

Original article

Molecular characterization of a 10-kDa buckwheat molecule reactive to allergic patients' IgE

Background: Using the sera from buckwheat (BW)-allergic patients, several putative causative molecules were reported. However, few molecules were determined on the molecular structure. We demonstrated in 2000 that the major allergen with 24 kDa (BW24KD) is a legumin-like storage protein.

Objective: The aim of this study was to isolate and characterize further a major allergen with 10 kDa by molecular cloning.

Methods and results: Buckwheat allergens were identified by immunoblotting analysis using sera from 14 allergic and two nonallergic individuals. We identified a protein with 10 kDa (BW10KD) that reacted with immunoglobulin E (IgE) more strongly than with IgG and IgA in 57% of the allergic patients but not with IgE in nonallergic individuals. Analyses were performed by N-terminal amino acid sequencing and molecular cloning. Physiological significance was assessed by an immunoblotting experiment showing that the reactivity of an allergic patient's serum IgE to BW10KD was competitively inhibited by natural BW extracts.

Conclusion: Molecular cloning experiments indicated that BW10KD as a BW allergen was a member of the 2S-albumin multigene family.

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Key words: allergen; amino acid sequence; buckwheat; immunoblot analysis; 2S-albumin.

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Accepted for publication 3 September 2003

Buckwheat (BW) allergens initiate severe symptoms including wheezing, urticaria, vomiting, and anaphylactic shock in patients with hypersensitivity to BW (1, 2). Several BW molecules recognized by serum immunoglobulin E (IgE) derived from allergic patients have been reported (3–15). Yanagihara reported proteins with molecular mass of 100, 50, and 17 kDa as BW allergens (11). Urisu et al. (8) characterized BW allergens by immunoblotting analysis using sera from 15 patients and described an allergen with a molecular mass of 24 kDa (BW24KD) as a major allergen of BW. Recently, we demonstrated that BW24KD was the β subunit of a legumin-like storage molecule (16). Regarding other putative allergic molecules, there are considerable variations in molecular weights. Yano et al. (12) also described proteins of 8–9 kDa, which had strong IgE-binding activity. Recent studies have verified that other BW allergens (19, 18, 16, 14, and 9 kDa) may be of importance (14, 15).

In this study, we focused on the characterization of a 10 kDa BW protein (BW10KD) that reacted with serum IgE more strongly than IgG or IgA in 57% of 14 allergic patients. N-terminal amino acid sequencing, cDNA

cloning, and serological analyses showed that BW10KD belongs to 2S-albumin and is a BW allergen.

Materials and methods

Patients

Fourteen BW-allergic patients, eight males (aged 4–68 years), six females (aged 3–34 years), and two nonallergic individuals (males: aged 22–25 years) were selected for this study according to case histories and radioallergosorbent tests (RAST). None of the patients had received hyposensitization therapy before collection of sera. Total serum IgE levels of patients were between 370 and 4393 IU/ml. Specific IgE level for BW was measured by an immunofluorometric method (<0.35 Ua/ml in case 7, 0.68 Ua/ml in case 8, <0.35 Ua/ml in case 9, <0.35 Ua/ml in case 10, 1.61 Ua/ml in case 11, 82.00 Ua/ml in case 12, 20.40 Ua/ml in case 13, 44.70 Ua/ml in case 14, by FEIA RAST, CAP System, Pharmacia, Uppsala, Sweden). Cases 1–6 were analyzed in BW24KD study as described in Ref. (16). All patients were positive on RAST scores of House dust 1, House dust 2, Der. f., Der. p., and Japanese cedar pollen (JCP). Laboratory data of nonallergic individuals showed that total serum IgE levels were between 40 and 123 IU/ml. Radioallergosorbent test scores of House dust 1, House dust 2, Der. f., and Der. p. were all negative among these individuals.

Buckwheat extracts

The extracts were prepared under a mild condition as described (17–19). Briefly, BW (15 g) was extracted in 100 ml of 0.125 M NaHCO₃ at 4°C for 1 h. After extraction, the supernatant was separated by centrifugation at 15 000 *g* for 30 min. The precipitates were re-extracted in 100 ml of 0.125 M NaHCO₃ at 4°C for 1 h. The combined supernatants were dialyzed against distilled water and used for the sample. Japanese cedar pollen extracts were prepared as described (18).

Antibodies and other reagents

Alkaline phosphatase (AP)-conjugated anti-human IgE antibody was obtained from Biosource (Camarillo, CA). Alkaline phosphatase-conjugated anti-human IgG and anti-human IgA antibodies were obtained from TAGO (Burlingame, CA). Horseradish peroxidase (HRP)-conjugate goat anti-human IgE was obtained from Biosource.

Immunoblotting analysis

Immunoblotting analysis was performed as described (16, 20). Briefly, the extracts were separated on 10 or 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto a polyvinylidene difluoride microporous membrane (PVDF, Immobilon, Millipore, MA). The membrane was incubated with patients' sera at a dilution of 1:3, followed by the addition of AP-conjugated anti-human IgG, IgE, or IgA (1:1000). Protein bands were visualized by reaction with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT; Promega, WI).

Chromatography

The BW extracts were loaded on an anion-exchange chromatographer (DEAE-TOYOPEARL 650M, Tosoh, Tokyo) that had been equilibrated with Na₂HPO₄ (20 mM, pH 7). After washing the column with the same buffer, the proteins were eluted with a linear gradient of NaCl ranging from 0 to 1 M NaCl in Na₂HPO₄ (20 mM, pH 7). A constant flow rate of 1 ml/min was maintained throughout this program. The peaks were detected at 300 nm.

Amino acid sequencing

SDS-PAGE was performed as described and transferred onto a PVDF membrane using the Sartorius semi-dry system. A band of 10 kDa was cut out, and the membrane was washed in distilled water. The N-terminal amino acid sequence of the sample was determined with a Model 473A protein sequencer (PE Applied Biosystems, Chiba).

cDNA cloning

Based on the N-terminal amino acid sequence of the 10-kDa buckwheat-allergic protein, a degenerate primer (5'-MGNAAR CARATGMGNATGATGGA-3') was synthesized (M=A/C, N=A/T/G/C, R=A/G; Invitrogen, Tokyo). 3' RACE-PCR (rapid amplification of c-DNA ends-polymerase chain reaction) amplification was carried out in a final reaction volume of 20 µl containing 0.5 µl of cDNA solution, 50 pmol of the degenerate primer, 10 pmol of the NotI-oligo-dt primer for the reverse direction, 0.2 mM each dNTPs, 1× PCR buffer, 1.5 mM MgCl₂, and one unit

KOD plus polymerase (TOYOBO) using a Takara PCR thermal cycler MP:TP3000. Amplification parameters were 94°C for 2 min, followed by 40 cycles with 94°C for 30 s, 60°C for 30 min, and 68°C for 90 s. A 68°C incubation for 10 min as a final extension step was included. The PCR product was cloned at the EcoRV blunt-ends of a pBlueScript vector (IH0503). Employing the 500 bp PCR fragment prepared from IH0503 by using the dT primer as a 3'-end probe, a specific clone IH0201 was identified from a cDNA library, which was constructed from poly(A)+RNA extracted from immature seeds of 14 DPA (14 days after pollination; Ref. 21). To obtain the 5'-end region of the full-length BW10KD cDNA clone (Fe2SA1), RACE-PCR was performed using the SMART PCR cDNA synthesis kit with a primer, 5'-CATTGCTCAAGTTGT GGCTCCAT-3', which corresponds to the internal site of the IH0503 partial clone.

DNA sequencing

Cy5 dye terminator kit (Pharmacia) was used. Nucleotide sequences of the insert were determined with an automatic DNA sequencer (ALF express DNA sequencer; Pharmacia).

Computer analysis

Homology search was done using the CLUSTAL-W system for protein sequence alignment and DDBJ-GenBank-EMBL for gene sequence alignment. The sequences were aligned using the computer program BoxShade (http://www.ch.embnet.org/software/BOX_form.html).

Results

Immunoblotting analysis of buckwheat extracts

The BW were electrophoresed under a reduced condition and immunoblotted by sera from 14 allergic patients or two nonallergic individuals as primary antibodies and AP-conjugated anti-human IgG, IgE, or IgA antibody as a secondary antibody. As shown in Fig. 1A, the 24-kDa protein (BW24KD, legumin-like storage molecule; Ref. 16) was visible as the most prominent band, which was recognized equally by IgG, IgA, or IgE. The blotting pattern of IgG, IgA, or IgE was almost identical although it was not exactly the same in every individual. The 10-kDa protein (BW10KD) was also identified as a characteristic protein that was strongly reactive to IgE rather than to IgG or IgA in 57% of allergic individuals (Fig. 1A). The densitometric analysis was represented in cases 1–5 (Fig. 1B). In the case of nonallergic subjects, BW10KD was weakly reactive to IgG and IgA but not to IgE. IgE-reactive molecules with less than 10 kDa were not detected in this analysis.

Purification of BW10KD

To purify the BW10KD, the BW was fractionated by anion-exchange chromatography on a column of DEAE-TOYOPEARL 650M (Fig. 2A). SDS-PAGE analysis of these fractions under reducing conditions revealed that the 10-kDa molecule was contained in the vicinity of fraction

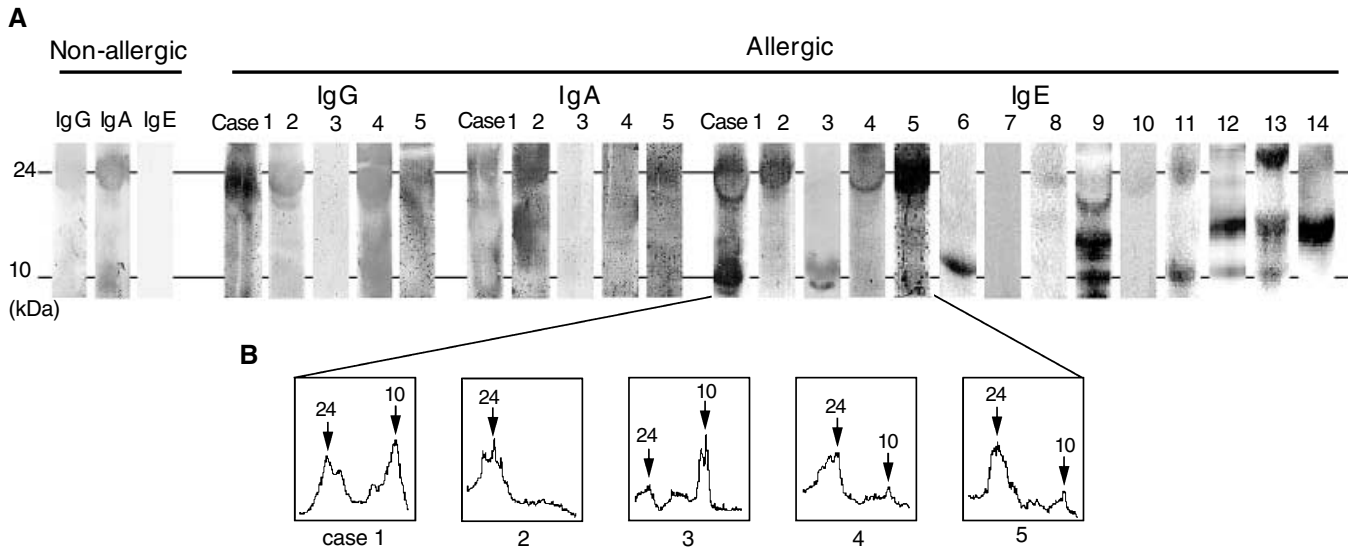


Figure 1. Immunoblotting analysis using sera from allergic and nonallergic individuals. (A) The BW were electrophoresed in 15% SDS-PAGE under reducing conditions. The binding of serum was detected by AP-conjugated anti-human IgG, IgE, or IgA in cases 1–5, whereas cases 6–14 were measured by HRP-conjugated anti-human IgE. (B) The intensity of the IgE bands was determined by a densitometer (Image Master, Pharmacia Biotech AB, Uppsala, Sweden).

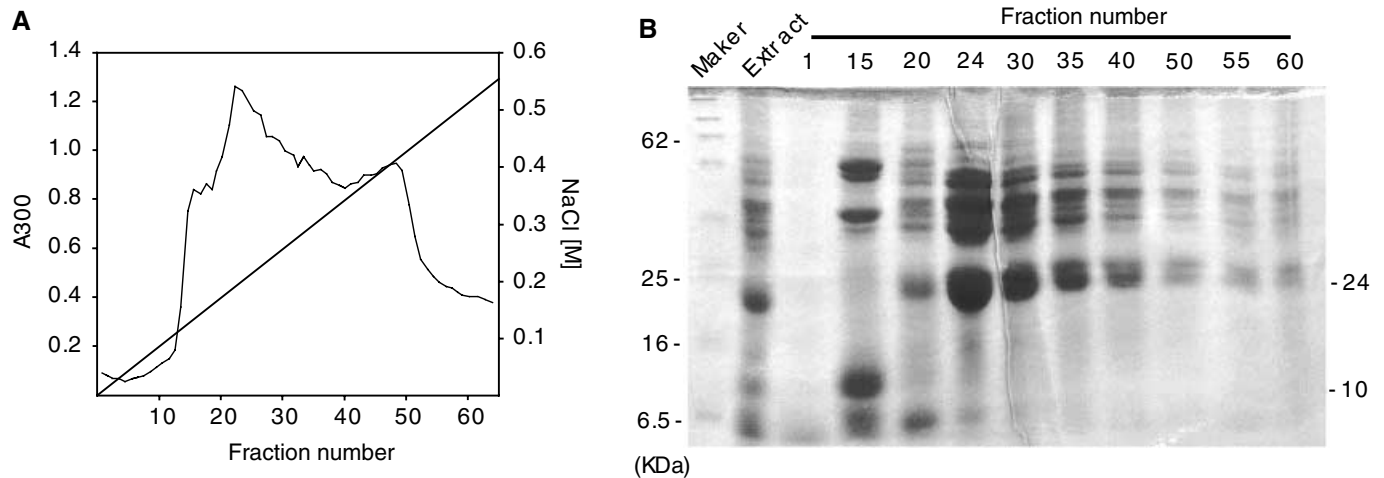


Figure 2. Purification of BW10KD. (A) Anion-exchange chromatography of BW on a column of DEAE-TOYOPEARL 650M. (B) SDS-PAGE on fractions separated by anion-exchange chromatography. The fractions were electrophoresed in 15% SDS-PAGE under a reducing condition. The gel was stained with Coomassie Brilliant Blue (CBB).

No. 15 (Fig. 2B). As the BW10KD was strongly detected in fraction nos. 11–20, these fractions were pooled for further analysis.

Identification of BW10KD

The samples were separated by 15% SDS-PAGE under reducing conditions and transferred onto the PVDF membrane. Since the band of BW10KD was resolved as a broad band, the BW10KD-blotted membrane strip was cut into two pieces of upper and lower portions. Each sample was independently determined on the amino acid sequence. Both samples showed an identical N-terminal

amino acid sequence: DSQMRSKXRKQMRMMEP QLE. Based on this result, we searched a specific cDNA clone and identified a cDNA clone, Fe2SA1 (accession no. AB055892). The Fe2SA1 contained an inserted fragment of 628 bp, of which the open reading frame encoded 133 amino acids.

The predicted amino acid sequence of Fe2SA1 is homologous to 2S-albumin of the Brazil nut at 36.2% (accession no. S14947), cotton at 28.5% (accession no. T09878), pumpkin at 32.5% (accession no. T10257), sesame at 26.7% (accession no. Q9AUD1), peanut (Arah2) at 23.7% (accession no. Q941R0), sunflower at 29.7% (accession no. P23110), rape at 23.1% (accession

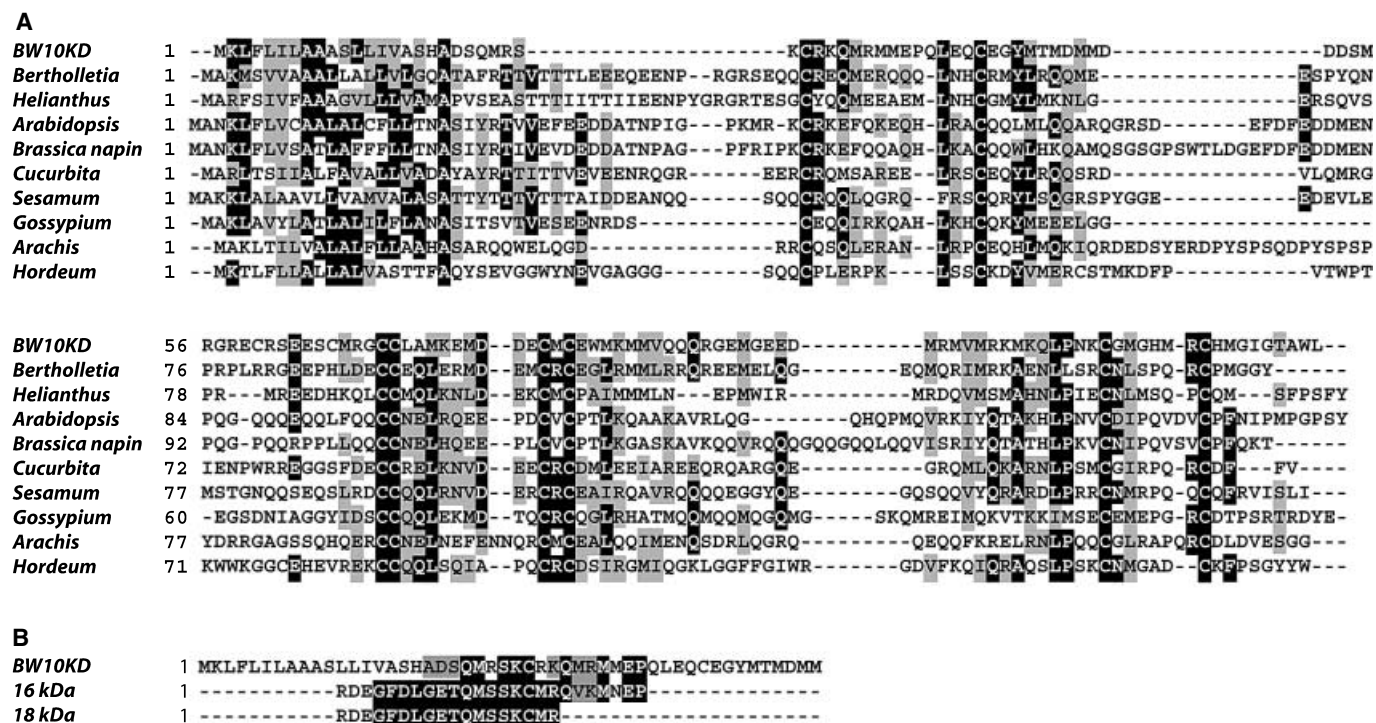


Figure 3. Sequence analysis of BW10KD. Fe2SA1 (accession no. AB055892) represents the full-length cDNA sequence of BW10KD, which was accepted by DDBJ–GenBank–EMBL. The sequences were aligned using BoxShade; identical or similar residues are shown in black or gray shaded boxes, respectively. (A) Alignment of amino acid sequence deduced from the cDNA sequences of Fe2SA1 and 2S-albumin genes. The following sequences were analyzed: BW10KD (*Fagopyrum esculentum*, accession no. AB055892), *Bertholletia excelsa* (2S-albumin, accession no. S14947), *Cucurbita* sp. (prepro2S-albumin, accession no. T10257), *Sesamum indicum* (2S-albumin precursor, accession no. Q9AUD1), *Gossypium hirsutum* (2S-albumin storage protein, accession no. T09878), *Arabidopsis thaliana* (2S-albumin, accession no. P15457), *Brassica napus* (napin; seed storage protein, accession no. P17333), *Helianthus annuus* (2S-albumin accession no. P23110), *Arachis hypogaea* (allergen AraH6, accession no. Q941R0), and *Hordeum vulgare* (hordoin-doline-ab, accession no. Q9M4E4). (B) N-terminal amino acid sequence of 10 kDa (this paper), 16 kDa (14), and 18 kDa (15) BW proteins.

no. P17333), *Arabidopsis thaliana* at 25.4% (accession no. P15457), and barley at 22.9% (accession no. Q9M4E4) (Fig. 3A).

BW16KD reported by Park et al. (14) and BW18KD reported by Yoshimasu et al. (15) show an almost identical N-terminal amino acid sequence, to which Fe2SA1 shows significant homology.

Thus, Fe2SA1 shows strong homology to 2S-albumin of various plants. These molecules are characteristic for its high content of methionine (Fe2SA1: 18.5% methionine). The position of cysteine is conserved among these 2S-albumins.

Competition inhibition assay

To confirm the reactivity of a patient's serum IgE to natural BW10KD of BW, the patient serum (case 6) was preincubated with either the BW or unrelated cedar pollen extracts for 1 h at 4°C (Fig. 4). Immunoblotting was then performed in an identical manner. The natural BW completely inhibited the reactivity of the patient's serum IgE to SDS-PAGE-separated BW10KD, while the JCP extracts did

not. Thus, an allergic patient's serum IgE reacted to the binding epitope of BW10KD, irrespective of whether the form was nylon-membrane-fixed or water-soluble, showing that BW10KD is a BW allergen.

Discussion

We have previously showed that BW24KD is a major allergic molecule of BW and a legumin-like storage protein. In this study, we identified a characteristic molecule with a smaller molecular weight than BW24KD, namely, a 10-kDa molecule that is strongly recognized with a patient's IgE but hardly with IgG or IgA.

We studied the 10-kDa molecule (BW10KD) by using antisera from 14 BW-allergic patients. Eight out of 14 patients showed IgE-reactivity to BW10KD. These patients showed almost no reactivity to BW10KD-blotting patterns in IgG or IgA (Fig. 1A). Employing the antisera of case 6, IgE-reactivity to BW10KD was completely absorbed with the whole extracts of BW but not with those of cedar pollen (Fig. 4).

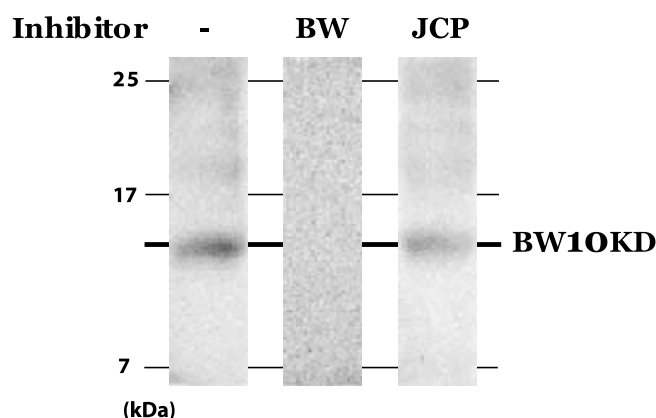


Figure 4. Competitive inhibition experiment using SDS-PAGE-separated BW10KD and buckwheat (BW) extracts on the reactivity of BW-allergic patient's sera. The purified BW10KD was electrophoresed in 15% SDS-PAGE under a reducing condition and transferred onto a PVDF membrane. The serum (100 μ l) from an allergic patient (case 6) was preincubated with 200 μ l BW or JCP extracts for 1 h at 4°C, and immunoblotting analysis was then performed. The filter was stained with HRP-conjugated anti-human IgE. LAS-1000 (Fuji Film, Tokyo) were used as an image analyzer.

Based on the N-terminal amino acid sequence of BW10KD, we isolated a cDNA clone, IH0503 (21). This experiment demonstrated that BW10KD was a member of the 2S-albumin family. A homology search using the computer program Clustal-W found that Fe2SA1 shows 24.1–35.6% identity to storage proteins classified in the 2S-albumin from *Bertholletia excelsa*, *Gossypium hirsutum*, *Cucurbita* sp., *Brassica napus*, *Arabidopsis thaliana*, *Sesamum indicum*, *Lupinus angustifolius*, *Arachis hypogaea*, and *Hordeum vulgare* (Fig. 3A). These plants range from the dicot to the monocot species in angiosperm. However,

Urisu et al. (8) pointed out that the majority of subjects with allergies to BW did not develop immediate hypersensitivity after ingestion of these plants. Therefore, the allergic epitopes are present at a characteristic location in the sequence of BW10KD but not in the sequence of the homologue of the other plants. The 16- or 18-kDa molecule of BW was also reported as an allergen by Park et al. (14) or Yoshimasu et al. (15). The N-terminal amino acid sequences of these molecules are partially identical to that of BW10KD (Fig. 3B), indicating that these molecules also belong to the 2S-albumin family. These results suggested that the allergic epitopes of BW10KD might exist somewhere in a common sequence among 10, 16, and 18 kDa 2S-albumin family members but not in the regions homologous to other plants.

In order to examine whether the patient's IgE reacts to the BW10KD, we prepared the recombinant BW10KD with an *Escherichia coli* gene expression system (pMal-cR1; New England BioLabs, Beverly, MA).

In the immunoblotting analysis using patients' antisera, the recombinant BW10KD strongly showed positive development in two cases (cases 11 and 12) but not in others (data not shown). This experiment suggested that the IgE-reactive epitopes were probably variable among patients and that some patients might respond to the epitopes that include the sugar moieties.

Thus, this study demonstrated that the BW 2S-albumins including BW10KD played a role to induce the IgE response in the majority of BW-allergic patients.

In the cases of walnuts (22) and peanuts (23), the 2S-albumin is known as a major allergen. Thus, it is important to examine the distribution and structure of allergy-responsible epitopes in the 2S-albumin family of plants.

These results may be useful information for the prevention of or therapeutics for BW allergy.

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