

Application of the 16-kDa buckwheat 2 S storage albumin protein for diagnosis of clinical reactivity

Soo-Young Choi, MSc*; Jung-Ho Sohn, BSc*; Yong-Won Lee, MD*†; Eun-Kyung Lee, MSc*; Chein-Soo Hong, MD, PhD*†; and Jung-Won Park, MD, PhD*†

Background: The 16-kDa protein of buckwheat (BW) has been implicated as a major allergen in BW allergy.

Objective: To characterize the 16-kDa allergen and evaluate its clinical significance as an indicator of BW allergy.

Methods: Complementary DNA from the 16-kDa allergen was cloned and expressed in *Escherichia coli*. Allergenicity was confirmed with IgE immunoblotting or with an enzyme-linked immunosorbent assay. The clinical utility of the recombinant protein (r16 kDa) for diagnosis of BW reactivity was evaluated in 18 BW-allergic and in 20 asymptomatic BW-sensitized subjects.

Results: The 16-kDa allergen, composed of 127 amino acids, has 50% homology to the reported 8-kDa BW allergen, which belongs to the 2 S storage albumin. The r16-kDa protein can inhibit specific IgE (sIgE) antibody binding to the native BW 16-kDa allergen but minimally inhibited sIgE binding to crude BW extract. Approximately 77.8% of patients with the BW allergy produced sIgE antibodies to the r16-kDa protein, compared with a complete lack of reactivity in the 20 asymptomatic BW-sensitized subjects. The areas of the receiver operating characteristic curves for the skin prick test (mean, 0.93; 95% confidence interval, 0.85 to approximately 1.01; $P < .001$) and the r16-kDa enzyme-linked immunosorbent assay (mean, 0.93; 95% confidence interval, 0.84 to approximately 1.01; $P < .001$) were higher than the area of the BW IgE measurement curve determined by ImmunoCAP (a system for assaying serum IgE) (mean, 0.80; 95% confidence interval, 0.66 to approximately 0.94; $P = .002$).

Conclusions: The 16-kDa allergen belongs to the 2 S storage albumin. Measurement of r16-kDa sIgE was more discriminating than measurement of ImmunoCAP sIgE in whole BW extracts for the diagnosis of clinical reactivity to BW.

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INTRODUCTION

Buckwheat (BW) (*Fagopyrum esculentum*) belongs to the Polygonaceae group of weeds. Most BW products have previously been used in feeding livestock and poultry. The major use of BW is for human food.¹ Buckwheat is becoming more popular in many countries as a health food product^{2,3} and is frequently used in food products, such as pancakes, bagels, breads, breakfast cereals, and noodles, and is being used as a stuffing material to fill pillows. However, many cases of allergic reactions to BW have been reported in Korea, Japan, the United States, and other Western countries.^{4–9}

The 24-, 19-, 18- to 16-, 8-, and 9-kDa proteins of BW have been recognized as major allergens. The 24-kDa BW protein has been identified as the β subunit of 11 S globulin,^{10–12} the 7- to 9-kDa BW allergen as vicilin,^{8,13,14} and the 8-kDa BW protein as 2 S albumin.¹⁵ The specific IgE (sIgE) antibodies to the 24-kDa allergen can be found in BW-allergic and asymptomatic subjects, making them a poor

candidate for the diagnosis of BW reactivity.^{8,16} It has been reported that the N-terminus of the 16-kDa protein is 1 of the major BW allergens⁸; this has been confirmed by other investigators.^{16,17} Interestingly, sIgE antibodies to the 16- and 19-kDa allergens are found exclusively in patients who complain of allergic symptoms after ingestion of BW but not in asymptomatic subjects with BW sensitization,^{8,16} suggesting that measurement of sIgE antibodies against these major allergens may be especially useful for the diagnosis of BW reactivity.

In this study, we first deduced the full amino acid sequence of the 16-kDa allergen from its complementary DNA (cDNA) sequence and evaluated the allergenicity of recombinant 16-kDa (r16-kDa) allergen and then applied it to the diagnosis of BW reactivity in 18 BW-allergic and in 20 asymptomatic BW-sensitized subjects.

METHODS

Subjects

Eighteen BW-allergic patients and 20 asymptomatic BW-sensitized subjects, who visited Severance Hospital Allergy-Asthma Clinic, Seoul, Korea, from March 1992 to September 2006, for evaluation of food allergies, were enrolled in this study. The medical ethics committee of Yonsei University, Seoul, approved this study (No. 4-2006-0204). These subjects were selected based on their mean wheal size in response to the BW skin prick test. Four allergic patients were diagnosed

* Institute of Allergy, Yonsei University College of Medicine, Seoul, Korea.

† Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Korea.

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as having BW allergy by a double-blind, placebo-controlled, food challenge (DBPCFC) and the other 14 recognized allergy symptoms immediately after BW ingestion. For the DBPCFC analysis, a capsule was used as a vehicle control and lactose powder was used as a placebo. Initially, 50 mg of dried BW flour was administered and the doses were doubled until the patients complained of symptoms. If the patients did not complain of symptoms after ingestion of 5 g of dried BW flour, an open challenge test with boiled BW was given.

The clinical features of the BW-allergic patients are summarized in Table 1. Asymptomatic BW-sensitized subjects did not complain of any symptoms after the ingestion of BW, even though they had a positive skin response (Bencard Ltd, Worthing, England) of a mean wheal size larger than 4 mm. Two of the asymptomatic sensitized subjects were remitted from BW allergy. Skin tests, serum sampling, and the collection of patient history regarding BW allergy were performed at the same time. Serum samples were donated by the subjects and frozen at -70°C until further use.

cDNA Cloning

Total RNA was extracted from the immature seeds of BW using an RNA synthesis kit (Qiagen, Germantown, Maryland). To obtain the complete 3' end of the sequence, we used rapid amplification of cDNA ends–polymerase chain reaction (PCR); sense primer was synthesized based on the N-terminal amino acid sequence of the 16-kDa BW protein,^{8,17} 5'-MGN GAY GAR GGN TTY GAY YTN GG-3', and an oligo (dT) conjugated adaptor was used for the antisense primer (5'-CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTT-3'). 3' Rapid amplification of cDNA ends–PCR was performed in a 20- μL reaction mixture containing 0.5 μL of cDNA, 50 pmol of the degenerated sense primer, 2 pmol of the adaptor-conjugated antisense primer, 2.5-mmol/L each deoxyribonucleotide triphosphate, PCR buffer, and 1 U *Taq* DNA polymerase (Intron Biotechnology, Sungnam, Kyungkido, Korea). Reaction conditions were 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute, and a final extension step of 72°C for 7 minutes. The reverse transcription PCR product was cloned with a cloning kit (pCR II-TOPO TA; Invitrogen, Carlsbad, California), and nucleotide sequences were determined with a BigDye Terminator Cyclic Sequencing reaction system (ABI 3100 Genetic Analyzer; Applied Biosystems, Foster City, California). The clone representing the correct nucleotide sequence for the 19-kDa BW protein was selected and used as a template for expression cloning.

Expression of cDNA Clones

For expression of cDNA clones, PCR was performed again using primers for the enzyme sites of *NdeI* and *XhoI*. Forward and reverse primers were designed: forward, 5'-CAC ATA TGA GNG AYG AGG GNT TYG ATT TNG-3'; and reverse, 5'-CAA CTC GAG TCA CAA AAT ACC GAT TTC C-3'. Polymerase chain reaction was performed in 50 μL of PCR buffer containing 2.5-mmol/L each deoxyribonucleotide

triphosphate, 50 pmol of the forward primer, 10 pmol of the reverse primer, 1.25 U of the *i-Pfu* DNA polymerase (Intron Biotechnology), and 1 μL of subcloned DNA as template. Five annealing precycles at 54°C were followed by 30 cycles of amplification (94°C for 20 seconds, 59°C for 15 seconds, and 72°C for 30 seconds). We used the pET-21a vector (Novagen, Madison, Wisconsin) to express the protein. The pET-21a vector was treated with restriction enzymes *NdeI* and *XhoI*, then ligated with the resultant cDNA. The ligated product was transformed in BL21 cells and incubated at 37°C overnight. After incubation, we selected an objective colony by restriction enzyme treatment. This single colony was inoculated into 3 mL of LB medium containing ampicillin and incubated overnight with shaking at 37°C . The following morning, this solution was used to inoculate 50 mL of YT medium 2X and incubated with shaking at 37°C until the optical density of liquid medium at 600 nm reached 0.4 to 1.0 (approximately 2 to 3 hours). After incubation, a sample was removed for the uninduced control, then 1-mmol/L isopropyl- β -D-thiogalactopyranoside (Sigma, St Louis, Missouri) was added to the remainder and incubated at 30°C for 4 hours.

Purification of the r16-kDa BW Allergen

The induced and control flasks were placed on ice for 5 minutes, and then the cells were harvested by centrifugation at 5,000g for 5 minutes at 4°C . The pellets were thawed on ice for 15 minutes and were resuspended in lysis buffer (100-mmol/L sodium dihydrogen phosphate, 10-mmol/L Tris base and concentrated hydrochloric acid, and 8M urea, pH 8.0) under denaturing conditions at 5 mL/g wet weight. For complete lysis, cells were stirred for 30 to 60 minutes at room temperature and centrifuged at 10,000g for 20 to 30 minutes to pellet cellular debris. Based on an affinity purification kit (Ni-NTA; Qiagen), 1 mL of the 50% slurry was added to 4 mL of cleared lysate and gently mixed by shaking for 1 hour at room temperature. The lysate-resin mixture was carefully loaded into an empty column and the flow-through was collected for sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. After washing twice with wash buffer (5-fold resin volume, 100-mmol/L sodium dihydrogen phosphate, 10-mmol/L Tris base and concentrated hydrochloric acid, and 8M urea, pH 6.3), the recombinant protein was eluted with elution buffers of pH 5.9 and 4.5. The eluates were dialyzed with phosphate-buffered saline (PBS) before use. The protein concentrations of the recombinant allergen or the BW crude extracts were assayed with a protein assay kit (Bio-Rad, Hercules, California).

Partial Purification of the Natural 16-kDa Protein

Ion exchange chromatography (diethylaminoethanol) was used for the separation of crude BW allergen. The lyophilized crude BW extract (30 mg/200 mL) was injected into a column containing a 0.5M to approximately 0.25M sodium chloride solution. The target fraction was passed again by gel filtration chromatography (Sephacryl S-200; Pharmacia, Uppsala,

Table 1. Clinical Features of Buckwheat-Allergic Patients

Patient No./sex/age, y	Provocation (dose, mg)	CAP IgE level, kU/L	r16-kDa sIgE, AU/mL	SPT wheal size, mm	Other food	Symptoms
1/M/25	None	9.86	21.7	8	Peach	U/A, R, and GI
2/M/28	D (400)	9.97	52.4	19	None	U/A, R, and GI
3/M/19	None	>100.00	500.6	12	Crab	U/A and R
4/F/40	None	22.40	360.0	17	None	U/A and R
5/M/32	None	2.78	10.3	15	None	U/A
6/M/19	None	12.20	4.3	38	None	U/A and R
7/M/23	D (1,200)	6.40	61.5	19	None	U/A and R
8/M/22	None	4.84	16.3	10	None	U/A
9/M/22	D (600)	3.80	5.8	29	None	U/A and R
10/M/20	None	17.40	1061.0	16	None	U/A and R
11/M/11	None	3.80	694.0	33	Peach	U/A and R
12/M/44	None	0.53	16.0	14	None	U/A and GI
13/M/19	None	0.60	6.0	11	None	U/A and R
14/M/25	D (600)	1.66	20.8	30	None	U/A
15/M/20	None	>100.00	376.0	50	None	U/A and R
16/M/29	None	0.48	7.7	14	None	U/A and R
17/M/15	None	76.60	146.6	28	None	U/A and R
18/M/19	None	20.80	213.2	15	None	U/A and R

Abbreviations: AU, arbitrary unit; CAP, ImmunoCAP (a system for assaying serum IgE) (Pharmacia, Uppsala, Sweden); D, positive result to double-blind, placebo-controlled food challenge; GI, gastrointestinal; R, respiratory; r16-kDa, recombinant 16 kDa; sIgE, specific IgE; SPT, skin prick test; U/A, urticaria/angioedema.

Sweden), and the collected eluate (16-kDa protein) was dialyzed against PBS and then frozen at -70°C until use.

SDS–Polyacrylamide Gel Electrophoresis and IgE Immunoblotting

Antigens were dissolved in reducing sample buffer (12.5-mmol/L Tris–hydrogen chloride, pH 6.8, 5% glycerol, 0.4% SDS, 1% mercaptoethanol, and 0.02% bromophenol blue) and boiled at 100°C for 5 minutes. Allergens were separated by electrophoresis on 13.5% polyacrylamide gels (Hoeffer, San Francisco, California) and run at 50 V for 30 minutes and at 180 V for 2 hours. Proteins were transferred onto nitrocellulose membranes (pore size, $0.45\ \mu\text{m}$) (Amersham, Buckinghamshire, England) on a transfer unit (Hoeffer) in buffer (25-mmol/L Tris, 192-mmol/L glycine, and 20% methanol, pH 8.3) at 350 mA for 2 hours. The membranes were blocked by 5% nonfat dried milk in TBS-T (50-mmol/L Tris and 0.1% polysorbate [Tween], pH 7.5) and then incubated with 1:10 diluted atopic serum samples at 4°C overnight and washed with TBS-T. Membranes were incubated with 1:1,000 diluted alkaline phosphatase conjugated goat antihuman IgE (Sigma) for 60 minutes. After washing, the colorimetric reaction was developed by a substrate system (BCIP/NBT; Promega, Madison, Wisconsin).

Enzyme-Linked Immunosorbent Assay–Specific IgE Measurement and Inhibition Enzyme-Linked Immunosorbent Assay

The r16-kDa sIgE was assayed by enzyme-linked immunosorbent assay (ELISA). A 96-well polystyrene plate was coated with $50\ \mu\text{L}$ of $10\ \mu\text{g/mL}$ of allergens in 0.05M carbonate buffer, pH 9.6, overnight at 4°C . After washing

with PBS-T (137-mmol/L sodium chloride, 1.8-mmol/L potassium phosphate monobasic, 10-mmol/L disodium hydrogen phosphate, 27-mmol/L potassium chloride, and 0.1% polysorbate 20 [Tween 20], pH 7.4), each well was blocked with 1% bovine serum albumin in PBS-T. The sIgE antibodies were detected by incubating in $50\ \mu\text{L}$ of diluted BW atopic serum samples (1:10) for 1 hour at room temperature. After washing, 1:1,000 biotin-labeled goat antihuman IgE (Vector, Burlingame, California) was added to the well and incubated with 1:1,000 streptavidin-peroxidase (Sigma) for 30 minutes before washing again. The colorimetric reaction was developed with ABTS solution (25 mg of 2, 2-azino-bis-3-ethylbenzthiazoline-sulfonic acid in 50mM citrate phosphate buffer and $50\ \mu\text{L}$ of 30% hydrogen peroxide). The reaction was stopped by the addition of $100\ \mu\text{L}$ of 2mM sodium azide, and optical density was determined at 405 nm UV by an automated microplate spectrophotometer (Dynatec, Alexandria, California). The level of r16-kDa sIgE antibody was expressed in arbitrary units (AU). The pooled serum samples ($n = 5$; patients 3, 4, 15, 17, and 18 in Table 1) of high BW sIgE were assumed to have 1,000 AU per milliliter. The mean $\pm$ SD value of r16 sIgE from 15 nonatopic serum samples was 5.0 ± 1.8 AU.

For ELISA inhibition tests, atopic serum samples were preincubated with each concentration of allergen overnight at 4°C before ELISA measurement, as previously described.

Statistical Analysis

Statistical analysis was performed using a commercially available software program (SPSS, version 9.0; SPSS Inc, Chicago, Illinois). The differences in the mean values of

wheel size and levels of sIgE were analyzed with the Wilcoxon signed rank test. The relationship between r16-kDa sIgE and ImmunoCAP (CAP) (Pharmacia) (a system for assaying serum IgE) IgE was evaluated with Pearson product moment correlation analysis. Clinical validation of r16-kDa sIgE and CAP IgE measurements was done with receiver operating characteristic (ROC) curve analysis. $P < .05$ was considered statistically significantly different.

RESULTS

Full Amino Acid Sequence of the 16-kDa BW Allergen and Its Homology

Based on the reported *N*-terminal sequence of the 16-kDa BW allergen,^{8,17} we cloned the cDNA of the 16-kDa BW allergen and deduced its full amino acid sequence (GenBank accession No. AAX57578). The 16-kDa BW allergen consisted of 127 amino acids with an estimated molecular weight of 14.7 kDa. Homology tests to other food allergens were done through the National Center for Biological Information program. The 16-kDa BW allergen has 50% homology to the known 8-kDa BW allergen from 2 S albumin (GenBank accession No. BAB79444).¹⁵ This 16-kDa BW 2 S albumin protein also had homology to other known food allergens, such as the group 2 major allergen found in peanuts (Ara h 2, 34% homology),¹⁸ the group 6 major allergen, also found in peanuts (Ara h 6, 30% homology), the 2 S storage protein of the Brazil nut (Ber e 1, 18.9% homology), and the 2 S albumin of English walnut (Jug r 2, 30% homology).¹⁹

Allergenicity of the r16-kDa BW Allergen

The 16-kDa BW allergen was expressed in *Escherichia coli*. To see whether the r16-kDa allergen has comparable allergenicity to that of nonrecombinant BW, inhibition immunoblotting and inhibition ELISA were done with BW atopic pooled serum samples ($n = 5$; patients 3, 4, 15, 17, and 18 in Table 1). The partially purified 16-kDa allergen underwent electrophoresis and transferred to the membrane. The r16-kDa allergen inhibited the binding of sIgE to the native partially purified 16-kDa BW protein (Fig 1). The r16-kDa sIgE was dose dependent and completely inhibited by BW crude extract and by itself. However, sIgE binding to BW whole extract was not inhibited by the r16-kDa allergen (Fig 2). Although the 16-kDa BW allergen has mild homology with peanut allergens, and the possibility of cross-reactivity between BW and rice has been suggested,²⁰ binding of sIgE to the r16-kDa BW allergen was not inhibited by whole peanut or rice extracts.

Clinical Application of the Skin Prick Test and Measurement of CAP sIgE and r16-kDa sIgE in the Diagnosis of BW Reactivity

The BW-allergic patients had strong skin reactivity in skin prick tests (SPTs), higher CAP sIgE to BW crude extract, and sIgE to r16-kDa allergen ($P < .001$ for all). The ELISA sIgE to the r16 test was more discriminating than CAP sIgE to crude BW extract for the diagnosis of BW-allergic subjects.

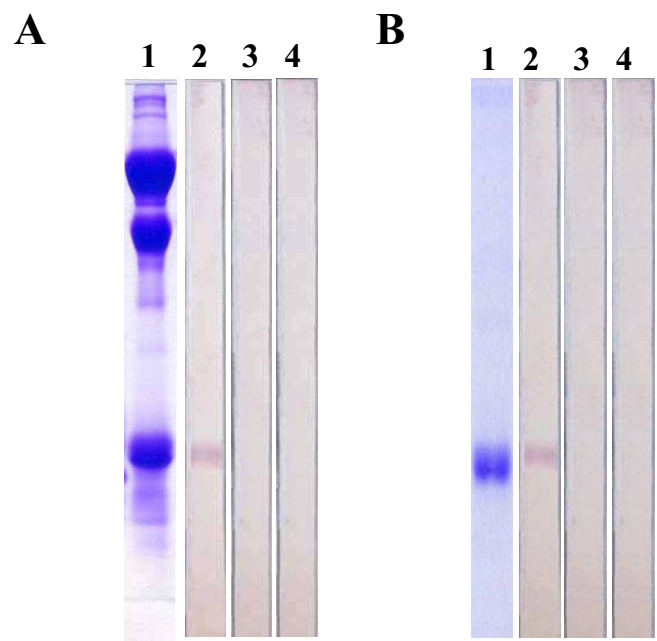


Figure 1. Inhibition immunoblotting with partially purified native 16-kDa buckwheat (BW) extract (A) and recombinant 16 kDa (r16 kDa) (B) using pooled serum of BW-allergic patients ($n = 5$; patients 3, 4, 15, 17, and 18 in Table 1). Lane 1, Commassie blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis results for partially purified 16-kDa BW extract (A) or r16 kDa (B); lane 2, IgE immunoblots to partially purified 16-kDa BW extract (A) or r16 kDa (B) without inhibitor; lane 3, IgE immunoblots with inhibitor (20 μ g/mL of r16 kDa); and lane 4, IgE immunoblots with inhibitor (20 μ g/mL of purified 16-kDa BW protein).

The optimal cutoffs for the differentiation of BW allergy from an asymptomatic sensitizer, shown in Figure 3, were determined by ROC curves: wheal diameter of SPT result, 12.8 mm; BW sIgE CAP result, 3.5 kU/L; and r16-kDa ELISA result, 10.0 AU per milliliter. The areas of the ROC curves for the SPT (mean, 0.93; 95% confidence interval, 0.85 to approximately 1.01; $P < .001$) and the r16-kDa BW ELISA test (mean, 0.93; 95% confidence interval, 0.84 to approximately 1.01; $P < .001$) were higher than the area of BW CAP IgE measurement (mean, 0.80; 95% confidence interval, 0.66 to approximately 0.94; $P = .002$). The levels of r16-kDa sIgE correlated with CAP sIgE in BW SPT-positive subjects ($r = 0.44$, $P = .006$), but not with CAP sIgE in BW-allergic subjects ($r = 0.33$, $P = .18$) or BW asymptomatic sensitized subjects ($r = -0.22$, $P = .36$) (Fig 4).

DISCUSSION

The 16-kDa protein has been implicated as a major BW allergen candidate.^{8,16,17} These previous studies have also shown the different patterns of sIgE binding to whole BW extract in BW-allergic and asymptomatic sensitized subjects. The sIgE antibodies to the 16- and 19-kDa BW allergens were found specifically in symptomatic subjects, whereas the

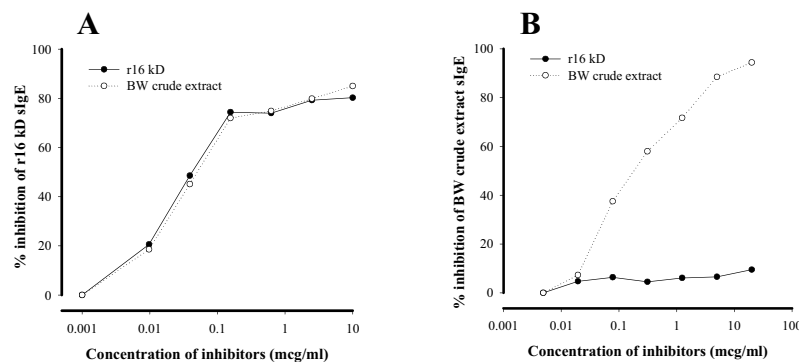


Figure 2. Enzyme-linked immunosorbent assay inhibition of recombinant 16 kDa (r16 kDa) binding specific IgE (sIgE) (A) and buckwheat (BW) crude extract binding sIgE (B) with pooled BW-allergic patient serum samples (patients 3, 4, 15, 17, and 18 in Table 1). The r16-kDa allergen is the inhibitor.

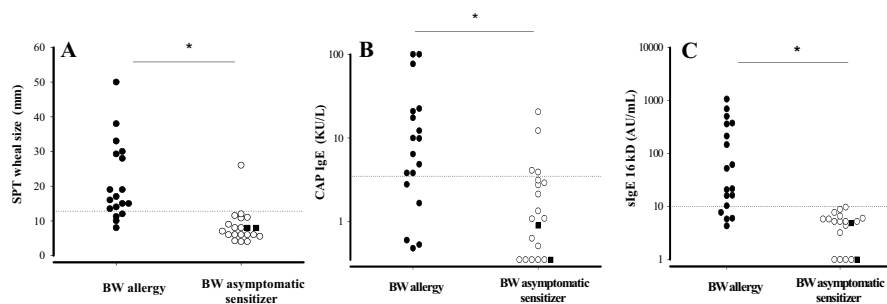


Figure 3. Mean wheal sizes resulting from the skin prick test (SPT) (A), levels of specific IgE (sIgE) to buckwheat (BW) crude extract determined by ImmunoCAP (CAP) (a system for assaying serum IgE) (Pharmacia, Uppsala, Sweden) (B), and sIgE to the recombinant 16-kDa (r16-kDa) allergen in BW allergy; patients ($n = 18$) and BW asymptomatic sensitized subjects ($n = 20$). AU indicates arbitrary unit; filled squares, subjects who were remitted from BW allergy; dotted lines, the optimal cutoffs determined from receiver operating characteristic curves for the differentiation of BW allergy from asymptomatic sensitizer; asterisk, $P < .001$.

sIgE antibodies to the 11 S globulin protein (molecular weight, 22 to approximately 24 kDa) were found nonspecifically in BW-allergic patients and asymptomatic sensitized subjects.^{8,16} To our knowledge, this study is the first to identify the full amino acid sequence of the 16-kDa BW allergen (GenBank accession No. AAX57578), and these results have been confirmed by another group (GenBank accession No. DQ304682).²¹

In food allergy detection, the poor positive predictive value of the SPT and sIgE measurements using whole extracts is well known, and DBPCFC is the gold standard for the diagnosis of food allergy. Because DBPCFC is difficult and dangerous, the application of discriminative sIgE levels using the CAP test or wheal size in the SPT has been successfully evaluated for the diagnosis of peanut, egg, and milk allergies.^{22,23} We found that all BW-allergic patients had strong SPT responses. Large wheal size (>8 mm) in the SPT was also predictive of clinical reactivity in milk, egg, and peanut allergies, with 95% accuracy.^{24–26} Furthermore, the r16-kDa sIgE levels were more discriminating in the diagnosis of BW reactivity than CAP sIgE measurements or the SPT with whole BW extracts. Recently, Beyer et al²⁷ reported that the measurement of sIgE of Ara h 2 peptides is useful for the

diagnosis of clinical reactivity to peanuts. These results suggest the possibility that the sIgE of major allergens or its peptides may have supplemental roles for the diagnosis of food allergy.

The autologous and heterologous inhibition immunoblottings for r16 kDa in this study proved the allergenicity of the r16-kDa allergen. Although the r16-kDa allergen was tagged with 6 histidines, our immunoblotting features suggest the histidine tag may not affect the allergenicity of r16 kDa. Other investigators²¹ have also reported sufficient allergenicity of the r16-kDa BW allergen expressed in *E coli*. We do not know why this 16-kDa allergen is more specific to the phenomenon of clinical reactivity to BW than the 24-kDa 13 S globulin or 9-kDa trypsin inhibitor allergens.⁸ Other groups^{16,21} have reported that the 16-kDa allergen is pepsin resistant and that the BW 19- and 24-kDa 11 S globulins are easily digested by pepsin. Many food allergens have conformational and sequential epitopes. Individuals who possess sIgE to conformational epitopes may tolerate foods denatured by cooking or digestive enzymes, whereas allergies to sequential epitopes may react to the food in any form.^{28,29} Subjects with allergies to conformational epitopes may tend to develop clinical tolerance.³⁰ Some clinically nonreactive

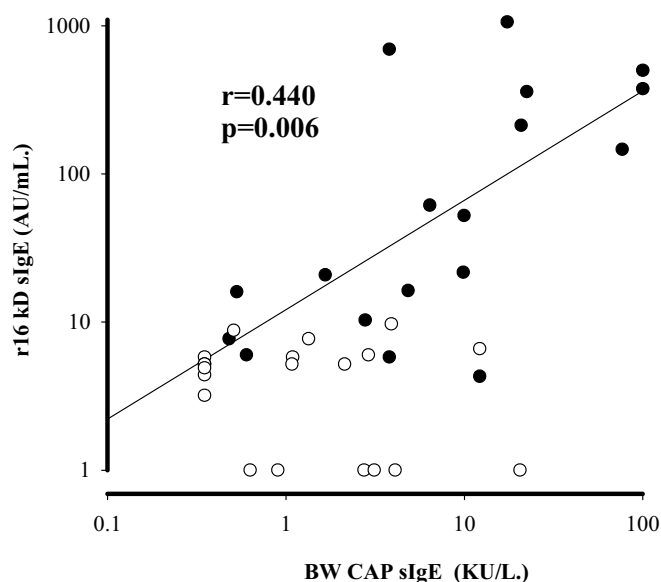


Figure 4. Correlation of ImmunoCAP (CAP) (a system for assaying serum IgE) (Pharmacia, Uppsala, Sweden) specific IgE (sIgE) to whole buckwheat (BW) extract and sIgE to the recombinant 16-kDa (r16-kDa) allergen. Filled circles represent BW-allergic patients; open circles, asymptomatic BW-sensitized subjects.

vegetable allergens have carbohydrate epitopes.^{31,32} The r16-kDa allergen was expressed in *E. coli*, which cannot glycosylate the expressed protein, except in the presence of carbohydrate epitopes.

The 16-kDa BW protein is not the only 2 S albumin allergen in BW. Researchers reported the 8-kDa 2 S albumin protein as the major allergen of BW and demonstrated the homology between the 16-kDa BW allergen and the 8-kDa BW 2 S albumin protein by comparing the N-termini of the 2 allergens¹⁵ and the full amino acid sequences²¹; the possibility of shared IgE epitopes should be considered. Unfortunately, native or recombinant 8-kDa BW allergen was not available for evaluation of cross allergenicity. But the recombinant 8-kDa BW, expressed in *E. coli*, bound to sIgE only in 2 of 14 BW-allergic patients,¹⁵ suggesting some BW-allergic patients may have sIgE to carbohydrate epitopes of the 10-kDa BW allergen.

Another report demonstrated that the N-terminus of the 16-kDa allergen has 80% homology with rice (*Oryza sativa*) dehydrin (GenBank accession No. OSU 60097), but our ELISA inhibition test excluded the possibility of cross-allergenicity between the BW 16-kDa allergen and *O. sativa* whole extract, a result that was also supported by a previous study. Yamada et al²⁰ reported the presence of cross-allergenicity between BW and rice in BW-tolerant subjects but not in BW-sensitive subjects, and, as previously discussed, the sIgE to the r16-kDa BW allergen is more specific to BW-reactive subjects. Clinically, we also did not detect a rice allergy in BW-allergic patients. We have described 2 peanut-allergic

patients of 19 BW-allergic patients who experienced frequent cosensitization to peanuts.⁸ Our results revealed a mild homology between the amino acid sequences of the BW 16-kDa allergen and major peanut or English walnut allergens. Just like peanuts and nuts, BW is also notorious for inducing severe anaphylaxis.^{4–8} Although our ELISA inhibition test excluded the presence of cross-allergenicity between the BW 16-kDa allergen and major peanut allergens, the possibility of cross allergenicity between other BW and peanut allergens was not eliminated.

In conclusion, we demonstrated that the 16-kDa BW allergen is a major allergen of BW belonging to 2 S albumin, with strong homology to 8-kDa BW 2 S albumin. The recombinant 16-kDa BW possessed allergenicity and might be especially useful for the diagnosis of the clinical reactivity to BW.

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Requests for reprints should be addressed to:
Jung-Won Park, MD, PhD
Institute of Allergy and Department of Internal Medicine
Yonsei University College of Medicine
Sudaemungu Schincheondong 134
Seoul 120-752, Korea
E-mail: parkjw@yumc.yonsei.ac.kr