

Immunological Characterization and Mutational Analysis of the Recombinant Protein BWp16, a Major Allergen in Buckwheat

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Buckwheat allergy is one of the most critical diseases manifested by severe and dangerous symptoms in Japan and other countries. We previously isolated the cDNA encoding protein BWp16, a member of the 2S albumin family with a conserved motif of 8 cysteine (Cys) residues. Comparison of the deduced amino acid sequences of BWp16 and related proteins in the 2S albumin family showed similarities between BWp16 and BW 8-kDa from buckwheat, Ara h 6 from peanuts and Ric c 1 from castor bean. Purified recombinant BWp16 (rBWp16) expressed in *Escherichia coli* was recognized by >80% of sera from patients with positive for IgE binding to buckwheat. Mutational analysis of rBWp16 revealed that 7 out of 10 mutants in the Cys residues showed weaker IgE binding to patient's serum than wild-type rBWp16 (rBWp16 WT). Mutations of Cys65 and Cys66 in rBWp16 decreased the pepsin digestibility of the protein, and an ELISA inhibition assay revealed a weaker inhibitory effect of rBWp16 C65S than that of rBWp16 WT. These results suggest that the Cys residues, especially Cys65, are involved in the allergenicity of rBWp16. Our findings provide new evidence for the role of Cys residues in 2S albumin family proteins and open the door to the production of hypoallergens and application to safe diagnostic methods and allergen-specific immunotherapy of buckwheat allergy.

Key words allergen; buckwheat; recombinant protein; 2S albumin; cysteine residue; immediate hypersensitivity reaction

Buckwheat (*Fagopyrum esculentum*) is consumed as not only a common pseudocereal in Japan, Korea, and other East Asian countries, but as a health food and substitute for wheat flour in Western countries. Since the first case report of buckwheat allergy in 1909,¹⁾ buckwheat has been recognized as one of most important food allergens manifested by severe and dangerous symptoms induced by ingestion or inhalation of a small amount of the flour or food products.^{2–4)} Buckwheat allergy is a type I immune response, *i.e.*, an immediate hypersensitivity reaction (IHR) that includes anaphylaxis mediated by a specific IgE antibody. In addition, buckwheat allergy is rarely outgrown, unlike allergy to cow's milk and hen's eggs. For these reasons, it is important to identify the allergen molecule and clarify the mechanism of buckwheat allergy for development of the accurate diagnosis and safer immunotherapy. Several lines of evidence have suggested that buckwheat contains a number of allergens to react with IgE from buckwheat allergy patients.^{5–7)} One of them, the 24-kDa protein Fag e 1, has been reported to be the major allergen responsible for buckwheat allergy based on the results of IgE binding assays with sera from patients with positive IgE binding activity to buckwheat.^{5,6,8)} On the other hand, Tanaka *et al.*⁹⁾ showed that the 16-kDa protein BWp16 is resistant to pepsin digestion and appeared to be responsible for IHR caused by buckwheat, whereas the 24-kDa protein was pepsin-sensitive and reacted with sera that had a positive buckwheat-specific IgE CAP RAST score, but was not responsible for IHR. Moreover, we have reported finding that >70% of sera from patients with positive IgE binding activity to buckwheat reacted with the band corresponding to BWp16, suggesting that BWp16 is a strong candidate for the major buckwheat allergen.¹⁰⁾ Recent isolation and sequence analysis of cDNA encoding BWp16 has shown that BWp16 consists of 149 amino acid residues and belongs to the 2S albumin family.^{5,10)} The 2S albumin family contains water-sol-

uble seed storage proteins isolated from cereals. They share a conserved motif of 8 cysteine (Cys) residues (...C...C...CC...CXC...C...C...).¹¹⁾ Since proteins that contain disulfide bonds derived from the Cys residues are generally resistant to proteolysis,¹²⁾ the disulfide bonds have been proposed to play an important role in the allergenicity of proteins.^{12,13)} In fact, some proteins in the 2S albumin family including proteins found in peanuts,¹⁴⁾ sesame seeds,¹⁵⁾ Brazil nuts,¹⁶⁾ and mustard,¹⁷⁾ are resistance to the proteolysis and are major allergens.

In vivo assays, such as skin tests and provocation tests, are traditional and common ways of diagnosing allergy. However, because of the critical symptoms of buckwheat allergy it is risky to diagnose it by means of such tests. In addition, advances in molecular biology have enabled us to produce and analyze recombinant allergens, which are uniform and can be standardized, as opposed to allergens from food extracts, which are mixtures of allergens and non-allergens. Thus, *in vitro* assays using well-characterized recombinant allergens should be established for the diagnosis of buckwheat allergy.

We previously generated recombinant BWp16 (rBWp16) in *Escherichia coli* (*E. coli*) and found that native BWp16 from buckwheat extracts and rBWp16 were immunologically equivalent.¹⁰⁾ In the present study we analyzed the IgE binding activity of rBWp16 to sera from patients with positive IgE binding activity to buckwheat. The results of the present study showed that ELISA with rBWp16 can be used for *in vitro* diagnosis of buckwheat allergy. Moreover, in the present study, we generated rBWp16 containing mutations in its Cys residues and analyzed the IgE binding activity and the resistance to pepsin digestion of the mutants to assess the role of the Cys residues in the allergenicity of BWp16 and to generate potentially hypoallergenic forms of this major buckwheat allergen.

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MATERIALS AND METHODS

Site-Directed Mutagenesis A Ser residue was substituted for each Cys by using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, CA, U.S.A.) according to the manufacturer's protocol. The synthetic oligonucleotides used in the site-directed mutagenesis are listed in Table 1. DNA sequences were determined by using a DNA analyzer (model 3730; Applied Biosystems, Foster City, CA, U.S.A.).

Expression of the Wild-Type and Cys Mutants of rBWp16 in *E. coli* BWp16 cDNA encoding amino acid residues 23—149 containing a mutation of each Cys to a Ser residue, referred to as rBWp16 C16S, C30S, C55S, C61S, C65S, C66S, C76S, C78S, C111S, and C118S, were cloned into the pGEX 6P-1 vector (GE Healthcare U.K. Ltd., Little

Table 1. The Synthetic Oligonucleotides Used in the Site-Directed Mutagenesis

Construct name	Sequence
BWp16-C16S	5'-CAGATGAGCTCAAAGAGCATGCGACAAGTGAAG-3'
BWp16-C30S	5'-GAGCCACATCTGAAGAAAAGCAACCGTTACATAGCC-3'
BWp16-C55S	5'-GTTGAAGGTGAAGGAAGCAAGAGTGAAGAGTCG-3'
BWp16-C61S	5'-CAAGAGTGAAGAGTCGAGCATGAGAGGATGCTGTG-3'
BWp16-C65S	5'-GTCGTGTATGAGAGGAAGCTGTGTGGCGATGAAG-3'
BWp16-C66S	5'-GTGTATGAGAGGATGCAGCGTGGCGATGAAGGAG-3'
BWp16-C76S	5'-GGAGATGGATGATGAAAGCGTTTGTGAGTGGATGAAG-3'
BWp16-C78S	5'-GGATGATGAATGTGTTAGCGAGTGGATGAAGATGATGG-3'
BWp16-C111S	5'-GGAATTGCCTAGTAAGAGCGGGCTTAGTGAATTGG-3'
BWp16-C118S	5'-GGCTTAGTGAATTGGAAAGCGGATCGAGAGGAATTCG-3'

The Cys was converted to Ser with single or double nucleotide substitutions from TGT or TGC to AGC (underlined).

Table 2. Characterization of the Sera from Patients with Positive IgE Binding to Buckwheat

Serum No.	Immediate hypersensitivity reaction	Age (year)	Sex	Major diagnosis/symptoms	Total IgE (IU/ml)	Buckwheat-specific IgE levels (IU/ml)	Buckwheat-specific IgE scores	Other allergen-specific IgE scores
001	Positive	34	M	Food allergy (buckwheat anaphylaxis), hay fever (cedar pollen)	278	5.31	3	—
002	Positive	7	M	Food allergy (buckwheat anaphylaxis), asthma	800	8.15	3	—
003	Positive	37	M	Food allergy (buckwheat anaphylaxis), atopic dermatitis, asthma	11000	11.7	3	—
004	Negative	26	F	Allergic rhinitis	1000	2.88	2	—
005	—	5	M	—	4459	—	4	av (2), om (2), ew (2), cr (2), sh (2)
006	—	11	M	—	5363	—	3	om (3), b (2), ew (3), m (5), pn (4)
007	—	—	—	—	491	>100	6	m (2), hd (2)
008	—	9	F	—	190	0.91	2	to (2), la (3)
009	—	3	M	—	2703	15.4	3	om (4), mi (5), dd (2), ew (6), w (5), se (4), pt (4), ap (3), ey (4), ch (3), me (3)
010	—	15	M	—	2650	4.88	3	mi (5), cd (3), dd (2), w (3), og (3), rg (3), fu (4), fm (2), jc (3), rw (3), yw (2)
011	—	2	F	—	1002	0.79	2	ew (3), m (5), w (3), se (2), po (4), al (2), sq (1)
012	—	—	—	Food allergy (milk, egg)	9600	52	5	cd (2), mi (5), hd (5), m (5), ew (3), pn (3), dd (5)
013	Positive	6	M	—	1270	3.6	3	mi (5), hd (5), pn (3), ce (5), ba (2), jc (3), r (3), st (2), ap (3), ki (3), me (2)
014	—	3	F	Food allergy	6510	28.4	4	m (6), ef (6), p (6), sb (5), w (6), r (3), cn (3), be (3), sq (5), oc (5), om (5)
015	Positive	2	M	Food allergy (buckwheat)	7340	7.13	3	p (3), sb (2), mi (5)
016	—	7	—	Food allergy (wheat anaphylaxis)	1090	3.2	2	—
017	—	6	—	—	566	2.2	2	—
018	—	6	—	—	10800	1.2	2	—
019	—	15	—	—	7580	9.5	3	—
020	—	3	—	Atopic dermatitis	8630	3.8	3	—
021	—	5	—	Food allergy (milk anaphylaxis)	4490	7.5	3	—
022	—	5	—	—	1510	3.6	3	—
023	—	5	—	Atopic dermatitis	864	7.5	3	—
024	—	7	—	Atopic dermatitis	18300	4.0	3	—
025	—	3	—	Atopic dermatitis	4580	4.2	3	—
026	—	7	—	Atopic dermatitis	22000	13.2	3	—
027	—	1	—	Atopic dermatitis	366	13.6	3	—
028	—	1	—	Severe atopic dermatitis	17500	37.8	4	—
029	—	7	—	Food allergy (peanut anaphylaxis)	10200	61.0	5	—

M, male; F, female. —, no data. Food allergy: al, almond; ap, apple; av, avocado; b, bee; be, beef; ba, banana; cd, cat dander; ce, cedar; ch, chicken meat; cn, coconuts; cr, crab; dd, dog dander; ew, egg white; ey, egg yolk; fm, fungi mix; fu, fungi; hd, house dust; jc, Japanese cypress; ki, kiwi; la, latex; m, milk; me, melon; mi, mite; oc, octopus; og, orchard grass; om, ovomucoid; pn, peanuts; po, pork; pt, potato; r, rice; rg, rye grass; rw, ragweed; sb, soybean; se, sesame; sh, shrimp; sq, squid; st, strawberry; to, tomato; w, wheat; yw, yellowweed. Buckwheat and other allergen-specific IgE, determined using the CAP-RAST system (Pharmacia).

Chalfont, U.K.) as described previously.¹⁰⁾ The 10 resulting plasmids for the Cys mutants were transformed to *E. coli* BL21 (DE3) pLysS cells. All recombinant mutated proteins and rBWp16 WT, except rBWp16 C16S, were expressed as described previously.¹⁰⁾ *E. coli* cells containing the mutant rBWp16 C16S construct were grown at 37 °C in 200 ml of Luria-Bertani (LB) medium containing 100 µg/ml ampicillin and 100 µg/ml chloramphenicol. When the absorbance of the culture reached at 600 nm 0.4, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM, and the cells were incubated for 12 h at 16 °C. The cells were then harvested, washed, and sonicated as described previously.¹⁸⁾ All of the rBWp16 proteins were purified and quantified the protein concentration as described previously,¹⁰⁾ and the rBWp16 proteins obtained were used for the following experiments.

Patients' Sera Sera from 29 Japanese patients whose buckwheat-specific IgE CAP RAST score was positive (#001 to #029, Table 2) and control sera from 3 healthy volunteers (N-1 to -3) were used in this study. Informed consent was obtained from all patients and volunteers. Our study was approved by the Ethical Review Committee of National Institute of Health Sciences.

SDS-PAGE and Immunoblotting Buckwheat seed extract was obtained by the method described in a previous report.⁹⁾ The buckwheat seed extract (10 µg/lane) was suspended in SDS sample buffer with standard reducing conditions, boiled at 95 °C for 3 min, electrophoresed on a 15% SDS-polyacrylamide gel (DRC, Tokyo, Japan), and transferred to 0.22-µm nitrocellulose membranes (S&S, Dassel, Germany). The membranes were blocked, cut into *ca.* 4-mm strips, and incubated as described previously.¹⁰⁾ The bound IgE antibodies were detected by using Konica ImmunoStain HRP-1000 (Konica Minolta, Tokyo, Japan) according to the manufacturer's protocol.

ELISA After coating 96-well plates (Nunc LockWell™ Module Plate, C8, M type, Cat # CN-446469, Nunc A/S, Denmark) with each rBWp16 protein (1.0 µg/50 µl of 50 mM sodium carbonate buffer [pH 9.6]/well) and incubating them at 4 °C overnight, they were washed with phosphate-buffered saline (PBS, pH 7.2) containing 0.05% Tween 20 (PBS-T), and the plates were blocked with 0.1% casein-PBS for 1 h at room temperature. The plates were then washed with PBS-T and incubated at 4 °C overnight with serum samples diluted to 5% (1:20 dilution) in 0.1% casein-PBS. The wells were washed with PBS-T containing 1 M NaCl 4 times, for colorimetric detection they were exposed to horseradish-peroxidase (HRP)-conjugated goat anti-human IgE antibodies (Nordic Immunology, 1:1000 dilution) in 0.1% casein-PBS for 1 h at room temperature and reacted with substrate solution (TMB reagent, Cat#555214; BD Biosciences, San Diego, CA, U.S.A.). Colorimetric intensity (OD450) was measured according to the manufacturer's protocol.

Inhibition ELISA Diluted patient's serum #001 and control serum (1:20 dilution) were pre-incubated at 4 °C overnight with an equal volume of serially diluted rBWp16 WT, C65S, or C66S in 0.2% casein-PBS or PBS. 96-well plates were coated with each rBWp16 protein (1.0 µg/50 µl of 50 mM sodium carbonate buffer [pH 9.6]/well) and incubated at 4 °C overnight. After washing with PBS-T, the plates were blocked with 0.1% casein-PBS for 1 h at room tempera-

ture, washed with PBS-T, and incubated with pre-incubated serum samples at 4 °C overnight. The wells were then washed, and detection was performed as described above.

Pepsin Digestion Test The pepsin digestion tests of rBWp16 WT, C65S, and C66S were performed as described previously.¹⁰⁾ Protein samples were suspended in the SDS sample buffer with standard reducing conditions, boiled at 95 °C for 3 min, electrophoresed on a 15% SDS-polyacrylamide gel (DRC, Tokyo, Japan) and stained with Coomassie brilliant blue (CBB). For Western blot analysis, the electrophoresed gels were transferred to PVDF membranes. The membranes were blocked with 0.5% casein-PBS for 2 h at room temperature, incubated with diluted serum #001 (1:4 dilution) for 1 h at room temperature and then transferred at 4 °C overnight. After washing with PBS-T, the membranes incubated for 1 h with HRP-conjugated goat anti-human IgE (1:500 dilution) at room temperature and washed again. The bound IgE antibodies were detected by using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Inc., Boston, U.S.A.) according to the manufacturer's protocol.

RESULTS

Comparison of the Deduced Amino Acid Sequences of BWp16 with Those of Other Proteins in 2S Albumin Family

The BWp16 cDNA encodes a 149-amino-acid-residue precursor protein with a 22-amino-acid-residue predicted signal peptide in the N-terminal region that is cleaved to generate a mature protein predicted to contain 127 amino acid residues.^{5,7,9,10)} We then designated Arg23 as Arg1, the first amino acid of the mature BWp16 (Fig. 1A). Alignment of the deduced amino acid sequences of BWp16 and other 2S albumin proteins revealed a conserved motif of 8 Cys residues, which is one of the characteristic features of members of the 2S albumin family (Fig. 1B). BWp16, BW 8-kDa, Ses i 2, and Car i 1 contained two extra Cys residues, which is unusual for 2S albumins. The phylogenetic tree indicated that BWp16 has sequence homology with the protein BW 8-kDa from buckwheat, Ara h 6 (*Arachis hypogaea*) from peanuts, and Ric c 1 (*Ricinus communis*) from castor bean (Fig. 1C).

ELISA of rBWp16 with Sera from Patients with Positive IgE Binding Activity to Buckwheat We previously reported finding that >70% of sera from patients with positive IgE binding activity to buckwheat reacted with BWp16 in buckwheat extract by Western blot analysis, and that BWp16 seemed to be the major allergen in buckwheat allergy.¹⁰⁾ To confirm this finding, we performed ELISA with rBWp16 and sera from Japanese patients with positive IgE binding activity to buckwheat. Twenty-four of the 29 sera (83%) were positive for IgE binding activity to rBWp16 (Figs. 2A, B). These results support our previous data showing that BWp16 in buckwheat extract reacted with more than 50% of the sera from buckwheat CAP-RAST IgE-positive patients and functions as a major allergen. On the other hand, although the buckwheat-specific IgE CAP RAST score of serum #004, #018, #019, #025, and #027 was positive, they had little increased level of IgE binding activity with rBWp16.

Western Blot Analysis of Buckwheat Extracts Using

Sera from Patients with Positive IgE Binding to Buckwheat Since buckwheat contains several different allergens,⁵⁻⁷⁾ we performed a Western blot analysis with buckwheat extracts to identify the allergen recognized by patients' sera #001 to #015. All sera except #003 and #004 reacted to the 15-kDa band corresponding to BWp16 (Fig. 3), but no bands of IgE binding with sera #003 and #004 were detected, even though the buckwheat-specific IgE CAP RAST scores of sera #003 and #004 were positive. Six of 15 (40%) sera

also showed IgE binding activity to the band corresponding to 24-kDa. One serum, #014, and one normal control serum, N-2, recognized the band corresponding to 19-kDa. These results indicate that a 15-kDa protein corresponding to BWp16 is a major allergen that binds to human IgE sera in >50% of patients and that the 24-kDa and 19-kDa proteins are minor buckwheat allergens than BWp16.

ELISA of Cys-Residue-Mutated rBWp16 with Patient's Serum The disulfide bonds derived from the Cys

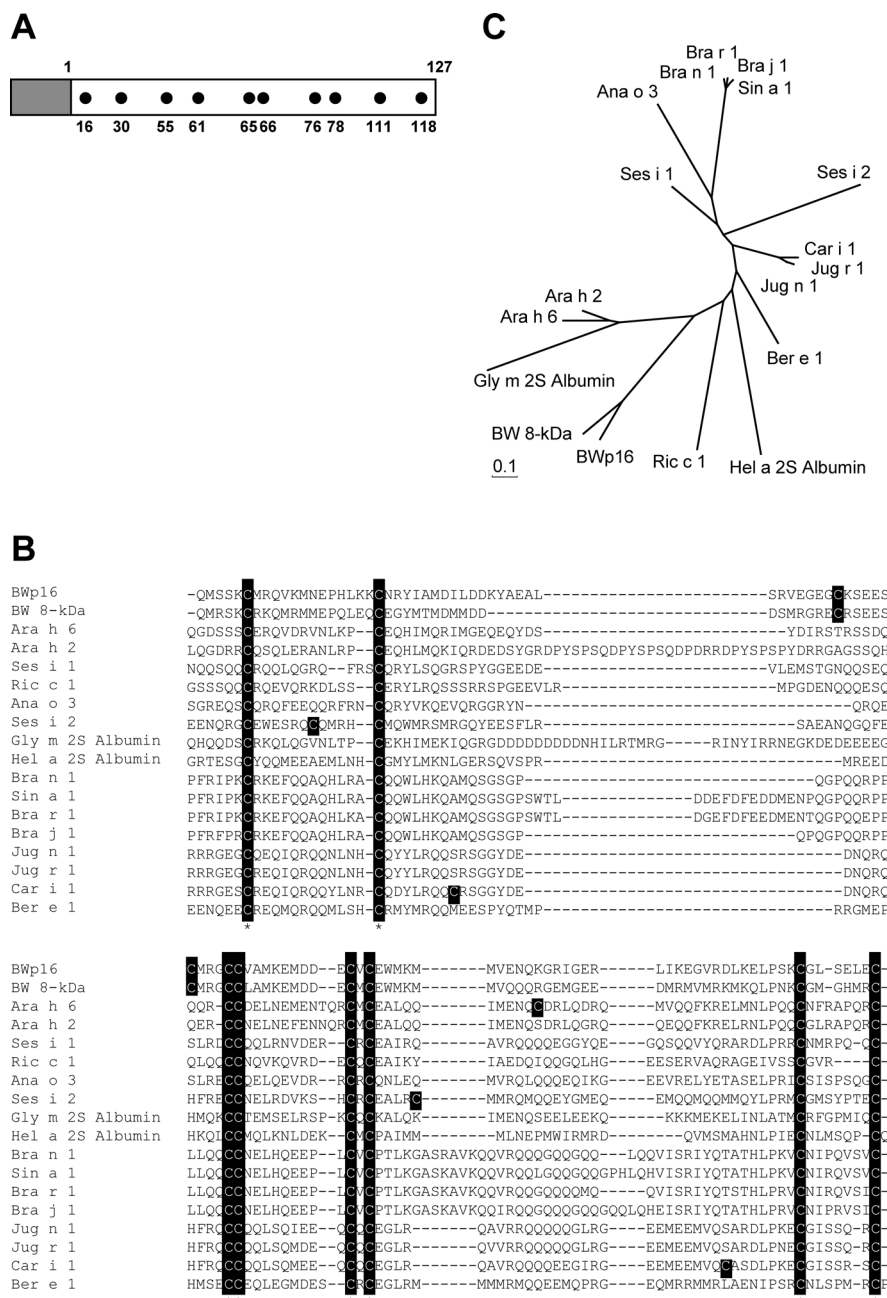


Fig. 1. Comparison of the Deduced Amino Acid Sequences of BWp16 and Related Proteins in the 2S Albumin Family

(A) Schematic representation of BWp16. The predicted signal peptide is illustrated as the gray portion of the bar. The white portion of the bar represents the mature BWp16. Ten Cys residues are shown as filled circles, and the numbers of the residues are shown at the bottom. (B) Alignment of the deduced amino acid sequences of BWp16 and members of the 2S albumin family protein. The conserved domains containing eight Cys residues of BWp16 and related 2S albumins have been aligned by using the ClustalW program. Gaps were introduced to optimize the alignment. The Cys residues are indicated in white against black background. Asterisks indicate the conserved amino acid residues. Abbreviations: Ana o 3, cashew (*Anacardium occidentale*); Ara h 2, peanuts (*Arachis hypogaea*); Ara h 6, peanuts (*Arachis hypogaea*); Ber e 1, Brazil nuts (*Bertholletia excelsa*); Bra j 1, oriental mustard (*Brassica juncea*); Bra n 1, canola (*Brassica napus*); Bra r 1, turnip (*Brassica rapa*); BW 8-kDa, buckwheat (*Fagopyrum esculentum*); Car i 1, pecan (*Carya illinoensis*); Gly m 2S Albumin, soybean (*Glycine max*); Hel a 2S Albumin, sunflower (*Helianthus annuus*); Jug n 1, black walnuts (*Juglans nigra*); Jug r 1, walnuts (*Juglans regia*); Ric c 1, castorbean (*Ricinus communis*); Ses i 1, sesame seeds (*Sesamum indicum*); Ses i 2, sesame seeds (*Sesamum indicum*); Sin a 1, white mustard (*Brassica alba*). (C) Phylogenetic tree of BWp16 and related proteins in the 2S albumin family. The tree was constructed from the matrix of sequence similarities calculated with the ClustalW program and illustrated by Tree View software. The scale indicates the relative branch lengths.

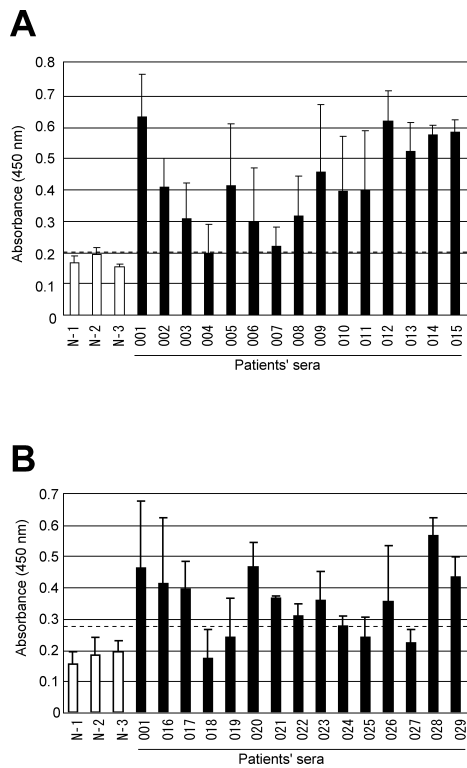


Fig. 2. ELISA of rBWp16 with Sera from Patients with Positive IgE Binding Activity to Buckwheat

Binding of specific IgE antibodies in the sera to rBWp16 (1 μ g/well) was analyzed by solid-phase ELISA. Values are means $\pm$ standard deviation (S.D.) of 3 independent experiments, each performed in duplicate. The ELISA was judged to be positive when the absorbance of the patient's serum was greater than average + 2S.D. of the control sera.¹⁰⁾ Error bars indicate S.D. of mean ($n=3$). (A) ELISA of rBWp16 with sera from patients with positive IgE binding activity to buckwheat, #001 to #015. Dashed line indicates the average + 2S.D. = 0.207. (B) ELISA of rBWp16 with sera from patients with positive IgE binding activity to buckwheat, #016 to #029 and #001. Dashed line indicates the average + 2S.D. = 0.268.

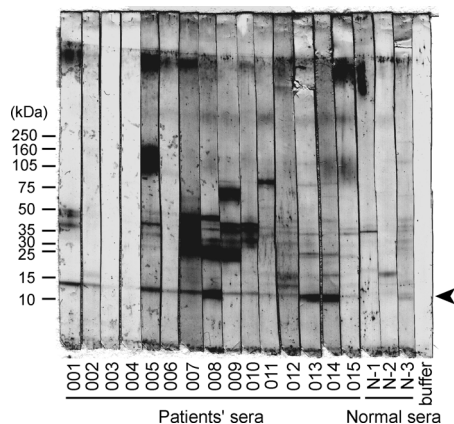


Fig. 3. Western Blot Analysis of Buckwheat Extracts with Sera from Patients with Positive IgE Binding Activity to Buckwheat

Buckwheat extract was subjected to SDS-PAGE and immunoblotted with sera from patients with positive IgE binding activity to buckwheat, #001 to #015, and control sera, N-1 to 3. The positions of the molecular weight markers are indicated on the left. The arrowhead indicates the band corresponding to BWp16.

residues are considered to play an important role in the allergenicity of proteins. It was reported that Der f2, which is the major house dust mite allergen, has 3 disulfide bonds, and is found that 2 of them are critical to the IgE binding capacity with sera from mite allergy patients.¹⁹⁾ To clarify the role of

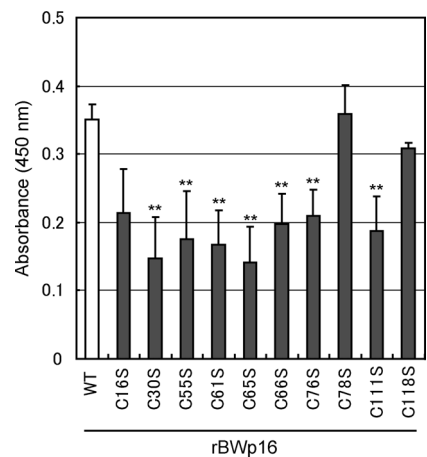


Fig. 4. ELISA of Cys-Residue-Mutated rBWp16 with Serum from Patient with Positive IgE Binding Activity to Buckwheat

Binding of serum from buckwheat allergy patient #001 to the rBWp16 (1.0 μ g/well) with and without mutation in the Cys residues to Ser residues was analyzed by solid-phase ELISA. Values are means $\pm$ standard deviation (S.D.) of 3 independent experiments, each performed in duplicate. The double asterisks indicate the p -values (Student's t -test) < 0.05.

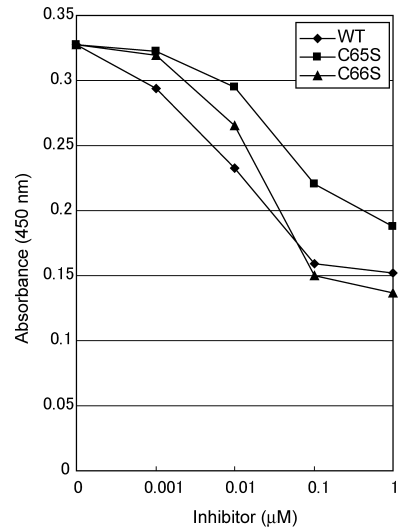


Fig. 5. Inhibition ELISA of rBWp16 WT, C65S, and C66S with Serum from Patient with Positive IgE Binding Activity to Buckwheat

Serum from buckwheat allergy patient #001 (1 : 20 dilution) was pre-incubated with different concentrations of rBWp16 with or without mutations in the Cys residues. The pre-incubated serum samples were transferred to rBWp16 WT-coated wells, and bound IgE was analyzed by ELISA. The 50% inhibitory concentration of IgE binding (IC_{50}) of rBWp16 WT, C65S, and C66S was also calculated. A representative result of two similar experiments is shown.

Cys residues in IgE binding activity to BWp16, we generated 10 variants of rBWp16 with a mutation at each Cys residue in which it was replaced by a Ser residue, and we refer to them as rBWp16 C16S, C30S, C55S, C61S, C65S, C66S, C76S, C78S, C111S, and C118S. We then analyzed the reactivity with IgE in serum #001 to wild-type and mutated rBWp16. Mutants rBWp16 C30S, C55S, C61S, C65S, C66S, C76S, and C111S showed decreased IgE binding activity in comparison with rBWp16 WT, suggesting that these Cys residues have possibility to play a role in the allergenicity of rBWp16 (Fig. 4).

Mutation of Cys65 and Cys66 in rBWp16 Decreases the Efficiency of ELISA Inhibition of the Binding of Pa-

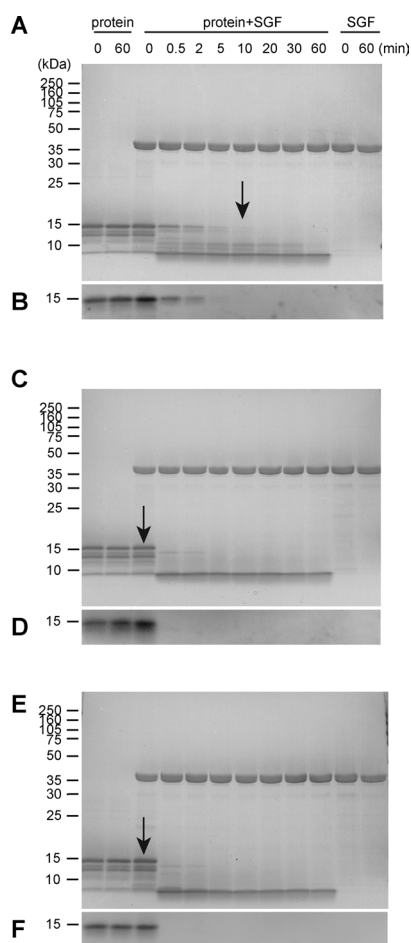


Fig. 6. SGF Digestion Profiles of rBWp16 WT, C65S, and C66S

The digestibility of rBWp16 was assessed with simulated gastric fluid (SGF, 0.076% pepsin-2 mg/ml NaCl-HCl, pH 1.2). The rBWp16 protein and SGF were mixed and incubated at 37 °C for 0, 0.5, 2, 5, 10, 20, 30, and 60 min, and the samples obtained were subjected to 15% SDS-PAGE. The positions of the molecular weight markers are indicated on the left. (A, C, E) CBB staining of rBWp16 digested with pepsin. (A) rBWp16 WT (C) rBWp16 C65S (E) rBWp16 C66S. The arrows indicate rBWp16. (B, D, F) Immunoblot of rBWp16 digested with pepsin, using serum #001 from patient with positive IgE binding activity to buckwheat. (B) rBWp16 WT (D) rBWp16 C65S (F) rBWp16 C66S.

tient's Serum to rBWp16 WT As shown in Fig. 4, 7 of the 10 Cys residues affected IgE binding activity to buckwheat. 2S albumin family proteins are members of prolamin superfamily that is characterized by a conserved motif of eight Cys residues as follows: C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C. CC and CXC are particular characteristics in this superfamily. According to the result of Fig. 4, decrease of IgE binding to the mutants C65S and C66S (which are CC motif constituents) was greater than that to the mutants C76S and C78S (which are CXC motif constituents). Furthermore, Cys65 seems to bind Cys30 and Cys66 to Cys111 by intra S-S bond by 3D prediction model by the full automatic prediction server ROKKY (<http://www.proteinsilico.org/roky/roky-p/>) (data not shown). The degree of decrease of IgE binding to the mutants C30S and C65S was significant and similar and that to mutants C111S and C66S was similar. Therefore, CC motif seems to be important for IgE binding of BWp16 by formation of conformational epitopes. We then selected the mutants rBWp16 C65S and C66S and assessed the competitive IgE binding inhibitory effect of these mutants by the ELISA inhibition method with serum #001,

which was from patient with positive IgE binding activity to buckwheat. The inhibition efficiency of rBWp16 C66S was slightly lower than that of rBWp16 WT (Fig. 5), whereas that of rBWp16 C65S was significantly lower than that of rBWp16 WT. The 50% inhibitory concentration (IC_{50}) value of rBWp16 WT, C65S, and C66S was 0.0116, 0.0348, and 0.0135 μ M, respectively, indicating that Cys65 is more important than Cys66 for IgE binding activity to BWp16.

Mutation of Cys65 and Cys66 in rBWp16 Affects the Stability of rBWp16 under SGF Digestion Conditions Stability to proteolytic attack in the gastrointestinal tract is an important factor in assessing the allergenicity of proteins. Stability also determines whether a food allergen is capable of causing severe symptom. Since 2S albumins were found to be highly stable to digestion by simulated gastric fluid (SGF) in previous studies of plant-food allergens,²⁰ we investigated the resistance of wild-type and mutated rBWp16 to proteolysis by SGF. rBWp16 WT was completely digested by exposure to SGF for 10 min (Figs. 6A, B), whereas rBWp16 C65S (Figs. 6C, D) and C66S (Figs. 6E, F) were immediately digested and entirely resolved within 30 s, indicating that Cys65 and Cys66 are key amino acids in the stability of rBWp16 to proteolysis.

DISCUSSION

The results of ELISA and Western blot analysis in this study indicated that BWp16 is a major allergen of buckwheat allergy (Figs. 2, 3). In a previous report 24-, 19-, and 16-kDa proteins were identified as strong candidates for major buckwheat allergens, and the 19-kDa buckwheat allergen, which has homology with the rice α -globulin protein was found to be specific for buckwheat allergy patients.⁵ On the other hand, the 24-kDa protein, not the 19-kDa protein, is proposed as a major allergen with high IgE binding frequency in buckwheat allergy patients.⁶ Park *et al.*⁵ suggested that the differences in immunoblotting features were due to different methods of protein extraction. However, our results indicated that both the 24- and 19-kDa proteins do not seem to be major allergen (Fig. 3). In fact, buckwheat proteins larger than BWp16 were pepsin-sensitive.⁹ Furthermore, BWp16 is reported to be pepsin-resistant and to be an IHR-related allergen derived from buckwheat, whereas the 24-kDa protein is pepsin-sensitive and not involved in IHR.⁹

Twenty-four of 29 sera from patients with CAP-RAST score positive IgE binding activity to buckwheat used in this study showed the positive IgE binding activity to rBWp16 (Fig. 2). Five sera which were derived from all patients with IHR were included in these 24 sera (Table 2). Tanaka *et al.*⁹ and Choi *et al.*²¹ reported that the BWp16 is related with the IHR of buckwheat allergy. Based on these results, we concluded that BWp16 is an important major allergen of great interest in the diagnosis of buckwheat allergy syndrome.

The results of this study showed that ELISA with rBWp16 was capable of substituting for the Western blot analysis with native extracts of buckwheat. ELISA with recombinant allergens is a more convenient and uniform way to diagnose allergy than immunoblotting assay with food extracts. This suggests that the ELISA with rBWp16 is an effective method for *in vitro* diagnosis of buckwheat allergy.

In this study we demonstrated that rBWp16 WT is very

stable to proteolysis by SGF, whereas mutated rBWp16 C65S and C66S were less stable (Fig. 6). This indicates that the single amino acid substitution and disruption of key disulfide bond affects digestibility to SGF and that substitutions of Cys65 and Cys66 reduce the stability of rBWp16. Moreover, the ELISA inhibition efficiency of rBWp16 C65S was lower than that of the rBWp16 WT, suggesting that the single amino acid substitution at Cys65 modulates its IgE binding capacity and the disulfide bond is important to IgE epitopes formation for the proteins (Fig. 5). The above findings indicate that the Cys residues, especially Cys65, affect the allergenicity of rBWp16. The recombinant house dust mite allergen with single or double Cys substitution showed their reduced IgE binding capacity to sera from house dust allergy patients, suggesting that Cys substitution affect the protein allergenicity.^{19,22} De Weerd *et al.*²³ also generated major rye grass pollen allergen Lol p1 with Cys substitution and showed their reduced IgE binding to grass pollen allergy patients. Moreover, site-directed mutagenesis has been used successively to produce low IgE-reactive recombinant forms of the popular allergens such as major apple allergen Mal d1 and birch pollen allergen Bet v1.^{24–26} These results have shown that replacement of only a few key amino acids within an allergen molecule can significantly reduce the human IgE reactivity of these allergenic proteins. Modified recombinant allergens exhibiting reduced allergenicity seem to decrease the risk of symptoms and to be useful for immunotherapy. Our results may be help of engineering hypoallergens for buckwheat allergy.

Alignment of BWp16 and other 2S albumin proteins and their phylogenetic tree indicate that BWp16 belongs to the 2S albumin family, which has a conserved motif consisting of 8 Cys residues. Prediction of the conformation of the BWp16 protein by the full automatic prediction server ROKKY (<http://www.proteinsilico.org/rokky/rokky-p/>) indicated the presence of two disulfide bonds, Cys30-Cys65 and Cys66-Cys111 (data not shown), and we predicted the location of two additional disulfide bonds in BWp16, Cys16-Cys76 and Cys78-Cys118, based on the known conserved disulfide bonds in the 2S albumin family determined by NMR spectroscopy.^{27–29} The difference of the inhibitory effect between Cys65 and Cys66 may be attributable to the difference in contribution to IgE binding activity by the two predicted disulfide bonds, Cys30-Cys65 and Cys66-Cys111.

In this study we demonstrated that the ELISA with rBWp16 can be used for *in vitro* diagnosis of buckwheat allergy. We also generated single Cys-residue-mutated BWp16 to analyze the role of Cys residues in the BWp16 allergen and suggested that at least one of the Cys residues on BWp16 molecule contributed to the allergenicity of the protein, either by participating in the formation of a conformational epitope or the stability of the protein. Further mutational analysis by using rBWp16 molecules containing double mutations in the Cys residues in which the disulfide bond is disrupted would be helpful to understand the role of Cys residues on the allergenicity of the protein. Application of this knowledge by the generation of low allergenicity forms of BWp16 may contribute to the development of safe and reliable diagnostic methods and allergen-specific immunotherapy of buckwheat allergy.

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