in freshly made buffer I (6 M urea, 2% w/v SDS, 0.05 M Tris(hydroxymethyl)aminomethane (Tris)-HCl pH 8.8, and 50% w/v glycerol) and buffer II (6 M urea, 2% w/v SDS, 0.375 M Tris-HCl pH 8.8, 20% w/v glycerol, and 2.5% w/v iodoacetamide). The IPG strip gels were used for second-dimension SDS-PAGE on a 12.5% acrylamide gel, as described above. All reagents for 2-DE were purchased from Bio-Rad.

Immunoblot analysis. Proteins were separated by SDS-PAGE or 2-DE and blotted onto polyvinylidene difluoride (PVDF) membranes (Biorad) in transfer buffer (25 mM Tris-HCl pH 8.2, 192 mM glycine, and 20% v/v methanol). The membranes were blocked with blocking buffer (5% w/v skim milk, Becton, Dickinson, Franklin Lakes, NJ, 50 mM Tris-HCl pH 8.0, 0.15 M NaCl, and 0.05% w/v polyoxyethylene sorbitan monolaurate, Tween 20), and incubated with serum in blocking buffer (1:200 dilution) for 1–18 h at 25 °C. Specific IgE binding was detected using a HRP (horseradish peroxidase)-conjugated goat anti-human IgE (ϵ chain) antibody (Bethyl Laboratories, Montgomery, AL). Peroxidase reactivity was visualized by 3,3'-diaminobenzidine (Dojindo, Kumamoto, Japan) as an HRP detection substrate.¹⁵ All other chemical reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Immunoblot band intensities on the PVDF membrane were quantified using Imagequant TL image analysis software (GE Healthcare UK).

Limited proteolysis. Four mg of lyophilized total dH protein was suspended in 250 µL of 70% v/v formic acid and added to 100 µL of 0.75 M cyanogen bromide dissolved in 70% v/v formic acid. The reaction mixture was kept at 4 °C for 24 h. Then 350 µL of 20% w/v tricarboxylic acid (TCA) was added to the solution, and the protein fragments were precipitated by centrifugation at 20,000 g for 5 min. The protein fragments were washed twice with 70% v/v ethanol and then dried.

N-Terminal sequence analysis. Honey proteins separated by SDS-PAGE were transferred onto a PVDF membrane (Bio-Rad), and then stained with Coomassie Brilliant Blue (CBB) R-250 (Wako). N-Terminal sequence analysis was carried out with a protein sequencer (Model PPSQ-31A; Shimadzu Scientific, Kyoto, Japan) using standard programs.

Peptide N-glycosidase F (PNGase F) digestion. N-Linked deglycosylation was conducted with PNGase F (Takara Bio, Japan). The protein, dissolved in water, was added to an equal volume of denaturing buffer (1 M Tris-HCl pH 8.6, 1% w/v SDS, and 1.5% v/v 2-mercaptoethanol) and boiled for 10 min at 100 °C. The denatured protein was added to 4 volumes of an enzyme mixture (1.25% w/v Nonidet P-40 and 0.05 mU/µL PNGase F) and incubated overnight at 37 °C.

Results

Characterization of total protein from honey

Total proteins from three honey samples containing hH, dH, and cH were applied to SDS-PAGE for separation of proteins in two ranges: from 10 to 50 kDa (Fig. 1A, lanes 1–4) and from 50 to 140 kDa (Fig. 1A, lanes 5–8). Of the dH and cH samples, dH was

chosen for detailed investigation because the overall band patterns were very similar (Fig. 1A). A major protein of 54 kDa (hH) or 60 kDa (dH and cH) was the main constituent of the total protein for each honey sample (Fig. 1A). The isoelectric point (PI) of the major proteins was approximately 5.5 by 2-DE analysis (Fig. 1B and C). Fifty- (dH and cH) and 56- (hH) kDa proteins, likely the degradation products of the major proteins, were found with slightly lower MW than the major proteins (Fig. 1B and C). Seventy kDa proteins were also found in all three samples by SDS-PAGE and 2-DE (Fig. 1A–C). In the upper range, two high MW (hw) proteins, of 138 and 110 kDa (hH) and of 138 and 116 kDa (dH and cH), were detected in slight amounts by SDS-PAGE (Fig. 1A, lanes 6–8). The hw proteins could not be resolved in 2-DE because of the pore size limit of the first-dimension gel. In the lower range, more than 20 proteins were found by 2-DE in the hH and dH total protein samples (Fig. 1B and C).

Deglycosylation of honey proteins

The major proteins and several other proteins showed smear patterns on SDS-PAGE and 2-DE (Fig. 1), indicating that they might be glycosylated. To clarify the glycosylation of honey proteins, hH and dH proteins were digested with PNGase F for cleavage of N-linked oligosaccharides. Four proteins in each honey of 54, 70, 110, and 138 kDa (hH) and of 60, 70, 116, and 138 kDa (dH) downshifted to 46, 62, 88, and 127 kDa and 52, 62, 100, and 127 kDa respectively (Fig. 2). These results suggest that the four proteins in each honey were N-linked glycoproteins.

Immunoblot experiments

In order to detect the IgE-binding proteins in the total proteins of the honey samples, immunoblot analyses were performed with serum from the honey-allergic patient. The serum displayed specific IgE binding to the major proteins of 54 kDa (hH) and 60 kDa (dH and cH) and two hw proteins of 138 and 110 kDa (hH) and of 138 and 116 kDa (dH and cH) (Fig. 3A). Furthermore, 2-DE and subsequent immunoblot analyses revealed that the 50- (hH) and 56-kDa (dH) proteins also specifically bound to patient serum IgE (Fig. 3B and C). Thus, four proteins from each honey were found to be IgE-binding proteins for the honey-allergic patient.

On the other hand, in at least three IgE-binding proteins, 54, 110, and 138 kDa (hH) and 60, 116, and 138 kDa (dH), the removed of N-linked oligosaccharide strongly reduced IgE-binding activity as compared to intact proteins (Fig. 3D, lanes 1–4).

Identification of a 60-kDa IgE-binding protein in dH

To identify three IgE-binding proteins (60, 116, and 138 kDa) in dH, these N-terminal sequences were directly analyzed by protein sequencing, but unfortunately the proteins could not be sequenced. We speculated that modifications of the N-terminal amino acid, such as acetylation and formylation, might inhibit Edman degradation. We focused on identifying the 60-kDa IgE-binding protein, and the lyophilized protein was directly fragmented by limited proteolysis with cyanogen bromide to deduce the unmodified fragments of the N-terminal residue. Three fragments, of 31, 20, and

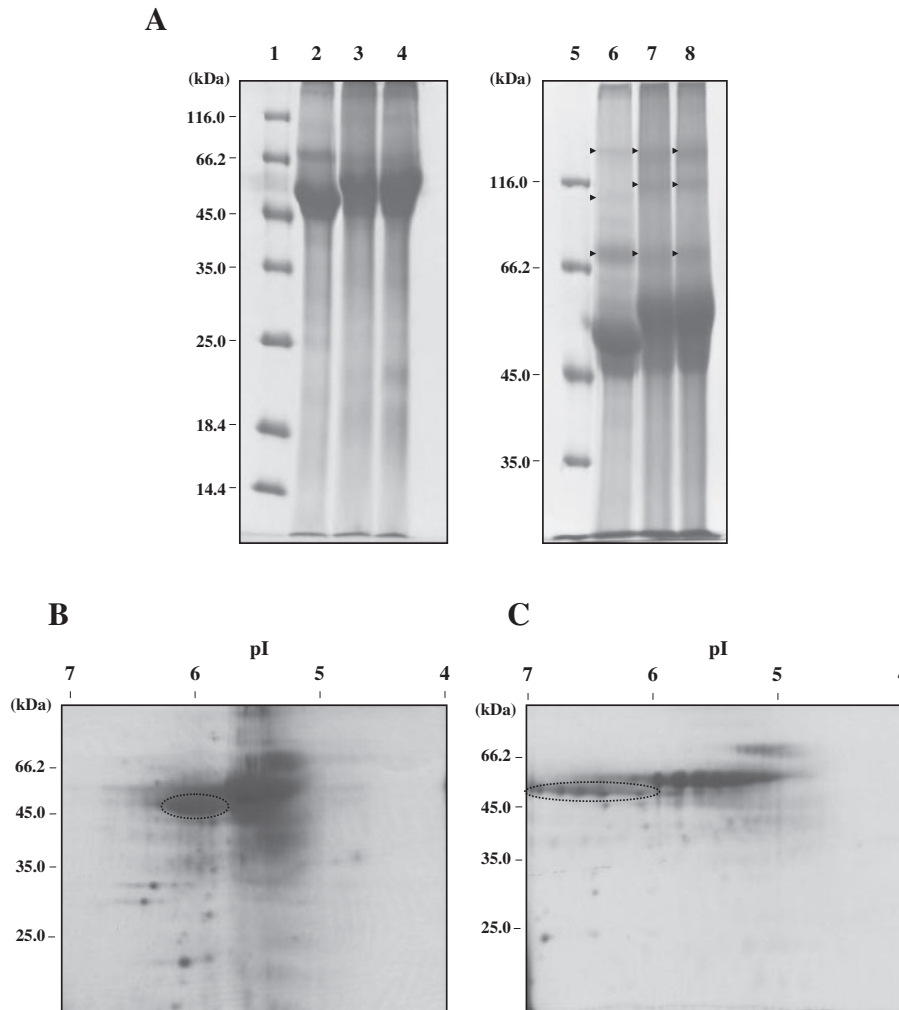


Fig. 1. Profiles of Total Honey Proteins.

(A), Honey proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (CBB R-250). Fifteen percent (lanes 1–4) and 10% (lanes 5–8) SDS-polyacrylamide gels show the separation of three total honey proteins: lanes 2 and 6, hH protein; lanes 3 and 7, dH protein; lanes 4 and 8, cH protein; lanes 1 and 5, MW marker (Fermentas, ING-2 Gel Standard). The positions of proteins with weak band intensity are shown by arrowheads. (B) and (C), The honey proteins from hH (B) and dH (C) were separated by 2-DE and stained with CBB R-250. The positions of the 50- and 56-kDa proteins are shown by broken ovals.

16 kDa, were derived (Fig. 4, lane 2), and subsequently sequenced. The N-terminal amino acid sequences of the 31-, 20-, and 16-kDa fragments were as follows: LRYNGVPSXLNVISKLVGDGGPLXQXYXD, ALSP-MTNNLYYSPXASTLLYYVNXXXF, and ALXPMX-NXLYYSPVASTNLYYVN respectively (X represents amino acid residues that were not clearly identified). The FASTA program revealed that all three fragments showed high homology to MRJP 1 from *Apis mellifera* (Gene accession, NP_001011579) (E values, 1.4×10^{-9} , 3×10^{-9} , 4.5×10^{-8} respectively).¹⁶⁾ For 13 methionine residues (positions 1, 6, 80, 149, 202, 240, 256, 261, 353, 357, 385, 358, and 403) in MRJP 1 composed of 421 amino acids, the N-terminal positions of the 31-, 20-, and 16-kDa fragments were Leu 81, Ala 257, and Ala 257 respectively. This indicates that the 60-kDa IgE-binding protein in dH is MRJP 1.

Three fragments, of 31, 20, and 16 kDa, were treated with PNGase F. Only the 31-kDa fragment was down-shifted to 24 kDa (Fig. 4, lane 3), and only the 31-kDa fragment maintained IgE-binding activity (Fig. 3D, lane 5).

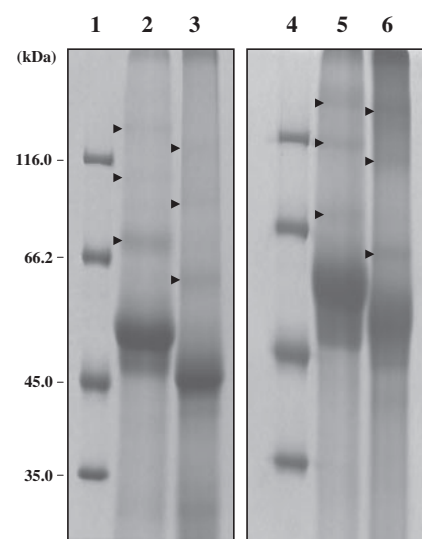


Fig. 2. N-Deglycosylation of Honey Proteins.

hH (lanes 2 and 3) and dH (lanes 5 and 6) proteins deglycosylated by PNGase F were separated by SDS-PAGE using 10% SDS-polyacrylamide gels and stained with CBB R-250: Lanes 2 and 5, non-treated PNGase F; lanes 3 and 6, treated PNGase F; lanes 1 and 4, MW marker (Fermentas, ING-2 Gel Standard). The positions of proteins with weak band intensity are shown by arrowheads.

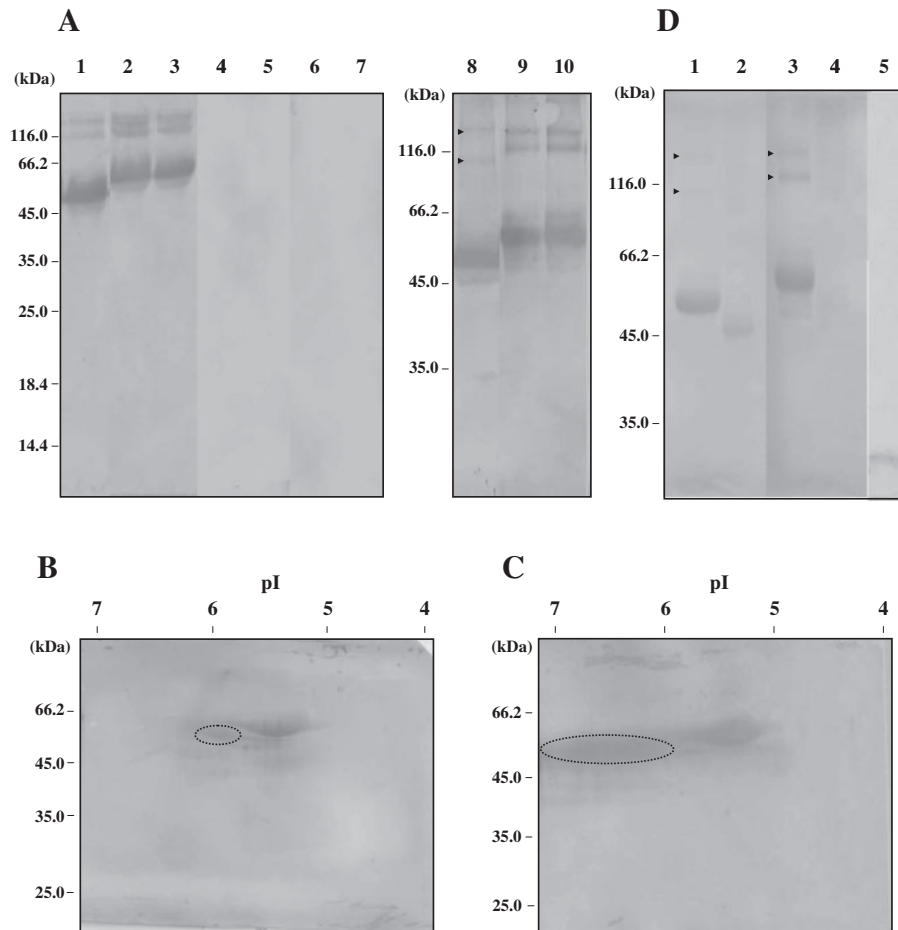


Fig. 3. Immunoblot Analysis of Total Protein from Honey.

(A), Immunoblot analysis on 15% (lanes 1–7) and 10% (lanes 8–10) SDS-polyacrylamide gels show the separation of three honey proteins: lanes 1, 4, 6, and 8, hH protein; lanes 2, 5, 7, and 9, dH protein; lanes 3 and 10, cH protein. Lanes 1–3 and lanes 8–10 were immunoblotted with serum from the honey-allergic patient, whereas lanes 4–5 and 6–7 were immunoblotted with sera from two persons not allergic to honey. (B) and (C), The honey proteins from hH (B) and dH (C) were separated by 2-DE and subsequently immunoblotted with serum of the honey-allergic patient. The positions of the 50- and 56-kDa IgE-binding proteins are shown by broken ovals. (D), Immunoblot analysis of *N*-deglycosylated honey protein and limited proteolysis fragment on 10% SDS-polyacrylamide gels: lanes 1 and 2, hH protein; lanes 3 and 4, dH protein; lane 5, limited proteolytic fragments of 60-kDa IgE-binding protein in dH. Lanes 2 and 4 were immunoblotted for honey proteins digested with PNGase F for cleavage of *N*-linked oligosaccharide. The positions of the IgE binding proteins with weak band intensity are shown by arrowheads.

Discussion

N-Terminal sequence analyses revealed that all three fragments (31, 20, and 16 kDa), products of limited proteolysis, showed high homology to MRJP 1. Previously, Won *et al.* identified the major protein (59 kDa) in honey as MRJP 1.¹⁷⁾ Their report supports our result that the 60-kDa IgE-binding protein (the major protein) in dH is MRJP 1. Furthermore, the 20- and 16-kDa fragments unexpectedly showed similar *N*-terminal sequences. We speculate that the 20-kDa fragment was derived from the 60-kDa IgE-binding protein whereas the 16-kDa fragment was derived from the 56-kDa IgE-binding protein. Fresh honey tends to contain little 56-kDa IgE-binding protein (data not shown). Thus, the 56-kDa IgE-binding protein may be a C-terminal degradation product of the 60-kDa IgE-binding protein.

Bauer *et al.* detected 54- and 60-kDa proteins in several honey samples as IgE-binding proteins in honey allergy.²⁾ The MW of these proteins matched the 54-(hH) and 60-kDa (dH and cH) IgE-binding proteins in our results (Fig. 3). Won *et al.* also identified two major proteins of different MW (56 and 59 kDa) from two

honey samples, both as MRJP 1.¹⁷⁾ Furthermore, Lee *et al.* found that MW differences in the proteins depended upon the species of honey bee; the major protein of 56 kDa is produced by *A. cerana* (the Asiatic honey bee), whereas that of 59 kDa is from *A. mellifera* (the Western honey bee).¹⁸⁾ Accordingly, we suggest that the 54-kDa IgE-binding protein in hH is also MRJP 1, derived from the Asiatic honey bee.

Most proteins from plants and bees are *N*-glycosylated independently of their localization.^{19,20)} In this study, at least three IgE-binding proteins were *N*-glycosylated (Fig. 2). Furthermore, the result of *N*-deglycosylation treatment implies that the *N*-glycosylation site(s) of the 60-kDa IgE-binding protein are in the limited proteolysis fragment of 31 kDa (Fig. 4). According to the MRJP 1 DNA sequence, consensus sequences for *N*-glycosylation (Asn-X-Ser/Thr, X excludes Pro or Asp) were found at Asn 23, 144 and 177, and Asn 144 and 177 locate in 31-kDa fragment. These results suggest that either asparagine residue or both are a MRJP 1 *N*-glycosylation sites in dH.

We estimate that the 54- and 60-kDa IgE-binding proteins are MRJP 1. The MRJP 1 protein tends to form

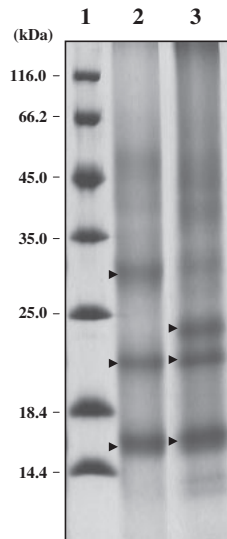


Fig. 4. Limited Proteolysis of dH Protein and N-Deglycosylation of a Fragment of It.

dH protein treated with cyanogen bromide (lane 2) and limited proteolysis fragments treated with PNGase F (lane 3) were separated by SDS-PAGE using 15% SDS-polyacrylamide gels, and were stained with CBB R-250. Lane 1 shows MW marker (Fermentas, ING-2 Gel Standard). The positions of the fragments derived from MRJP 1 are shown by arrowheads.

homo- and hetero-oligomers,^{10,11} and thus the two hw IgE-binding proteins (138 and 116 kDa) in dH might also be oligomerized MRJP 1, but we found that IgE binding affinity varied between hw and MRJP 1. The proportion of MRJP 1 to 138 kDa (116 kDa) IgE-binding protein was 18.1 to 1 (16.4 to 1) by SDS-PAGE analysis (Fig. 1A), whereas the IgE binding ratio was 5.5 to 1 (5.1 to 1) by immunoblot analysis (Fig. 3A). The difference in IgE binding affinity suggests that hw IgE-binding proteins are not oligomerized MRJP 1.

Deglycosylation of the three IgE-binding proteins drastically reduced binding ability to IgE (Fig. 3D). This suggests that carbohydrates in the IgE-binding proteins were the major epitope for the honey-allergic patient IgE. Recently, several studies of cross-reactive IgE, recognizing carbohydrate epitopes, were described for allergies to timothy grass, tomato, celery, and olive.^{21–24} Therefore, the honey-allergic patient might recognize several IgE-binding proteins at a time due to the cross-reactivity for carbohydrate epitopes. On the other hand, the deglycosylated 54-kDa IgE-binding protein in hH maintains binding ability to IgE (Fig. 3, lane 2), and a limited proteolysis fragment of 31 kDa from the 60-kDa IgE-binding protein in dH reduced IgE-binding ability (Fig. 3D, lane 5). This suggests that the protein region was also a fraction of epitope of the MRJP 1 for the honey-allergic patient IgE.

In this study, we detected four IgE-binding proteins in each honey and found for the first time that the 60-kDa IgE-binding protein is MRJP 1. However the IgE inhibition experiments such as ELISA-inhibition assay

are necessary to evaluate the IgE binding specificity of those proteins. Our efforts are currently focused on purification of the IgE-binding proteins to perform ELISA-inhibition assay and sequence analyses of hw IgE-binding proteins.

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