

Original article

Identification and characterization of the major allergens of buckwheat

Background: Buckwheat (BW) has been recognized as a common food allergen in Korea, Japan, and other countries. Until now, serologic findings of BW food-allergic patients and its major allergenic components have not been clarified. In this study, we analyzed the serologic findings of BW food allergy and characterized its major allergenic components.

Methods: Nineteen BW-allergic subjects with symptoms after BW ingestion and 15 asymptomatic control subjects with positive skin prick test to BW were recruited. BW-specific IgE was measured with the Pharmacia CAP kit. Allergenic components of BW were analyzed by IgE immunoblotting, periodate oxidation, two-dimensional PAGE, and sequencing of N-terminal amino acids.

Results: From the BW-allergic patients and asymptomatic controls, the sensitivity (100%), specificity (53%), and negative (100%) and positive predictive values (73%) of Pharmacia CAP specific IgE for diagnosis were estimated. The prevalence of IgE binding to 24-kDa (pI 8.3), 16-kDa (pI 5.6), and 9-kDa (pI 5.0/6.0) allergens was higher than 50% in BW-allergic and asymptomatic subjects. However, the specific IgE to split 19-kDa (pI 6.5/7.0) allergens were more specifically found in BW-allergic patients than in asymptomatic subjects (78% vs 7%). N-terminal amino-acid sequences of 19-kDa and 16-kDa allergens showed moderate and weak homology to the 19-kDa globulin protein of rice and α -amylase/trypsin inhibitor of millet, respectively. The N-terminus of the 9-kDa isoallergens were not different from each other and were identified as the reported trypsin inhibitors of BW. Attenuation of the IgE binding to the 9-kDa allergen was found with periodate oxidation.

Conclusions: The allergens of 24, 19, 16, and 9 kDa are strong candidates to be major allergens, and the 19-kDa allergen was relatively specific for BW-allergic patients. Moreover, measurement of BW-specific IgE and the features of immunoblotting should be very useful tools in the diagnosis of BW allergy.

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Buckwheat (BW) (*Fagopyrum esculentum*) belongs to the Polygonaceae family of plants, and its seeds can be used as food material. BW can be cultivated under more unfavorable conditions than rice, as at high altitudes, and it is sometimes used as a substitute for common cereals, although it is not botanically a cereal. BW noodles, jelly, and dumplings are favorite foodstuffs in Korea, Japan, and other east Asian countries. BW is also used in batter for cake, pancake, or crepes, and there is growing interest in buckwheat products as a health food and as a substitute for wheat flour in gluten-allergic persons in Western countries (1). Many cases of BW-allergic subjects have been reported in Korea, Japan, and the USA and other Western countries (2–7). In addition to the food allergy, BW causes occupational asthma in noodle makers, (8), bakers (9), packing workers (10), and workers in pancake restaurants (11). Its

hulls are used to stuff pillows in Asian countries, and it may be an aggravating factor of bronchial asthma in these areas.

The clinical study of BW allergy suggests that it is an IgE-mediated hypersensitivity reaction (2–12). However, its major allergenic components are still controversial (13, 14), although the 24-kDa allergen has been proposed as Fag e 1 (15, 16).

In this study, we analyzed the clinical findings of BW food-allergic patients and also identified its major allergens with the sera of BW-allergic patients and asymptomatic control subjects.

Material and methods

Subjects

Nineteen allergic subjects who complained of allergic

symptoms after ingestion of BW-containing noodle or jelly were enrolled. Among them, eight subjects were confirmed by double-blind, placebo-controlled food challenge (DBPCFC), and two patients were diagnosed by the open challenge test that followed the DBPCFC. The challenge test was performed in young male patients who wanted to confirm the diagnosis before military recruitment. Others were not challenged because of the history of severe allergic symptoms to BW. The mean age of BW-allergic patients was 23.5 ± 5.8 years, and all subjects were male. Another 15 respiratory allergic patients with positive skin responsiveness to BW but not complaining of allergic symptoms after BW ingestion were also enrolled as an asymptomatic control group. Their mean age was 28.1 ± 12.5 years, and the male:female ratio was 9:7. The skin prick test (SPT) was done with BW extract (Bencard Co, UK) with a histamine positive control (1 mg/ml). If the wheal size of buckwheat was larger than the histamine positive control, we considered it positive. The patients' sera were stored at -20°C until use. BW-specific IgE was measured by the Pharmacia Uni-CAP System (Pharmacia-Kabbi, Uppsala, Sweden) according to the manufacturer's guidelines.

DBPCFC

In DBPCFC, we used a capsule as the vehicle and lactose powder as a placebo. Initially, we administered 50 mg of BW flour and increased the dose by doubling it until the patients complained of symptoms. If the patients did not complain of symptoms after ingestion of 5 g of dried BW flour, the open challenge test with boiled BW was undertaken.

BW extract

Fifty grams of BW flour was defatted with ethylether, and it was then extracted in 100 ml of phosphate-buffered saline (137 mM NaCl, 1.8 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 27 mM KCl, pH 7.4) for 72 h at 4°C under constant stirring. The extract was centrifuged at 50 000 g for 1 h at 4°C , and the supernatant was dialyzed (the cutoff molecular weight was 1 kDa; Spectrum, Houston, TX, USA) against distilled water for 48 h. The dialyzed supernatant was lyophilized and stored at -20°C until use.

SDS-PAGE

SDS-PAGE was carried out by the methods of Laemmli (17). For allergen isolation, 5 mg/ml of BW crude antigen was dissolved in reducing sample buffer (12.5 mM Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, 1% 2-mercaptoethanol, and 0.02% bromophenol blue) and boiled at 100°C for 5 min. We also performed SDS-PAGE in a nonreducing condition in which the BW extract was dissolved in nonreducing sample buffer

(12.5 mM Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, and 0.02% bromophenol blue). Two sets of protein molecular markers from Pharmacia (the smallest marker was 14.4 kDa; Uppsala, Sweden) and Gibco BRL (the smallest marker was 10 kDa; Grand Island, NY, USA) were used for estimation of the molecular weights of identified BW allergens. For a preparative sheet, 200 μg of BW antigen was separated in 13.5% polyacrylamide gels with a 4% polyacrylamide stacking gel (Mighty Small electrophoresis unit; Hoeffer, San Francisco, CA, USA) at 50 V for 30 min and 180 V for 2 h.

IgE immunoblotting

Transfer of the proteins onto nitrocellulose membranes (pore size: 0.45 μm ; Amersham, Buckinghamshire, UK) was performed on a Transpor TE 42 Unit (Hoeffer, San Francisco, CA, USA) in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3) at 350 mA for 2 h. The membrane was sliced into 4-mm widths, if required. The sliced membranes were blocked by 5% nonfat dried milk in TBS-T (50 mM Tris with 0.1% Tween, pH 7.5) and then incubated with patient sera (1:10 diluted in TBS-T, 0.02% NaN_3) at 4°C for 18 h and washed with TBS-T. Goat antihuman IgE conjugated with alkaline phosphatase (Sigma, St Louis, MO, USA) was diluted 1:1000 in TBS-T before incubation with the blots for 90 min. After washing with TBS-T, the bound goat antibodies were detected with the BCIP/NBT system (Promega, Madison, WI, USA).

Two-dimensional PAGE

BW extract was dissolved in isoelectric focusing sample buffer (8 M urea, 0.1% ampholyte, 2% triton X-100, 1% 2-mercaptoethanol, and 0.04% bromophenol blue). An amount of 50 μg of BW extract was applied per lane of denaturing isoelectric focusing (IEF) gel, which was already focused with 150 V for 30 min. The BW extracts were then focused at 200 V for 150 min. After focusing, each lane of IEF gel was cut into 4-mm widths and incubated in equilibration buffer (5% 2-mercaptoethanol, 62.5 mM Tris-HCl, 2.3% SDS, and 10% glycerol) for 30 min at room temperature and applied onto polyacrylamide stacking gel. The SDS-PAGE and immunoblotting were then carried out as previously described with pooled sera ($n=5$).

Periodate oxidation

Periodate oxidation of carbohydrate structures was performed after blotting onto nitrocellulose membranes by incubation in 10 mmol/l sodium acetate buffer (pH 5.0) containing 50 mmol/l NaIO_4 . This was

performed in the dark at room temperature for 2 h. The reaction was stopped by addition of sorbitol to a final concentration of 0.015 mol/l.

N-terminal sequencing analysis

For N-terminal sequencing, two-dimensional PAGE was blotted to a polyvinylidene difluoride (PVDF) membrane (pore size: 0.45 µm; MSI, Westboro, MA, USA). The PVDF membrane was stained by 0.1% Coomassie brilliant blue in 50% methanol, destained in 50% methanol, and air-dried. The protein spot was excised, and microsequencing was done by the Precise Protein system (Applied Biosystems, Weiterstadt, Germany).

Results

Clinical features of BW-allergic patients and results of DBPCFC

BW-allergic patients frequently complained of urticaria (89.5%), dyspnea (84.2%), facial angioedema (52.6%), and gastrointestinal symptoms such as vomiting and abdominal pain (47.4%). In addition, two patients (nos. 6 and 12) lost consciousness after BW ingestion (Table 1). Two BW-allergic patients had complained of allergic reactions to peanuts at the time of diagnosis. We performed DBPCFC in 10 patients. Eight patients showed positive responses to BW by intake of less than 5 g of dried BW powder. Two subjects (nos. 5 and 8), who were negative in DBPCFC, elicited allergic symptoms by open challenge with boiled BW (Table 1).

Results of SPT and Pharmacia CAP specific IgE

All BW-allergic patients showed strong reactivity with a wheal larger than 8 mm in SPT to BW, and BW-specific IgE was detected with the Pharmacia CAP system in all allergic patients. In 15 asymptomatic subjects, BW-specific IgE was detected in seven subjects. When we estimated the sensitivity and positive predictive values of Pharmacia CAP BW-specific IgE on the basis of the BW-allergic patients (as true positive group) and asymptomatic subjects (as true negative group), its sensitivity, specificity, negative predictive value, and positive predictive values were 100%, 53%, 100%, and 73%, respectively.

Immunoblotting features of BW-allergic patients and asymptomatic subjects

We performed SDS-PAGE with reducing and non-reducing status of BW extract, respectively. At nonreducing status, the 24-kDa and 16-kDa allergenic components had disappeared. We identified eight allergens such as those of 9 kDa (pI 5.0/6.0), 14 kDa, 16 kDa (pI 5.6), 19 kDa (pI 6.5/7.0), 24 kDa (pI 8.3) and 30 kDa (pI 6.3), 43 kDa (pI 7.7), and 67 kDa (pI 7.0) in the BW extract with one-dimensional and two-dimensional SDS-PAGE immunoblotting (Figs. 1 and 2). The prevalence of IgE binding to the 24-kDa (68.4%), split 19-kDa (73.7%), 16-kDa (73.7%), and 9-kDa (52.6%) antigens was higher than 50% in BW-allergic sera (Fig. 1A). Other allergens, such as those of 30 kDa (26%), 43 kDa (21%), and 67 kDa (26%), were also found in BW-allergic subjects. The asymptomatic sera also had a high prevalence of IgE binding to antigens of 24 kDa (80%), 16 kDa (66.7%), and 9 kDa

Table 1. Clinical features of BW-allergic patients

Patient no.	Age (years)	Sex	Challenge	CAP IgE (kU/l)	Other food	Angioedema	Urticaria	Dyspnea	G-I	LOC
1	44	M	–	0.53	–	–	–	–	+	–
2	25	M	D	1.66	–	+	+	–	–	–
3	20	M	D	1.73	Peanut	+	+	+	–	–
4	23	M	D	6.40	–	–	+	+	–	–
5	23	M	O	5.38	–	+	+	+	+	–
6	20	M	–	6.29	–	+	+	+	–	+
7	25	M	–	53.5	–	–	+	+	+	–
8	20	M	O	10.6	–	+	+	+	+	–
9	24	M	–	7.03	–	–	+	+	+	–
10	28	M	D	9.97	–	–	+	+	+	–
11	21	M	D	3.21	–	+	+	+	+	–
12	18	M	–	47.6	Peanut	–	+	+	+	+
13	22	M	D	3.80	–	–	+	+	+	–
14	32	M	–	2.78	–	–	+	–	–	–
15	23	M	–	4.84	–	+	+	–	–	–
16	21	M	D	9.31	–	+	+	+	–	–
17	20	M	–	12.60	–	+	+	+	–	–
18	21	M	–	17.40	–	+	+	+	–	–
19	19	M	D	0.99	–	+	+	+	+	–
Total						11	18	15	10	2

G-I: gastrointestinal symptoms; LOC: loss of consciousness; D: positive result to DBPCFC; O: positive result to open challenge test.

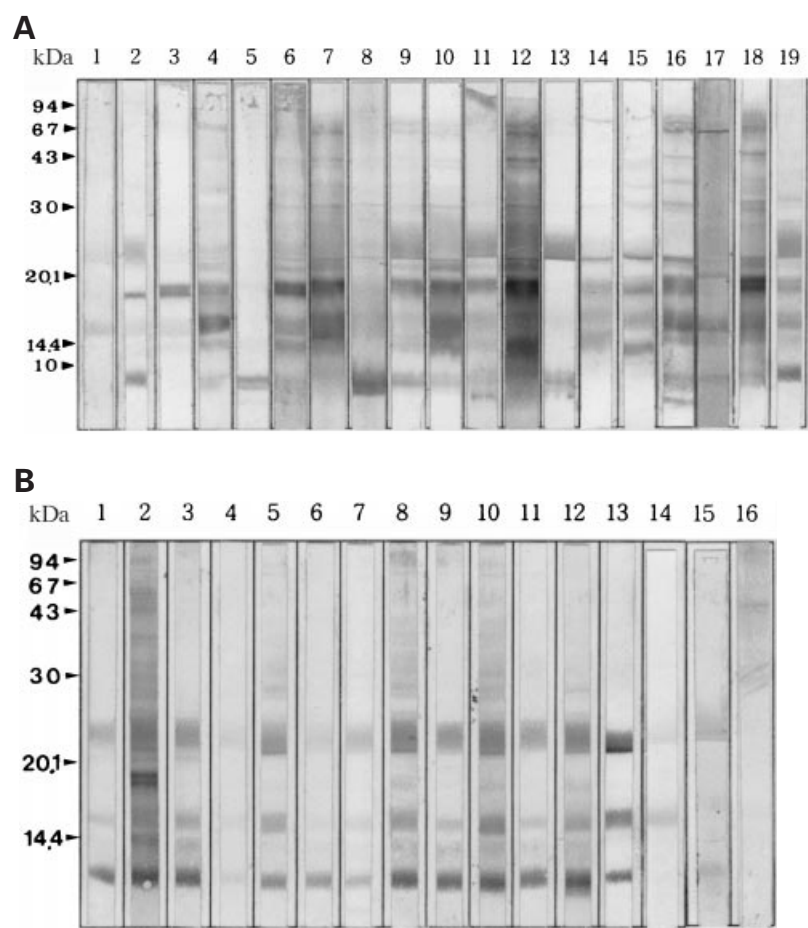


Figure 1. A) IgE immunoblotting features of 19 BW-allergic subjects; B) asymptomatic control subjects (lanes 1–15) and nonatopic control (lane 16).

(80%) (Fig. 1B). Only one asymptomatic subject had specific IgE to the 19-kDa allergen. Therefore, the split 19-kDa allergens were relatively specific in BW-allergic patients.

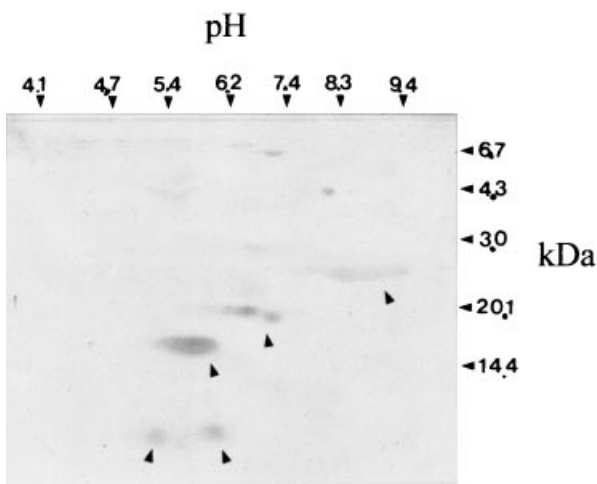


Figure 2. Two-dimensional SDS-PAGE and IgE immunoblotting features of BW allergens with atopic pooled sera ($n=5$). Arrowheads indicate identified allergens of BW.

Periodate oxidation for the carbohydrate determinant of BW

The presence of the carbohydrate IgE determinant of BW allergens was analyzed with periodate oxidative treatment. The IgE-binding activity to the 9-kDa allergen in the transferred NC membrane markedly decreased after periodate treatment, and that of the 16-kDa allergen was partially decreased. But the IgE-binding activities of the 24-kDa and split 19-kDa allergens were unchanged (Fig. 3).

N-terminal sequencing of BW major allergens

We excised the corresponding protein spots of 19, 16, and 9 kDa from the Coomassie brilliant blue-stained PVDF membrane and microsequenced its N-terminus (Table 2). The N-termini of two 9-kDa isoallergens (pI 5.0 and pI 6.0) were not different from each other up to the 17th amino acid. We could identify the N-terminus of the 19-kDa (pI 6.5) allergen up to the 14th amino acid. The N-terminus of the other 19-kDa allergen (pI 7.0) was identified up to the 4th amino acid and was not different from the previous one. The N-terminus of the 16-kDa allergen was identified up to the 25th amino acid. We performed a homology study with the

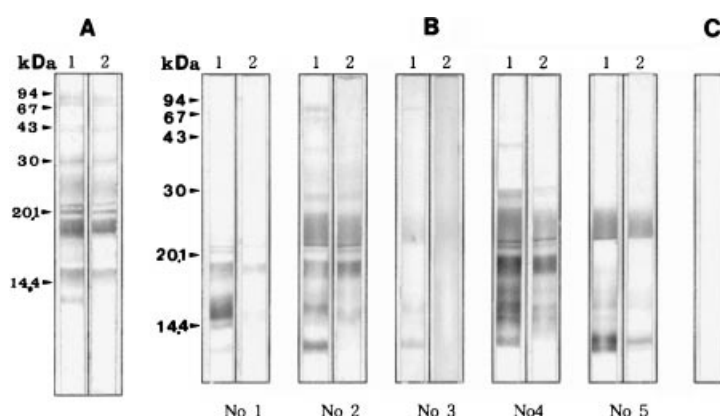


Figure 3. Immunoblotting features of periodate-oxidated BW allergens with pooled sera (A), individual allergic patients' sera (B), and nonatopic control (C). B) Nos. 1–5 indicate individual atopic subjects. Lane 1) untreated BW control; lane 2) periodate-oxidated BW.

database of the National Center for Biological Information. The N-terminal amino-acid sequence of the 19-kDa allergen had considerable homology with the 19-kDa α -globulin protein of rice (homology rate: 50%), which belongs to the 2S storage albumin family. The 16-kDa allergen also had homology of rather low stringency (homology rate: 38%) with the α -amylase/trypsin inhibitor of finger millet. The N-terminus of the isoallergenic 9-kDa allergen was identical to the reported trypsin inhibitors of BW (18).

Discussion

BW frequently elicits severe allergic symptoms (2–7). According to the results of 128 open-challenge positive cases of Korean children, BW was the most common causative food allergen (3), and about 0.2% of Japanese children had allergy to BW (4). However, little is known about its clinical features and the serologic findings of BW-allergic patients. Moreover, there is also some controversy over its causative major allergens (12–15). Clinical manifestations in our patients revealed that BW elicited severe allergic symptoms such as loss of consciousness, dyspnea, and facial edema. In addition, during the DBPCFC and open-challenge test, we also observed anaphylactic shock in two patients, who recovered after subcutaneous injection of epinephrine and infusion of saline. These results were consistent with those of other investigators who also confirmed the extremely strong allergenicity of BW (2, 5–7). In our study, BW-allergic patients had very strong skin reactivity. The wheal size of challenge-positive patients was larger than 16 mm.

Practically, the SPT, the measurement of specific IgE, and the positive history of severe adverse reactions to BW were very helpful in the diagnosis of BW allergy. We performed DBPCFC in 10 symptomatic patients, and all showed positive results in DBPCFC or subsequent open-challenge test. Considering the severe allergic symptoms evoked by BW, SPT and *in vitro* diagnostic tests may be very helpful in the diagnosis of BW. But some patients with strong skin reactivity and a high concentration of specific IgE did not have allergic symptoms to BW.

Many allergens such as those of 67, 43, 24, split 19, 16, 14, and 9 kDa were found in BW-allergic and asymptomatic control subjects. Among them, the split 19-kDa allergens were specifically found in BW-allergic patients, and other allergens were found in both BW-allergic and asymptomatic subjects. Other investigators suggested that the 24-kDa allergen, the β chain of 11S globulin, was the major allergen of BW in view of the results of immunoblotting and the RAST inhibition study (15). However, the prevalence of IgE-binding 24-kDa allergens was no different between BW-allergic patients' sera and RAST-positive subjects' sera in their study (15). There were some different points in immunoblotting features between their study and ours. They could not identify the 19-kDa allergens.

We do not know why the immunoblotting features were different. One possible cause is the difference in the osmolality of extraction buffers. We extracted the BW allergen with PBS solution which contained 0.8% NaCl, and they used Cocca's solution, which contains 0.5% NaCl. The differences in osmolality may have influenced the composition of the BW extract. But our

Table 2. N-terminal amino-acid sequences of BW allergens

MW	Sequences
19 kDa (pI 6.5)	Gly-Asp-Tyr-Pro-Leu-Glu-X-Cys-Arg-Gln-Lys-Ile-Glu-His
16 kDa (pI 5.6)	Arg-Asp-Glu-Gly-Phe-Asp-Leu-Gly-Glu-Thr-Gln-Met-Ser-Ser-Lys-Cys-Met-Arg-Gln-Val-Lys-Met-Asn-Glu-Pro
9 kDa (pI 5.0/6.0)	Ser-Asp-Lys-Pro-Gln-Gln-Leu-Leu-Glu-Glu-CysArg-Tyr-Leu-X-Arg-Iso

SDS-PAGE protein-staining feature was not different from the previous report. Protein staining of the SDS-PAGE gel showed that the 19-kDa allergens were tiny compared to the 24-kDa allergen. Therefore, we thought that the dosage of BW extract used in SDS-PAGE might be important for the immunoblotting features. In addition, the concentration of BW-specific IgE in the allergic patients was significantly higher than in the asymptomatic subjects, and that difference may have caused the differences in the features of immunoblotting. Moreover, the results of immunoblotting could vary markedly according to the methods used in the process.

Other investigators also reported asymptomatic subjects with a high concentration of BW-specific IgE and BW cross-reactivity with latex, rice, potato, and grass-pollen allergens (6, 19, 20). Only two BW-allergic patients in this study complained of allergic symptoms to peanut, suggesting that the true BW allergen might be less cross-reactive to other vegetable foodstuffs. The N-terminal amino-acid sequences suggest 19-kDa and 16-kDa allergens as the 2S storage albumin family and cereal trypsin/ α -amylase inhibitor family, respectively. The 2S storage albumin has been reported to be the major allergen of oriental mustard (Bra j 1) and yellow mustard (Sin a 1) (21, 22). But the inhibition immunoblotting experiment with recombinant Sin a 1 and Bra j 1, which were kindly donated by Dr Rodriguez, did not show cross-reactivity between the 19-kDa allergen of BW and these two recombinant 2S storage albumins (data not shown).

The cereal trypsin/ α -amylase inhibitors are also

known to be the major allergens of rice, barley (Hor v 1), and wheat (23). But we think that further studies are needed for more precise characterization of these major allergens of BW and to determine whether cross-reactivity is present or not. The 9-kDa allergen has been reported as a BW allergen (13), and the N-terminal sequence data showed it to be the isoallergenic trypsin inhibitor of BW, BWI-2a/BWI-2b (18). It consists of 51 amino acids and shares all amino acids except for the C-terminal tripeptide. BW is not easily digested, possibly due to the presence of the protease inhibitors; more than seven trypsin inhibitors are present in BW (24). The IgE-binding activity of the 9-kDa allergens decreased markedly after periodate oxidation, and this result suggested the presence of the carbohydrate IgE determinant in the 9-kDa allergens. Cross-reactive carbohydrate determinants were reported in the variable food allergens, and many of them were clinically insignificant (25).

Our results suggested that the allergens of 24, 19, 16, and 9 kDa are strong candidates to be major allergens of BW, while the 19-kDa allergen was relatively specific to the BW-allergic patients with clinical symptoms. We also suggest that measurement of BW-specific IgE and clarification of features of immunoblotting, in addition to the history of severe adverse reactions to BW, are very important in the diagnosis of BW allergy.

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